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Limitations on the determination of the stoichiometry and equilibrium constants of weak complexes by computer fitting methods: experimental verification

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Abstract

We present experimental evidence showing that computer fitting methods are inadequate for the study of weak complexations, whenever a certain equation relates the reaction parameters. In such cases, one must resort to direct (non-computational) methods for the determination of the stoichiometry of the interaction and the equilibrium constants. Our data refer to complex formation between cyclodextrins and diphenylpolyenes, and we show that differentiation between the 1/1 and 2/1 stoichiometries, by computer fitting, is not always possible. The direct method employed here to determine the correct interaction parameters was primarily time-resolved fluorescence spectroscopy.

1. Introduction

In a recent report [1], we discussed the ambiguities inherent in the determination by means of computer fittings of the stoichiometry of weak complexations and inclusion compound formations, when a certain relationship exists among the reaction parameters. Here we present experimental verification of the conclusions of the aforementioned previous work [1]. More specifically, we will discuss the inclusion compound formation between cyclodextrins (CD) and fluorophores of the type all-*trans*-α,ω-diphenylpolyenes (DPP).

In brief, the findings of our previous work are the following. Let Eq. (1) describe the

$$\mathrm{CD{-}DPP} + \mathrm{CD} \overset{K_1}{\rightleftharpoons} \mathrm{CD{-}DPP} \qquad (1)$$

1/1 stoichiometry of a complexation reaction between CD and the fluorophore DPP, with an equilibrium constant K_1 and the product is the complex CD–DPP. The experimental variable in this case is the overall fluorescence quantum yield Φ_{exp}, measured during the course of the complexation, i.e., during the addition of CD to the solution of DPP, while keeping the [DPP] constant. The calculated magnitude of Φ, on the other hand, is expressed by Eq. (2) [2]. In

$$\Phi = \frac{(\Phi_{\mathrm{f}} + K_1\Phi_1[\mathrm{CD}])}{(1 + K_1[\mathrm{CD}])} \qquad (2)$$

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this equation, Φ_f is the fluorescence quantum yield of the free DPP, i.e., before the fluorophore is associated with CD, and its value can be measured in a solution of DPP in the pure solvent. Φ_1 is the quantum yield of what we call the 1/1 complex, CD–DPP. Evidently, by computer fitting Eq. (2) to the experimental data Φ_{exp}, one can determine the parameters involved in the complexation reaction, viz. K_1 and Φ_1.

If the reaction (1) is followed by reaction (3), we have the formation of the 2/1

$$\text{CD–DPP} + \text{CD} \overset{K_2}{\rightleftharpoons} \text{CD}_2\text{–DPP} \tag{3}$$

complex, CD_2–DPP, the equilibrium constant is K_2. In a procedure similar to that used for the derivation of Eq. (2) [2], we have obtained, from Eqs. (1) and (3), Eq. (4). In this equation, Φ_2 is the fluorescence quantum yield of the 2/1 complex CD_2–DPP while all the symbols

$$\Phi = \frac{\left(\Phi_f + K_1\Phi_1\Phi_1[\text{CD}] + K_1K_2\Phi_2[\text{CD}]^2\right)}{\left(1 + K_1[\text{CD}] + K_1K_2[\text{CD}]^2\right)} \tag{4}$$

have the previously assigned meanings. Again by fitting Eq. (4) to the experimental data, one can determine the reaction parameters K_1, K_2, Φ_1 and Φ_2 and therefore establish the nature of the complexation interaction. The main point of our studies in Ref. [1] was that whenever Eq. (5)

$$\frac{K_1}{K_2} = \frac{(\Phi_2 - \Phi_f)^2}{(\Phi_1 - \Phi_f)(\Phi_2 - \Phi_1)} \tag{5}$$

is satisfied, Eqs. (2) and (4) become identical and it is therefore impossible to distinguish the 1/1 and 2/1 complexation reactions by computer fitting. In other words, by fitting either Eq. (2) or Eq. (4) to the experimental data, one obtains an equally good fit and cannot differentiate between the two reaction models, viz. 1/1 or 2/1.

