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Correction for Light Absorption in Fluorescence Studies of Protein–Ligand Interactions

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It is shown that absorption of the excitation light can lead to substantial systematic errors in fluorescence measurements of equilibrium constants for formation of protein–ligand complexes. The assumptions about the optical arrangement of the fluorescence spectrometer involved in the calculation of the correction of this absorption are discussed. A general semiempirical correction procedure which can be used for (calculated) absorbance values as high as 5 is described. The importance of choosing the excitation wavelength so as to minimize the necessity for these corrections is emphasized.

KEY WORDS: fluorescence; protein–ligand complexes; inner filter effect; equilibrium constants; absorption correction.

Fluorescence spectroscopy has proven to be a widely useful technique for the study of protein–ligand interactions (1–2). In particular, it often affords a simple method for the measurement of equilibrium constants for formation of protein–ligand complexes. A number of instrumental and photophysical variables affect the accuracy of fluorescence measurements (2–6), of which the most important in the context of equilibrium constant determinations is the absorption of the excitation beam, the primary absorption or inner-filter effect ((7); see also (8)). If a significant part of the total absorption is due to the species—usually the ligand—whose concentration is varied in the course of the experiment, the primary absorption effect will distort the concentration dependence of the fluorescence, leading to an incorrect value for the equilibrium constant.

The form of the correction factor required to compensate for this absorption effect has been discussed in detail by Parker (3), Holland *et al.* (9), and Lloyd (10). A number of authors have described the use of primary absorption effect corrections in biochemical systems (11–17), often using approximate corrections valid only under specific conditions, such as relatively low absorbance.

The purpose of this paper is to describe a simple, general procedure for correcting for primary absorbance effects in fluorescence measurements of equilibrium constants for protein–ligand complex formation.

THEORY

We shall consider the case where the only instrumental correction required is that for the primary absorption. We therefore assume that (a) any absorption of emitted fluorescence is negligible and (b) effects of light scattering, refractive index changes, and emission anisotropy are negligible. It is usually possible to ensure the validity of the first assumption by a suitable choice of emission wavelength; if necessary a correction factor analogous to that for primary absorption can be applied

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(10). The emission from fluorophores in or bound to a macromolecule is likely to be anisotropic, and this can lead to substantial errors in quantum yield and in the shape of the emission spectrum ((18) and refs. therein). However, for the present limited purposes of estimating the equilibrium constant for formation of a protein–ligand complex, these effects can be largely ignored, since the concentration dependence of the observed intensity will only be slightly affected.

The "right-angle" geometry adopted in the majority of commercial fluorescence spectrometers is shown in Fig. 1. The excitation beam is directed along the x axis, and the fluorescence detected is that emitted in the y direction from within some "observation window" between the points X_1 and X_2. The distances of those points from the front face of the cell, as fractions of the total path length, are denoted W_1 and W_2.

The corrected fluorescence, F_{corr}, is defined as the observed fluorescence, F_{obs}, divided by the "average fluorescence yield" across the observation window X_1 to X_2.

$$F_{corr} = F_{obs} \Big/ \frac{\int_{W_1}^{W_2} I(W)E(W)dW}{(W_2 - W_1)}. \quad [1]$$

The term $I(W)$ represents the intensity of the excitation beam at a point W on the x axis in the observation window, the intensity of fluorescence emitted from point W being taken to be proportional to $I(W)$. $E(W)$ then represents the efficiency with which this fluorescence is detected. If we define the absorption correction factor, C, by $F_{obs} = F_{corr} \times C$, then

$$C = \frac{\int_{W_1}^{W_2} I(W)E(W)dW}{(W_2 - W_1)}. \quad [2]$$

From the Beer–Lambert law,

$$I(W) = I_0 10^{-AW} \quad [3]$$

where A is the absorbance of the sample at the excitation wavelength for the path length used. (Note that this absorbance should be measured using the same bandwidth and spectral distribution as used for the excitation beam in the fluorescence measurements.)

