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On the determination of the stoichiometry and equilibrium constants of weak complexations

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Abstract

In this Letter, we present a criticism of the use of the (non-linear) least-squares fitting methods, as well as the so-called double-reciprocal plots, for determination of the stoichiometry and equilibrium constants of reactions involving weak complex formations. We show that, contrary to what is generally believed, these methods do not always suggest the correct parameters of such reactions. In particular, differentiation between $1/1$ and $2/1$ stoichiometries is impossible in many cases. The reason is that, in these cases, the fitting equations corresponding to the $1/1$ and $2/1$ stoichiometries turn out to be equivalent. Additional independent experimental evidence is therefore necessary in order to obtain reliable values for the complexation parameters.

1. Introduction

Among weak complexation reactions and formation of inclusion compounds, the most frequently encountered situations in real systems involve $1/1$ and $2/1$ stoichiometries [1–11]. These non-covalent bindings between reactants A and B are described by Eq. (1) for the $1/1$ stoichiometry, and by the combination of Eq. (1) with Eq. (2) for the $2/1$. K_1 and K_2 stand for the corresponding equilibrium constants.

$$\mathrm{A} + \mathrm{B} \overset{K_1}{\rightleftharpoons} \mathrm{AB}, \tag{1}$$

$$\mathrm{AB} + \mathrm{A} \overset{K_2}{\rightleftharpoons} \mathrm{A_2B}. \tag{2}$$

The progress of such reactions can be monitored by means of the experimentally determined value of some spectroscopic variable P of one of the reactants, say B, according to Eq. (3) for the $1/1$ and Eq. (4) for the $2/1$ [11].

$$P = \frac{\left(P_\mathrm{f} + K_1 P_1[A]\right)}{\left(1 + K_1[A]\right)}, \tag{3}$$

$$P = \frac{\left(P_\mathrm{f} + K_1 P_1[A] + K_1 K_2 P_2[A]^2\right)}{\left(1 + K_1[A] + K_1 K_2[A]^2\right)}. \tag{4}$$

In Eqs. (3) and (4) P_f, P_1 and P_2 indicate the value of P, (P_f) when B is unbound (free), (P_1) when B is bound to A as AB and (P_2) when B partakes in the $2/1$ complex $\mathrm{A_2B}$. [A] stands for the molar concentration of species A. In this kind of study one keeps the concentration of reactant B constant and measures P as a function of added A. The experimental data can be subsequently used to fit them to the corresponding Eqs. (3) and (4), and thus determine the parameters K_1 and K_2. Note that P can be any

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convenient spectroscopic quantity depending on the nature of species B, e.g. fluorescence quantum yield (Φ), absorption coefficient (ε), chemical shift (δ), etc.

In the course our current studies on the formation of inclusion compounds between cyclodextrins (CD) and the fluorophore all-*trans*-1,6-diphenyl-1,3,5-hexatriene (DPH), we have found that in some cases either Eq. (3) or Eq. (4) could be equally well fitted to the experimental data, viz. Φ of DPH vs. [CD], the fits were equally good, indicating that the stoichiometry of the complexation reaction could be either 1/1 or 2/1. From independent experiments, however, such as fluorescence lifetime measurements, we were able to determine the correct, unique stoichiometry of the reaction. These findings prompted us to examine Eqs. (3) and (4), i.e. to investigate the conditions under which these two equations become equivalent. This would explain why fitting the different Eq. (3) and (4), to the same set of data produces equally good fittings.

2. Results and discussion

2.1. Mathematical treatment

Our purpose was to determine the conditions under which Eqs. (3) and (4) become equivalent. This means that if we write Eq. (3) as Eq. (5),

$$P = \frac{P_f + K_1 P_1[\mathrm{A}]}{1 + K_1[\mathrm{A}]} = \frac{L + M[\mathrm{A}]}{1 + N[\mathrm{A}]}, \tag{5}$$

where L, M and N are constants, then Eq. (4) will be equivalent with Eq. (5) if the right-hand sides of both equations are identical, viz.