Similarly, it was shown [1] that neither the method of the so-called Benessi–Hildebrand double reciprocal plot [3], i.e., $1/(\Phi - \Phi_f)$ versus $1/[\text{CD}]$ [4], can distinguish between the 1/1 and 2/1 stoichiometries. Indeed, the equations corresponding to the double reciprocal plots are Eqs. (6) and (7) below, for the 1/1 and 2/1 stoichiometries, respectively, but these two

$$\frac{1}{(\Phi - \Phi_f)} = \frac{1}{K_1(\Phi_1 - \Phi_f)[\text{CD}]} + \frac{1}{(\Phi_1 - \Phi_f)} \tag{6}$$

$$\frac{1}{(\Phi - \Phi_f)} = \frac{\left(1 + K_1[\text{CD}] + K_1K_2[\text{CD}]^2\right)}{\left[K_1(\Phi_1 - \Phi_f)[\text{CD}] + K_1K_2(\Phi_2 - \Phi_f)[\text{CD}]^2\right]} \tag{7}$$

equations also become identical whenever Eq. (5) is satisfied [1]. Therefore when the Benessi–Hildebrand plot produces a straight line the stoichiometry may be either 1/1 or 2/1. If, however, the plot is not linear then the stoichiometry cannot be 1/1.

An additional problem arises from the fact that in order to examine whether Eq. (5) is satisfied, the parameters K_1, K_2, Φ_1 and Φ_2 must be known, which means that the stoichiometry of the complexation reaction must have been first determined. Evidently, a vicious circle. Therefore, in real experiments the following procedure is recommended. Both Eqs. (2) and (4) are fitted to the experimental data. If the goodness of the fit is clearly better in the one fit than in the other, then the equation of the good fit, either Eq. (2) or Eq. (4), and consequently the corresponding stoichiometry 1/1 or 2/1, are assigned to the complexation. Similar ambiguities arise when a double reciprocal plot, according to Eq. (6), produces a straight line. This does not necessarily mean that the stoichiometry is 1/1, it can very well be 2/1, if Eq. (5) is satisfied. In such situations, when both fits are good, or double reciprocal plots are inconclusive, other direct experimental methods must be employed in order to determine the correct stoichiometry of the reaction. For example, when the reactants and products fluoresce, alternative methods may include fluorescence parameters, as the ones employed in the present study. It should be emphasized at this point that the majority of complexation reactions and inclusion compound formations involve 1/1 or 2/1 mechanisms [5–13], it is therefore imperative that one takes into account the conclusions of this article and follow the procedure suggested here. Note that the ambiguity arising between

the 1/1 and 2/1 stoichiometries does not arise with other stoichiometries, e.g., 2/2, 2/3, etc. [1]. Presently we report on three different complexations between CD and DPP. Each case represents a different situation where the computer fitting clearly points to one stoichiometry (either 1/1 or 2/1), or the results are ambiguous, and therefore additional experimental evidence is required.

2. Experimental

The origin and purity of all materials, viz. α- and β-cyclodextrin (α-CD, β-CD), permethylated-β-cyclodextrin (PMβ-CD), all-*trans*-1,6-diphenyl-1,3,5-hexatriene (DPH) and all-*trans*-1,4-diphenyl-1,3-butadiene (DPB), as well as the methods employed, have been discussed in previous publications [8,14]. Since diphenylpolyenes are practically insoluble in pure water, but are soluble in quite a few solvents made up of water and some other water miscible compound, all experiments were performed in a 60:40 water/1,2-ethanediol mixed solvent (hereafter referred simply as 'mixed solvent') which solubilized adequate amounts of DPP. The complexations between DPP and CD were monitored through the fluorescence intensity of DPP, by adding, in a titration-like fashion, the cyclodextrin to the diphenylpolyene dissolved in the mixed solvent, while keeping the concentration of the fluorophore (DPP) constant, viz. 1.3×10^{-6} M for DPB and 8.0×10^{-8} M for DPH. The molecular volumes (V_{mol}) needed in order to calculate the rotational correlation times of the various molecules and then their fluorescence anisotropies (see Eq. (8)) were either obtained from crystallographic data or estimated from the molar volumes of their constituents. Thus, V_{mol}(DPH) = 235 cm^3 and V_{mol}(DPB) = 208.4 were estimated from critical tables, while V_{mol}(α-CD) = 761 [15], V_{mol}(β-CD) = 944.5 [16] and V_{mol}(PMβ-CD) = 1361 [17] cm^3 were taken from crystallographic data. The viscosity of the mixed solvent, equal to 3 cP, was found from critical tables. Finally, note that the experimental data for β-CD do not extend beyond [β-CD] ≅ 0.01 M, because of the low solubility of this particular cyclodextrin in the mixed solvent.