The detection efficiency function, $E(W)$, is much less easily defined. It will depend on the collimation of the excitation beam, on the observation window, and on the uniformity of sensitivity across the detector surface. $E(W)$ will thus depend critically on the sample-compartment optics, and will vary considerably from one spectrometer to another, as well as varying with the width of the emission slit(s). In view of this uncertainty, three distinct approaches to the evaluation of Eq. [2] have been used:

(i) Assume that the observed fluorescence originates from a single point in the sample cell. We then simply need to correct for the intensity of the excitation beam at this point, giving

$$C = 10^{-AW_0} \quad [4]$$

where W_0 is the fraction of the total path length the excitation beam must travel to reach the point from which the fluorescence is observed. Clearly the assumption of point-source fluorescence can never be strictly justified, particularly since, for maximum sensitivity, relatively large slit-widths are commonly used on the emission side.

(ii) Assume that the efficiency of detection of the fluorescence is uniform across the observation window (i.e., E is independent of

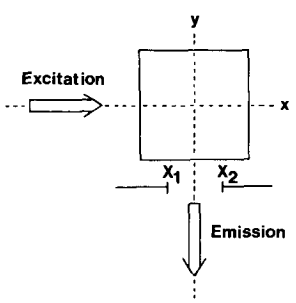

FIG. 1. The experimental arrangement for the measurement of fluorescence considered in the text.

W). Equation [2] then becomes (4,9)

$$C = \frac{10^{-AW_1} - 10^{-AW_2}}{2.303A(W_2 - W_1)} \qquad [5]$$

and the correction factor can be calculated from a knowledge of the absorbance and of the position and size of the observation window. If the dominant contribution to the absorbance comes from the ligand, A can be replaced by $\epsilon_L L_T$, where L_T is the total ligand concentration and ϵ_L its molar absorption coefficient at the excitation wavelength.

Uniform efficiency of detection requires, among other things, a uniform observation angle across the window; again, this can never be strictly true. Holland *et al.* (9) have described an optical arrangement which satisfies the uniform efficiency criterion to within 1%, but the optics of most commercial instruments are quite different. For example, the Perkin–Elmer instrument used in the present work has a lens adjacent to the sample cell on the emission side.

For sufficiently small values of $(W_2\text{-}W_1)$, Eq. [5] gives values of C identical to those given by Eq. [4]. For low absorbance, Eq. [5] can be simplified to

$$C = \frac{1 - 10^{-A}}{2.303A}. \qquad [6]$$

This is equivalent to Eq. [5] with $W_1 = 0$, $W_2 = 1$; for low enough absorbance, the absorption outside the window W_1-W_2 becomes insignificant. For $A < 0.3$, Eq. [6] gives values of C within 2% of those given by Eq. [5].

(iii) Determine the form of $E(W)$ empirically by curve fitting. This approach was used by Ehrenberg *et al.* (11), who described $E(W)$ by a polynomial. Rather than determine $E(W)$ for each instrument setting, we have applied the curve fitting directly to the determination of C, as described under Results and Discussion. For this purpose we use the equation

$$C = \frac{(e^{-aL_Td} - e^{-aL_T})}{aL_T(1 - d)} \qquad [7]$$

where a and d are parameters determined by curve fitting. The parameter a is related to, but not numerically equal to, ϵ_L, the molar absorption coefficient of the ligand at the excitation wavelength. With optimum values of a and d, Eq. [7] gives values of C within 0.1% of those given by Eq. [5] up to an absorbance of 2.0. In addition, Eq. [7] provides a satisfactory empirical description of the variation of C with L_T under conditions where neither the point-source nor the uniform sensitivity assumptions are likely to be valid (see below). For low absorbance, setting $d = 0$, Eq. [7] becomes identical to Eq. [6], with $aL_T = 2.303A$.

In fact, for sufficiently low absorbance, all three approaches give closely similar values for C and provide equivalent procedures for applying the primary absorption correction; for $A < 0.2$ Eqs. [4], [5], [6], and [7] give values for C which agree within 1%. At higher absorbance, on the other hand, the applicability of the assumptions about $E(W)$ to the particular spectrometer being used will determine whether Eq. [4], [5], or [6] can be used.