$$\frac{L + M[\mathrm{A}]}{1 + N[\mathrm{A}]} = \frac{P_f + K_1 P_1[\mathrm{A}] + K_1 K_2 P_2[\mathrm{A}]^2}{1 + K_1[\mathrm{A}] + K_1 K_2[\mathrm{A}]^2}. \tag{6}$$

Using straightforward algebraic manipulations and separation of terms of equal powers of [A], the above identity (6) takes the following form:

$$\begin{aligned} L + (K_1 L + M)[\mathrm{A}] + K_1(LK_2 + M)[\mathrm{A}]^2 \\ + MK_1 K_2[\mathrm{A}]^3 = P_f + (K_1 P_1 + P_f N)[\mathrm{A}] \\ + K_1(K_2 P_2 + P_1 N)[\mathrm{A}]^2 + K_1 K_2 P_2 N[\mathrm{A}]^3. \end{aligned} \tag{7}$$

Setting identical the terms of equal powers of [A] in the above identity (7) we come up with Eq. (8) for L, M and N.

$$\begin{aligned} &L = P_f \text{ (from the identity of the coefficients of the zero} \\ &\text{powers of } [A] \text{ on both sides of (7))} \\ &M = P_2 N \text{ (from the identity of the} \\ &\text{coefficients of the third powers of } [A]) \\ &N = K_1(P_1 - P_f)/(P_2 - P_f) \text{ (from the identity of} \\ &\text{the coefficients of the first powers of } [A]). \end{aligned} \tag{8}$$

However, the identity (7) will be valid only if the coefficients of the second powers of [A] are also identical on both sides of (7), i.e.

$$K_1(LK_2 + M) = K_1(K_2 P_2 + P_1 N). \tag{9}$$

Finally, substituting L, M and N from Eq. (8) into Eq. (9) we obtain the interesting Eq. (10),

$$\frac{K_1}{K_2} = \frac{(P_2 - P_f)^2}{(P_1 - P_f)(P_2 - P_1)}, \tag{10}$$

which interrelates the experimental parameters K_1, K_2, P_f, P_1, P_2. Clearly, whenever Eq. (10) is satisfied Eqs. (3) and (4) are equivalent and therefore computer fitting of either model 1/1 (Eq. (3)), or model 2/1 (Eq. (4)), to the experimental data will give equally good fits. Consequently, any conclusion about the nature of a complexation reaction, viz., stoichiometry and values of parameters, is rendered unreliable.

Eq. (10) deserves further investigation. Thus, we firstly note that the value of the parameter P_f which corresponds to the unbound (free) species B, is independent of the complexation reaction; therefore this parameter can be always treated as a constant for any particular complex formation. Secondly, solving the quadratic Eq. (10) for P_1 or P_2, with P_f assumed constant, we find that P_1 and P_2 have positive real (non-imaginary) values only when $K_1/K_2 \geq 4$. The behavior of Eq. (10) is shown (Fig. 1) in the 3-D depiction of the surface generated by this equation for $P_f = 0.1$. Note that only the upper part of this surface represents realizable experimental situations, because only this part of the surface corre-