3. Results and discussion

Our data are comprised of fluorescence quantum yields (Φ), fluorescence anisotropies (r) and fluorescence lifetimes (τ) versus [CD], for the systems DPH/PMβ-CD, DPB/α-CD and DPB/β-CD all dissolved in the mixed solvent. Each case represents a different situation where the computer fitting clearly points to a 2/1 stoichiometry (case of DPB/α-CD), or the results are ambiguous, either 1/1 or 2/1 (cases of DPH/PMβ-CD and DPB/β-CD). The direct experimental methods employed to distinguish between 1/1 and 2/1 in the latter two complexations were the analysis of the fluorescence decay and of the fluorescence anisotropy of DPP.

3.1. DPH / PMβ-CD

The non-linear fits of Eqs. (2) and (4) to the experimental data produced equally good fittings

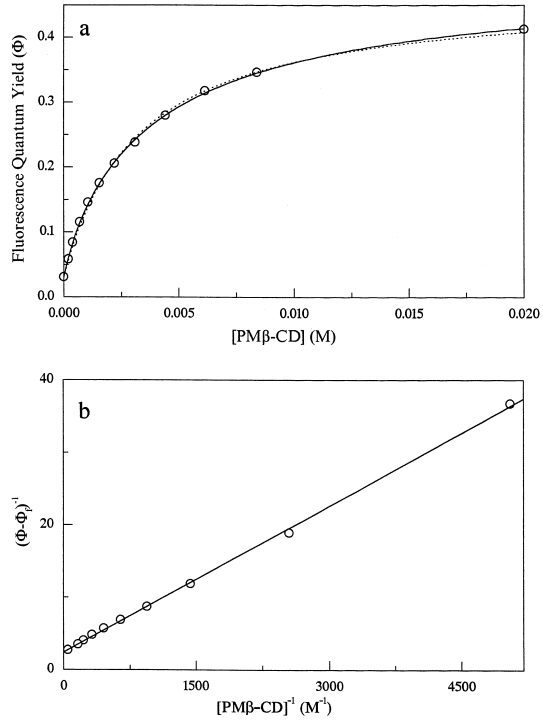

Fig. 1. DPH/PMβ-CD system. (a) Non-linear fits of Eqs. (2) and (4) to the experimental data; dotted line 1/1 stoichiometry, i.e., Eq. (2); solid line 2/1 stoichiometry, i.e., Eq. (4). (b) Double reciprocal plot according to Eq. (6).

Table 1
Experimental equilibrium parameters

System	Stoich.	K_1 (M^{-1})	K_2 (M^{-1})	K_1/K_2	Φ_f	Φ_1	Φ_2	R^a	Eq. (12)	Double Recip. plot
DPH/PMβ	2/1	1180	214	5.5	0.031	0.0165	0.490	4.8	s[b]	$R^2 = 0.9996$
DPB/α-CD	2/1	211	21	10	0.011	0.086	0.254	45	n-s	$R^2 = 0.9848$
DPB/β-CD	1/1	508	-	-	0.011	0.034	-	-	-	$R^2$0.9996