MATERIALS AND METHODS

Materials. Dihydrofolate reductase was purified from *Lactobacillus casei* MTX/R (19). The enzyme concentration was determined by absorption measurements and by fluorimetric titration with methotrexate (19). Nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate (ϵNADP$^+$),[1] folate, tryptophan, and *p*-aminobenzoyl-L-glutamate were from Sigma.

Fluorescence spectroscopy. Measurements were carried out using a Perkin–Elmer MPF-44A fluorescence spectrometer. Experiments were conducted at 25°C using 1-cm-path length quartz cells; ligands were added as microliter volumes of concentrated stock solutions. Ligand binding to dihydrofolate reductase was studied in 15 mM Bistris [2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-

[1] Abbreviations used: ϵNADP$^+$, nicotinamide 1,N^6-ethenoadenine dinucleotide phosphate; Bistris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol.

propanediol], pH 6.0, containing 0.5 M KCl, unless otherwise noted.

Data analysis. This was carried out in two stages. The raw data were first corrected for dilution, by calculating the true enzyme and ligand concentrations at each data point, and for the primary absorption effect, as discussed below. The second stage was the estimation of the equilibrium constant for complex formation from the corrected fluorescence data (20,21). For a simple binding process

$$E + L \rightleftharpoons EL \quad [8]$$

$$K' = [EL]/[E][L]. \quad [9]$$

For measurements of protein fluorescence,

$$\frac{[EL]}{E_T} = \frac{F_0 - F}{F_0 - F_\infty} \quad [10]$$

where $[EL]$ is the concentration of complex at a ligand concentration giving fluorescence intensity F, E_T is the total enzyme concentration, and F_0 and F_∞ denote the fluorescence intensity at zero and saturating ligand concentrations, respectively. Similarly, when ligand fluorescence is measured

$$\frac{[EL]}{E_T} = \frac{F - F_L}{F_\infty - F_L} \quad [11]$$

where F_L is the fluorescence of an equal concentration of ligand in the absence of protein. From the mass-action equation [Eq. 9],

$$[EL] = \tfrac{1}{2}[1/K' + E_T + L_T - (\{1/K' + E_T + L_T\}^2 - 4E_TL_T)^{1/2}] \quad [12]$$

and by combining Eq. [12] with Eq. [10] or [11], an expression for the corrected fluorescence as a function of L_T can be obtained.[2] The two unknowns, K' and F_∞, were determined by nonlinear regression, using 10–30 values of F and L_T. The regression analysis employed modifications of the programs of Batchelor (23), based on the algorithm of Marquardt (22). Initial values of K' were estimated from $1/K' = L_{50} - E_T/2$, where L_{50} is the ligand concentration at which 50% of the fluorescence change has occurred, and initial values of F_∞ either from a double-reciprocal plot ($1/F$ vs $1/L_T$) or simply from the measured fluorescence at the highest value of L_T used. (With a robust minimization routine and a sufficient data set, very accurate initial values are not required.) The standard errors quoted for K' are those estimated by the regression program; as discussed earlier (20), these are close to those estimated from replicate determinations.

[2] If there is more than one binding site per protein molecule, not only will Eq. [12] require modification but Eqs. [10] and [11] may also (24).

RESULTS AND DISCUSSION

Choice of Experimental Conditions to Minimize Inner-Filter Effects

In an experiment designed to measure an equilibrium constant, the enzyme and ligand concentrations to be used are governed by the magnitude of the equilibrium constant. If a good estimate of K' is to be obtained, one must ensure $E_T \leqslant 3/K'$, and ligand concentrations up to at least $10/K'$ must be employed. The experimental variables which can be manipulated to minimize the primary absorption effect are thus limited to the path length and the excitation wavelength. Short path length cells can be valuable (25), although some care is necessary to ensure accurate and reproducible positioning of the cell.