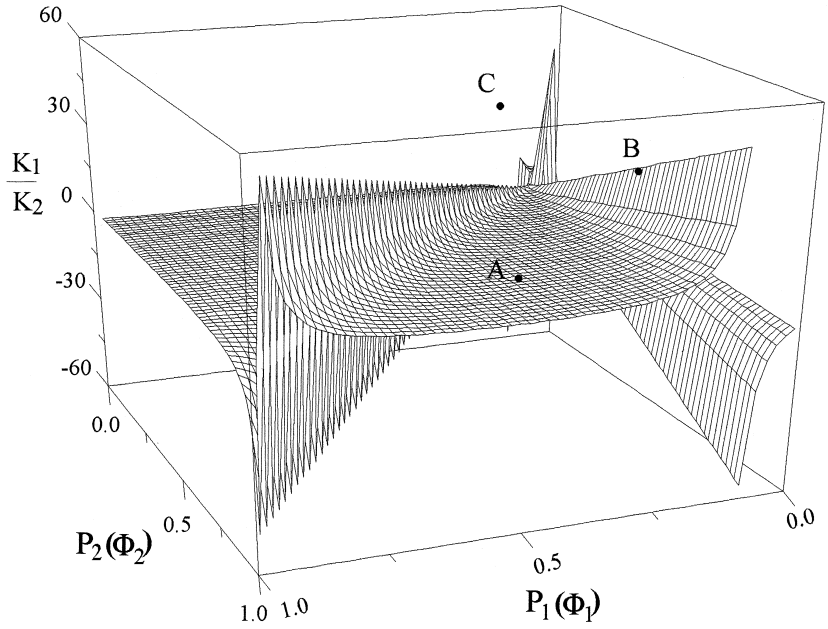

Fig. 1. 3-D graph of Eq. (10). The upper part of this surface corresponds to combinations of K_1/K_2, $P_1(\Phi_1)$ and $P_2(\Phi_2)$ which satisfy Eq. (10) and also to experimentally realizable situations, viz., positive and real values of the parameters (see text). Points A, B, C indicate the parameters, K_1/K_2, $P_1(\Phi_1)$ and $P_2(\Phi_2)$ for the corresponding synthetic data (see Fig. 2).

sponds to $K_1/K_2 \geq 4$. Thirdly, the algebraic expression of Eq. (10) is such that the part of the surface for $K_1/K_2 \geq 4$, corresponds to both cases, either $P_f > P_1 > P_2$, or $P_f < P_1 < P_2$. The former case is realized when addition of A reduces the magnitude of the parameter P, e.g. fluorescence quenching, while the latter when addition of A increases the magnitude of P.

2.2. Computer simulations

In the following we will show, by means of synthetic data, that whenever Eq. (10) is satisfied it is indeed impossible to differentiate between the $1/1$ and the $2/1$ models. To simplify matters, we will employ a specific example, viz., the case of complex formation between a cyclodextrin and DPH, while as P we will take the fluorescence quantum yield Φ of DPH. From our earlier studies [12], we know that when CD is added to a water/glycol solution of DPH the fluorescence quantum yield of the latter increases, therefore we have the case $\Phi_f < \Phi_1 < \Phi_2$ ($P_f < P_1 < P_2$ in the previous notation). Our synthetic data, which consists of calculated values of Φ at various CD concentrations, was produced by means of Eq. (4) corresponding to the $2/1$ model. In Eq. (4) we have replaced [A] by [CD] and P_f, P_1, P_2 by Φ_f, Φ_1, Φ_2 respectively, in all simulated data Φ_f was taken equal to 0.1. To this synthetic data, which corresponds to the $2/1$ model, we tried to fit both Eq. (3) for the $1/1$ and Eq. (4) for the $2/1$ model. For this reason we have chosen three examples corresponding to points A, B and C in Fig. 1. Points A and B lie on the upper part of the surface of Eq. (10), i.e. Eq. (10) is satisfied, and according to the previous discussion distinction between the two models $1/1$ and $2/1$ should be impossible. Point C, on the other hand, lies way above the surface, i.e. Eq. (10) is not satisfied, and therefore distinction between the two models should be feasible.

Inspection of Fig. 2, which shows the $1/1$ and $2/1$ computer fits to the synthetic data obtained using the $2/1$ model, confirms that for the data corresponding to points A and B, which satisfy Eq. (10), both Eqs. (3) and (4) can be equally well fitted (panels A and B in Fig. 2). This confirms that it is impossible to determine the correct stoichiometry