[a] $R = (\Phi_2\text{-}\Phi_f)^2/[(\Phi_2\text{-}\Phi_1)(\Phi_1\text{-}\Phi_f)]$.
[b] Satisfied; non-satisfied.

shown in Fig. 1a, with parameters listed in Table 1. Similarly, the double reciprocal plot $1/(\Phi - \Phi_f)$ versus $1/[CD]$ gave the straight line of Fig. 1b with $R^2 = 0.9996$. In view of the previous discussion, these results do not distinguish between the 1/1 and 2/1 stoichiometries, therefore we must use additional experimental evidence in order to determine the stoichiometry of this complexation. The alternative experimental method employed here to determine directly (no computer fitting) the stoichiometry of the complexation was the analysis of the fluorescence decay. From these analyses, we found three fluorescence lifetimes equal to 0.355, 2.3 and 6.1 ns, and also the relative fluorescence percentages of the corresponding three species present in the solution at various concentrations of added CD (see Table 2). The data in Table 2 show that upon addition of PMβ–CD the percentage of the species with $\tau =$ 0.355 ns, is diminished from 100% to 3%, the percentage of the species with $\tau = 2.3$ ns first rises up to 47% and then drops to ca. 24%, while the percentage of the species with $\tau = 6.1$ ns rises continuously from 0 up to 73%. These results clearly suggest that initially the 1/1 complex, (PMβ-CD)–DPH, is formed with $\tau_1 = 2.3$ ns, at the expense of the free DPH, with $\tau_f = 0.355$ ns. Upon addition of more cyclodextrin, (PMβ-CD)–DPH is changed to the 2/1 complex $(PM\beta\text{-}CD)_2$–DPH with $\tau_2 = 6.1$ ns. Thus, the lifetime measurements have definitely proved the presence of two different species in the reaction solution, plus of course the free DPH. Therefore we adopted the 2/1 stoichiometry and the parameters K_1, K_2, Φ_1 and Φ_2 obtained from the corresponding fitting (Fig. 1a and Table 1). Examination of Table 1 shows that for the system DPH/PMβ-CD the left side of Eq. (5), is $K_1/K_2 =$ 5.5, while the right side of Eq. (5), symbolized as R in Table 1, is $R = 4.8$. Considering the experimental errors involved in the measurements, it is clear that the two numbers, 5.5 and 4.8, are close enough to confirm the validity of Eq. (5).

To further confirm that the stoichiometry is indeed 2/1 we have measured the fluorescence anisotropy r of the solution at various CD concentrations. The fluorescence anisotropy r of a solution containing n fluorescent species is given by the generalized

Table 2
Percentages of free DPH (τ_f), (PMβ-CD)–(DPH) (τ_{11}), $(PM\beta\text{-}CD)_2$–(DPH) (τ_{21}) as a function of the concentration of the added cyclodextrin

[PMβ–CD](M)	$\tau_f = 0.355$(ns, %)	$\tau_{11} = 2.30$(ns, %)	$\tau_{21} = 6.10$(ns, %)
0	100	0	0
2.37×10^{-4}	42	44	14
6.28×10^{-4}	25	50	25
1.34×10^{-3}	15	47	38
2.64×10^{-3}	10	42	48
4.75×10^{-3}	7	37	56
7.0×10^{-3}	4	28	68
1.0×10^{-2}	3	24	73

form of the Perrin equation, Eq. (8), in which r_o is the maximum anisotropy measured in a frozen

$$r = \chi_f \phi_f r_f + r_0 \sum_{ij} \frac{\chi_{ij}\phi_{ij}}{1 + \left[\tau_{ij}/\tau(\text{corr})_{ij}\right]} \qquad (8)$$