However, the most convenient way to minimize primary absorption effects is by an appropriate choice of excitation wavelength (25,26). If the absorbing species is the fluorophore itself, the use of an excitation wavelength on the red edge of the longest wavelength absorption band will often be more satisfactory than excitation at the absorption maximum. This is illustrated in Fig. 2, which shows the concentration dependence of the fluorescence of *N*-(*p*-aminobenzoyl)-L-glutamate for several excitation wavelengths. The measured fluorescence is shown directly in Fig. 2A, while in Fig. 2B it has been normalized by the molar absorption coefficient at the ex-

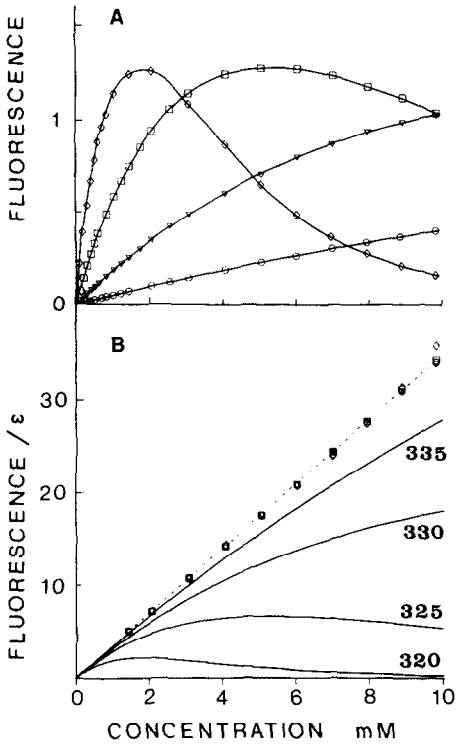

FIG. 2. The fluorescence of *p*-aminobenzoyl-L-glutamate as a function of its concentration, using various excitation wavelengths. (A) Fluorescence (arbitrary units) at excitation wavelengths of 320 (◇), 325 (□), 330 (▽), and 335 nm (○). The lines are the "best fits" of Eq. [13]. (B) The solid lines represent the data from (A), expressed as fluorescence divided by ϵ, the extinction coefficient at the excitation wavelength. In the absence of the inner-filter effect, these curves would follow the dashed line. The points (symbols as in (A)) show the experimental data after correction for the inner-filter effect as described in the text.

citation wavelength; in the absence of inner-filter effects, the data for all excitation wavelengths would fall on the straight (dashed) line in Fig. 2B. The concentration range shown, 0.1–10 mM, is that relevant to measurements of the binding of this compound to dihydrofolate reductase ($K' = 830\ M^{-1}$ (20)). On excitation at the absorption maximum, 274 nm ($\epsilon_{274} = 1.45 \times 10^4\ M^{-1}\ cm^{-1}$), essentially no fluorescence is detectable at concentrations > 0.5 mM (where $A > 7.25$). Even with excitation at 320 nm, the concentration dependence of fluorescence is still grossly nonlinear. However, by exciting further from the absorption maximum, at 335 nm, a more nearly linear concentration dependence is observed (Fig. 2) and the binding constant can be measured by working at this wavelength (20). When using this approach, precautions must be taken to ensure that the fluorescence observed is still that of the compound of interest, and not that of a fluorescent impurity with a longer wavelength absorption.

Correction for Primary Absorption Effects

Using the semiempirical Eq. [7] for the absorption correction term C, we have the general expression

$$F_{obs} = (F_{corr} + F_{blank}) \frac{\{e^{-aL_T d} - e^{-aL_T}\}}{aL_T(1 - d)} \quad [13]$$