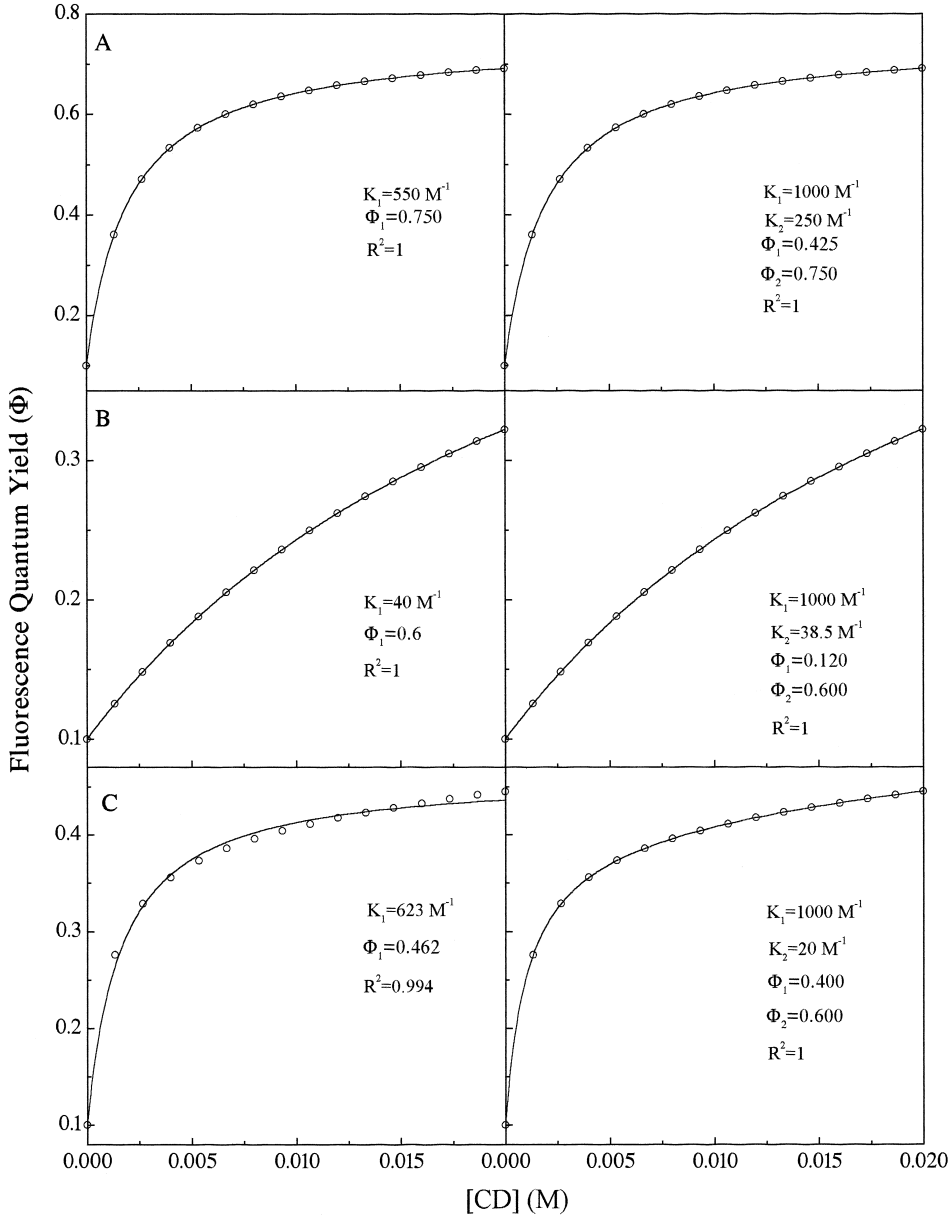

Fig. 2. Open circles are synthetic data obtained from Eq. (4) corresponding to model 2/1. Solid lines indicate computer fits to these synthetic data, by the 1/1 model (left panels) and the 2/1 model (right panels). The parameters used to obtain the synthetic data are: (A) $K_1/K_2 = 4$, $\Phi_1 = 0.425$, $\Phi_2 = 0.75$; (B) $K_1/K_2 = 26$, $\Phi_1 = 0.12$, $\Phi_2 = 0.6$; and (C) $K_1/K_2 = 4.16$, $\Phi_1 = 0.4$, $\Phi_2 = 0.6$, in all cases $\Phi_f = 0.1$. Parameters obtained from the fits, and values of R^2, are indicated on each panel.

when Eq. (10) is satisfied. On the contrary, the data corresponding to point C, which does not satisfy Eq. (10), can only be fitted by Eq. (4) (panels C in Fig. 2), therefore in such cases, viz., when Eq. (10) does not hold, the determination of the stoichiometry and of the equilibrium parameters is attainable.