where F_{blank} represents fluorescence from sources other than the compound of interest. The use of Eq. [13] can be illustrated by the correction of the data in Fig. 2. Here $F_{blank} = 0$ and, since the primary absorption effect comes from the fluorophore itself, $F_{corr} = kL_T$, where k is a coefficient of proportionality.[3] Values of k, a, and d were determined for each excitation wavelength by nonlinear regression analysis of the data in Fig. 2. Having determined a set of a and d values in this way, the correction factor for any concentration and any excitation wavelength can be calculated. Provided that Eq. [7] is able to describe the primary-absorption effect accurately, the corrected data points should fall on the straight line in Fig. 2B. It is clear that this does in fact happen. The corrected data point which lies furthest from the theoretical line is that for 19 mM *N*-(*p*-aminobenzoyl)-L-glutamate excited at 320 nm; here the calculated absorbance was 5.78, and yet correction by Eq. [7] gives a value within 3.2% of theory.

Equation [7] is thus able to correct accurately even for very large primary absorption

[3] This assumption of linearity of fluorescence with fluorophore concentration may not be justified in some systems, if there is self-association of the fluorophore in the relevant concentration range.

effects. However, it is clear from Fig. 2 that it is experimentally much more satisfactory to choose an excitation wavelength at which a much less drastic correction is required (here 335 nm).

The practical application of this correction procedure to measurements of binding constants is best illustrated by examples. Two kinds of experiments can be distinguished, depending on whether changes in ligand or protein fluorescence are measured. In each case, the ligand is added in small aliquots to the protein solution and is the absorbing species which necessitates the correction for primary absorption effects; the procedure used for determining the correction factor depends on whether the ligand is also the fluorophore.

Changes in Ligand Fluorescence

Figure 3 shows the data from an experiment in which the binding of ϵNADP$^+$ to dihydrofolate reductase was measured by following the increase in fluorescence of the ethenoadenine fluorophore on binding (cf. (21)). Figure 3A shows the fluorescence as a function of ligand concentration for the ligand alone and in the presence of the enzyme. In the absence of any primary absorption effect, the fluorescence in the absence of enzyme will be linear with ligand concentration, and the data in Fig. 3A can be analyzed on this assumption, using Eq. [11] and [12], to obtain a value for the binding constant. The value obtained is 2.32 ($\pm$0.75) $\times$ 10^4 M^{-1}; the theoretical curve calculated with this value of K' is shown in Fig. 3A. Although the fit of this curve to the data is probably acceptable, it is not good. It is in fact clear that the fluorescence in the absence of enzyme is not simply a linear function of ligand concentration—there is a significant primary absorption effect. By fitting the data in the absence of enzyme to Eq. [13], the values of a and d can be calculated, allowing the appropriate correction to be applied to the data obtained in the presence of the enzyme. The corrected data are shown in Fig. 3B, together with the theoretical curve calculated using the "best fit" value of K', 5.83 ($\pm$0.10) $\times$ 10^3 M^{-1}. The corrected data are clearly in much better agreement with the theoretical

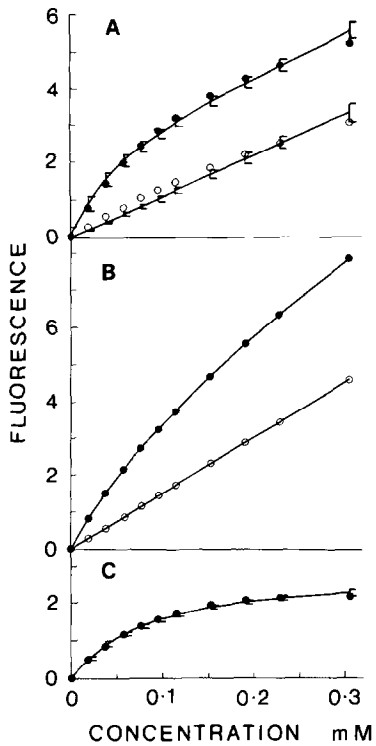

FIG. 3. Measurement of the binding of ϵNADP$^+$ to dihydrofolate reductase by using the increase in its fluorescence (excitation, 340 nm; emission, 400 nm) on binding. Solution conditions: 500 mM KCl, 50 mM citrate, pH 5.1. (A) Fluorescence in the absence (○) and presence (●) of the enzyme, uncorrected for inner-filter effects. The line through the data obtained in the presence of the enzyme is calculated for the "best fit" value of K', assuming *no* inner-filter effect. This assumption implies that the data in the absence of enzyme should be adequately described by a straight line. (B) Fluorescence in the absence (○) and presence (●) of the enzyme after correction for the inner-filter effect as described in the text (correction factors calculated by fitting the open circles in (A) to Eq. [13]). The line through the solid circles is calculated using the "best fit" value of K'; the open circles, having been corrected, are now adequately described by a straight line. (C) Data points derived by a point-by-point subtraction of data in the absence of enzyme from that in the presence of enzyme (open and closed circles of part A). The line is calculated using the "best fit" value of K'. In each case the brackets indicate the 95% confidence limits derived from the nonlinear regression analysis; in (B), the brackets are smaller than the symbols.

curve, and it is notable that the binding constant is not only much better determined but also differs by a factor of 4 from that estimated from the uncorrected data. Failure to take account of the primary absorption effect can thus lead to substantial systematic errors in the binding constant derived from fluorescence data.

One approach to the analysis of data such as this which has sometimes been used is to subtract, point by point, the fluorescence observed in the absence of enzyme from that observed in its presence. In the absence of any primary absorption effect, this procedure is of course correct (cf. Eq. [11]), but its application to uncorrected data will give erroneous results. (Absorption of the excitation beam produces the same *proportional* decrease in the fluorescence with and without enzyme, not the same *absolute* decrease.) Figure 3C shows data calculated by this subtraction procedure from that shown in Fig. 3A. The binding constant calculated by regression analysis is 1.29 (±0.1) $\times 10^4$ M^{-1}, a factor of 2.2 greater than that obtained by analysis of the properly corrected data.

Changes in Enzyme Fluorescence

When binding is followed by means of the quenching of the enzyme fluorescence accompanying complex formation, it is obviously important to correct for that part of the observed decrease in fluorescence which is due simply to the absorption of the excitation beam by the added ligand. When the ligand fluorescence is monitored, values of a and d in Eqs. [7] and [13] can be obtained from the measurements made in the absence of enzyme (see above). However, when the protein fluorescence is followed, a separate "calibration curve" must be obtained by measuring the effects of light absorption by the ligand on the fluorescence of a "standard" fluorophore. The only essential criteria for the choice of this standard are that it should fluoresce when excited at the wavelength to be used in the ligand-binding experiment and that it should not itself form a complex with the ligand(s) of interest. In view of the appreciable bandwidth often used for the excitation beam, it is also desirable that the standard should have an excitation spectrum of similar shape to that of the protein, and it is convenient if they have similar emission wavelengths. For these reasons, tryptophan has often been used as a standard in experiments of this kind. However, its photochemical lability is a significant disadvantage in this context, and we have sometimes found it more convenient to use *N*-(*p*-aminobenzoyl)-L-glutamate. Although this compound has an excitation spectrum which is less similar to that of the average protein, the same emission wavelength can be used and it is photochemically stable.

The procedure can be illustrated with reference to Fig. 4, which shows the quenching of the fluorescence of dihydrofolate reductase by folate. The uncorrected data, shown in Fig. 4B, can be fitted tolerably well by a theoretical binding curve with $K' = 3.45\ (\pm 0.4) \times 10^5$ M^{-1}. However, the decrease in fluorescence of the standard shown in Fig. 4A indicates that there is a significant primary absorption effect. In addition to the filter effect, the fluorescence of the standard (here tryptophan) will be decreased by simple dilution on addition of the ligand. The open circles in Fig. 4A show the standard fluorescence corrected for this dilution. These data can now be used to estimate the parameters a and d in the correction term by curve fitting using Eq. [13], but now with F_{corr} = constant (since the concentration of the standard is constant). The curve obtained with the optimum values of a and d is shown in Fig. 4A. The appropriate correction factors can then be calculated for each ligand concentration. The corrected enzyme fluorescence is shown in Fig. 4B, and the standard fluorescence in Fig. 4A; the latter data points are, as expected, scattered about a horizontal line. The corrected enzyme fluorescence gives a significantly better fit to the theoretical curve, with a value of $K' = 1.49\ (\pm 0.13) \times 10^5$ M^{-1}, a factor of 2.3 less than that obtained from the uncorrected data.