Now a problem arises because one cannot determine whether Eq. (10) is satisfied unless one has

already determined the equilibrium parameters, viz., K's and Φ's. The only way to escape from this vicious circle is to try to determine the stoichiometry of the complexation reaction by other, non-computational, direct methods. In our studies, we were able to overcome the above difficulty and determine the stoichiometry of the complexation reactions between CD and DPH, by analyzing the fluorescence decay

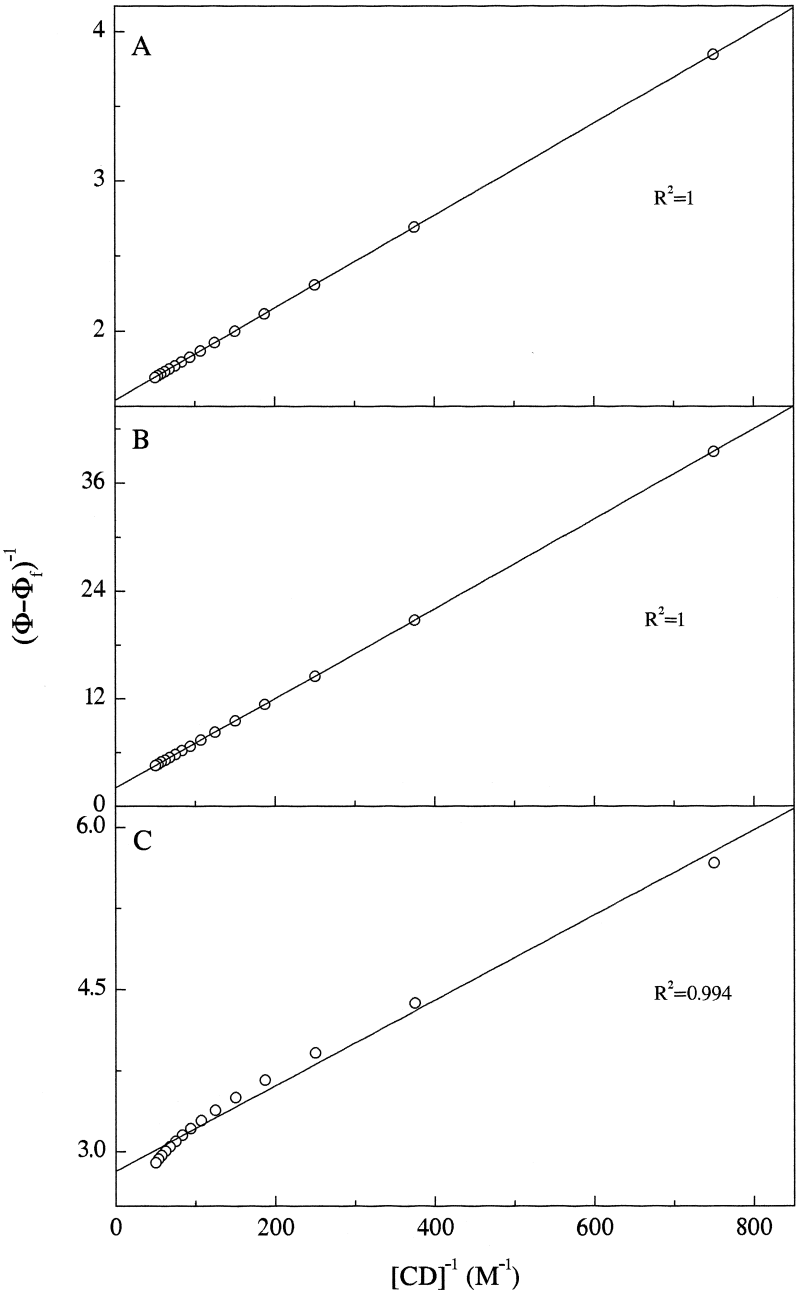

Fig. 3. Double-reciprocal plots of synthetic data A, B and C. The parameters used to obtain the synthetic data are the same as in the corresponding panels of Fig. 2.

of DPH at several cyclodextrin concentrations. In these experiments, when the stoichiometry is 1/1 only two lifetimes are observed, one due to the unbound DPH and the other due to the CD–DPH complex. Moreover, as the added [CD] is increased, and more CD–DPH is formed, the percentage of the DPH corresponding to the first lifetime decreases while the percentage of the second lifetime increases. If the stoichiometry is instead 2/1, then three fluorescence lifetimes are observed, viz., the previous two and that of the CD_2–DPH complex. Our results of these studies will be published elsewhere.