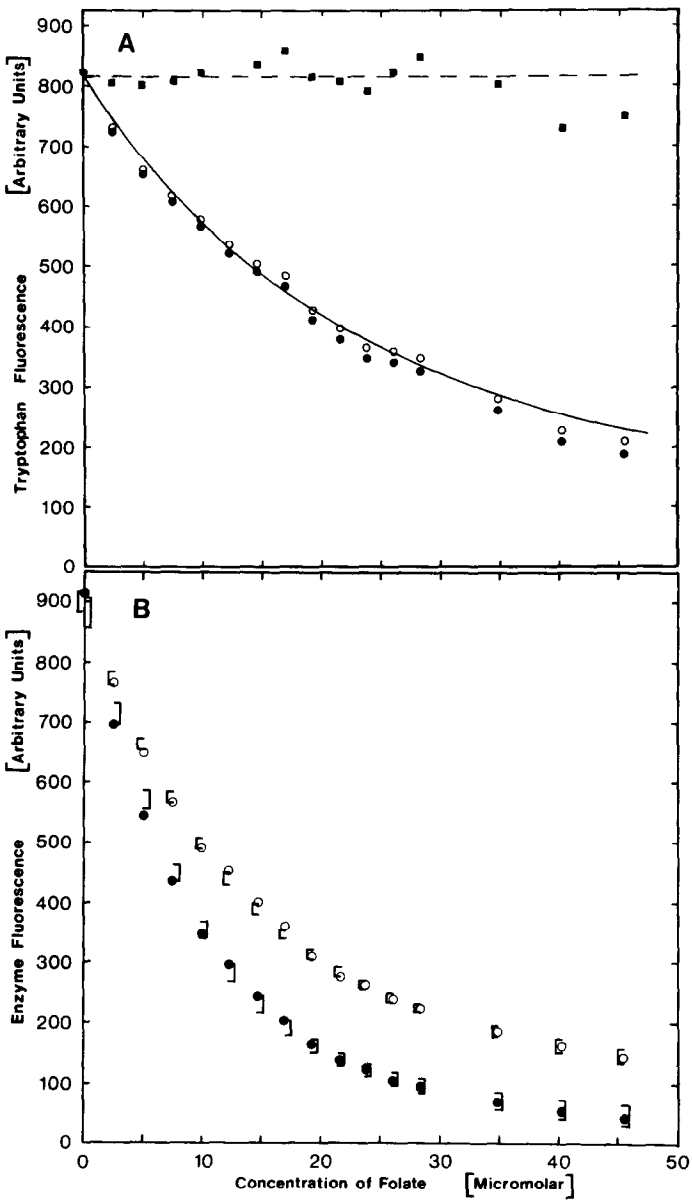

FIG. 4. The binding of folate to dihydrofolate reductase as measured by the quenching of the enzyme fluorescence (excitation, 290 nm; emission, 340 nm). (A) Standard (tryptophan) fluorescence, shown as raw data (●) and after correction for dilution (○). The solid line is the "best fit" to Eq. [13], and using the correction factors obtained from this fit the corrected data points (■) are seen to be scattered about the theoretical (dashed) line. (B) Enzyme fluoresence. Uncorrected (●) and corrected (○) data, with brackets indicating the 95% confidence limits from the nonlinear regression analysis.

CONCLUSIONS

The results presented here show that failure to correct properly for the primary absorption effect can lead to appreciable errors in the estimated equilibrium constant. These errors will tend to be greater in experiments involving enhancement of ligand fluorescence than in

those where the quenching of protein fluorescence is measured. In the former case, the largest primary absorption corrections must be applied to the largest signals, whereas in the latter case, if there is a substantial quenching, the large correction is applied to a small signal, and the absolute change in fluorescence due to the primary absorption effect is much less. The primary absorption effect can be minimized by proper choice of excitation wavelength, and if the absorbance is less than 0.2, any one of several equations can be used to calculate the correction factor from the extinction coefficient at the excitation wavelength. With larger absorption effects, the applicability of the assumptions involved in deriving the equations to the optical arrangement of the spectrometer must be considered. The simplest and most general solution is to use the semiempirical Eq. [7] in conjunction with "calibration curves."

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REFERENCES

1. Chen, R. L., and Edelhoch, H. (eds.) (1975–6) Biochemical Fluorescence: Concepts, Vols 1 and 2, Dekker, New York.
2. Udenfriend, S. (1969) Fluorescence Assay in Biology and Medicine, Vol. 2. Academic Press, New York.
3. Parker, C. A. (1968) Photoluminescence of Solutions, Elsevier, Amsterdam.
4. Guilbault, G. G. (1973) Practical Fluorescence, Dekker, New York.
5. Wehry, E. L. (ed.) (1976) Modern Fluorescence Spectroscopy, Vols. 1 and 2, Dekker, New York.
6. Miller J. N. (ed.) (1981) Standards in Fluorescence Spectrometry (UV Spectrometry Group), Chapman & Hall, London.
7. Parker C. A., and Rees, W. T. (1962) *Analyst* **87,** 83.
8. Stokes, G. G. (1852) *Phil. Trans.* **142,** 463.
9. Holland, J. F., Teets, R. E., Kelly, P. M., and Timnick, A. (1977) *Anal. Chem.* **49,** 706–710.
10. Lloyd, J. B. F. (1981) *In* Standards in Fluorescence Spectrometry (UV Spectrometry Group), (Miller, J. N., ed.), Chapman & Hall, London.
11. Ehrenberg, M., Cronvall, E., and Rigler, R. (1971) *FEBS Lett.* **18,** 199–203.
12. Brand, L., and Witholt, B. (1967) *in* Methods in Enzymology (Hirs, C. H. W., ed.), Vol. 11, pp. 776–856, Academic Press, New York.
13. Engel, G., Heider, H., Maelicke, A., von der Haar, F., and Cramer, F. (1972) *Eur. J. Biochem.* **29,** 257–262.
14. Helène, C., Brun, F., and Yaniv, M. (1971) *J. Mol. Biol.* **58,** 349–365.
15. Lamola, A. A., Eisinger, J., Blumberg, W. E., Kometani, T., and Burnham, B. F. (1977) *J. Lab. Clin. Med.* **89,** 881–890.
16. Mertens, M. L., and Kagi, J. H. R. (1979) *Anal. Biochem.* **96,** 448–455.
17. Chen, R. F. (1967) *Anal. Biochem.* **19,** 374–387.
18. Roberts, G. C. K. (1981) *In* Standards in Fluorescence Spectrometry (UV Spectrometry Group), (Miller, J. N., ed.), Chapman & Hall, London.
19. Dann, J. G., Ostler, G., Bjur R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V., and Harding, N. G. L. (1976) *Biochem. J.* **157,** 559–571.
20. Birdsall, B., Burgen, A. S. V., Rodrigues de Miranda, J., and Roberts, G. C. K. (1978) *Biochemistry* **17,** 2102–2110.
21. Birdsall, B., Burgen A. S. V., and Roberts, G. C. K. (1980) *Biochemistry* **19,** 3723–3731.
22. Batchelor, J. G. (1977) *Brit. J. Pharmacol.* **59,** 521P.
23. Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* **11,** 431.
24. Holbrook, J. J. (1972) *Biochem. J.* **128,** 921–931.
25. Chen, R. F., and Hayes, J. E. (1965) *Anal. Biochem.* **13,** 523–529.
26. Parker, C. A., and Barnes, W. J. (1957) *Analyst* **82,** 606–618.