2.3. Double-reciprocal plots

It is customary instead of fitting Eqs. (3) and (4)) to the experimental data, to employ what is called double-reciprocal plot methods. In these methods, of which the Benesi–Hildebrand [13] is the oldest but also the most frequently used one, use is made of the linear equivalent of Eq. (3) which is Eq. (11) [14]:

$$\frac{1}{\Phi-\Phi_f}=\frac{1}{K_1(\Phi_1-\Phi_f)[\mathrm{CD}]}+\frac{1}{\Phi_1-\Phi_f}. \quad (11)$$

Evidently, plotting $1/(\Phi-\Phi_f)$ versus $1/[\mathrm{CD}]$, (or more generally $1/(P-P_f)$ versus $1/[\mathrm{A}]$), a straight line results if the stoichiometry of the reaction is 1/1, otherwise the experimental points deviate from linearity. Moreover, if the stoichiometry is 1/1, the slope of the straight line is equal to $1/K_1(\Phi_1-\Phi_f)$, while its intercept with the axis of $1/(\Phi-\Phi_f)$ is $1/(\Phi_1-\Phi_f)$ and it is therefore possible to determine the equilibrium constant K_1. There are numerous occasions in the recent literature where such convenient double-reciprocal plots have been used [3,4,6,7,9–11,14–16]. In view of our previous discussion, it is expected that the methods of these double-reciprocal plots should also break down when Eq. (10) is satisfied. This is true as demonstrated in Fig. 3 which shows the double-reciprocal plots corresponding to the synthetic data of Fig. 2. Thus, the simulated data of points A and B, although they were obtained by means of Eq. (4) of the 2/1 model, nevertheless produce perfectly straight lines, erroneously suggesting 1/1 stoichiometry. The reason is that in these cases Eq. (10) is satisfied. This is not, however, the case with the simulated data of point C, for which Eq. (10) is not satisfied. In this case, the non-linearity of the double-reciprocal plot clearly, and correctly, suggests a stoichiometry different from 1/1. It should be emphasized here that in the present discussion we have dealt with the theoretical aspects of the method of the double-reciprocal plots. In real experimental situations, however, where usually only few points are measured in a rather restricted concentration range and where experimental uncertainties are not negligible, deviation from linearity of double-reciprocal plots is a rather doubtful criterion, which nevertheless is used in the literature very often [3,4,6,7,9–11,14–16].

Finally, it should be mentioned that contrary to the 2/1 stoichiometry, equations corresponding to the 2/2 stoichiometry cannot be reduced either to the equations for 1/1 or 2/1 stoichiometries, because the latter two do not depend on the concentration of the second reactant, viz., [B] (see Eqs. (3) and (4)), while equations for the 2/2 stoichiometry do depend on [B]. As for higher stoichiometries, e.g. 2/3, 3/3, etc., these are very unusual and therefore they have no practical interest.

3. Conclusions

The important conclusion of this Letter is that, in weak complexation reactions differentiation between 1/1 and 2/1 stoichiometries, by means of computer fitting methods or double-reciprocal plot techniques, is not always possible. More specifically, when Eq. (10) is satisfied one cannot distinguish, by the above methods, whether the reaction is a 1/1 or a 2/1 process. Moreover, since confirmation of the validity of Eq. (10) for a particular reaction requires previous knowledge of all the parameters involved in Eq. (10), i.e. requires complete knowledge of the nature of the reaction, it is impossible to know in advance whether or not Eq. (10) is satisfied and therefore whether or not differentiation between the two stoichiometries, 1/1 and 2/1, is feasible. Consequently, it is imperative that direct methods, e.g. determination of fluorescence lifetimes, be employed in order to determine the stoichiometry unambiguously. Simi-

lar confusion between 2/2 and 1/1 or 2/1 stoichiometries does not occur.

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