glass forming mixture ($r_o = 0.38$ for DPB and DPH) [18], $\tau(\text{corr})_{ij}$ stands for the rotational correlation time, χ_{ij} for the molar fraction and φ_{ij} for the ratio of the fluorescence quantum yield of species *ij* (e.g., CD_i–DPP_j) to the total fluorescence quantum yield [14]. (Note that since the complexes dealt with here have $j = 1$, in the text we have dropped subscript j and we symbolize K_{21} as K_2, Φ_{11} as Φ_1, τ_{21} as τ_2, χ_{ij} as χ_i, etc.) r_f is the fluorescence anisotropy of the free polyene in the mixed solvent, before the addition of any CD, and it was found equal to 0.306 for DPB and 0.15 for DPH. The rotational correlation time is calculated from the Einstein equation $\tau(\text{corr}) = \eta V/RT$ where η is the viscosity of the medium, V is the molar volume of the emitting species, R is the universal constant of the gases and T is the absolute temperature. Evidently, if the molar volume of the cyclodextrin is known from X-ray crystallography, and the viscosity of the solvent from the critical tables, one can calculate $\tau(\text{corr})$ assuming that the contribution of the polyene molecule to the molar volume of CD–DPP and CD_2–DPP is negligible, due to the fact that this molecule is practically buried inside the cavities of the CDs. On the other hand, the molar fraction χ_i of each species can be expressed in terms of the equilibrium constants ($\chi_i = [(\text{CD})_i - (\text{DPP})]/[\text{DPP}]_0$, where $[\text{DPP}]_0$ is the total polyene concentration), therefore using the parameters K_1, K_2, Φ_1 and Φ_2, extracted from the Φ versus [CD] fitting for the 2/1 stoichiometry (Fig. 1a, Table 1), we can calculate χ_i for each species present in the solution (see Fig. 2). Employing then these molar fractions, the fluorescence lifetimes known from the decay analysis, and the rotational correlation times calculated as described above, we have simulated by means of Eq. (8) the r versus [CD] curve, for the 2/1 stoichiometry. The simulated curve shown in the upper part of Fig. 2 indeed confirms the 2/1 stoichiometry of this complexation reaction. On the contrary, the r versus [CD] simulation corresponding to the 1/1 stoichiometry (dotted line in Fig. 2) does not agree at all with the experimental data.

3.2. DPB / α-CD

The non-linear fits of Eqs. (2) and (4) to the experimental data are shown in Fig. 3a and suggest that the stoichiometry is clearly 2/1, since only the fit of Eq. (4) is good, while that of Eq. (2) is unacceptable. The parameters extracted from the good fit are listed in Table 1. Similarly, the $1/(\Phi - \Phi_f)$ versus 1/[α-CD] double reciprocal plot shown in Fig. 3b excludes the possibility that this stoichiometry could be 1/1 since the plot is far from linear. The 2/1 stoichiometry of the DPB/α-CD

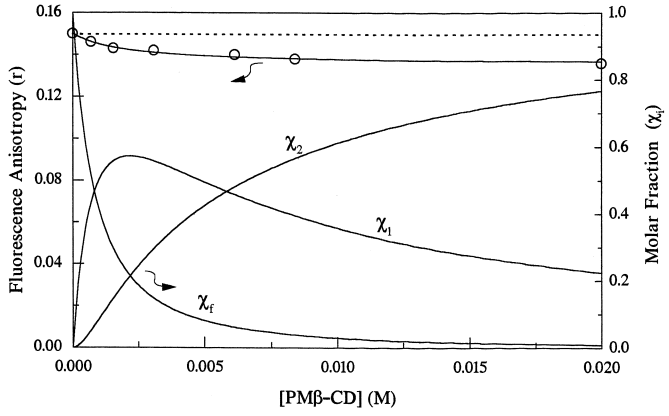

Fig. 2. Simulated molar fraction data χ [χ_f for free DPH; χ_1 for complex $(PM\beta\text{-}CD)_1$–$(DPH)_1$; χ_2 for complex $(PM\beta\text{-}CD)_2$–$(DPH)_1$] versus [PMβ-CD]. Upper part: Fluorescence anisotropy r versus [PMβ-CD]; solid line 2/1 stoichiometry, dotted line 1/1 stoichiometry, simulated according to Eq. (8) with parameters taken from Table 1; open circles, experimental data.

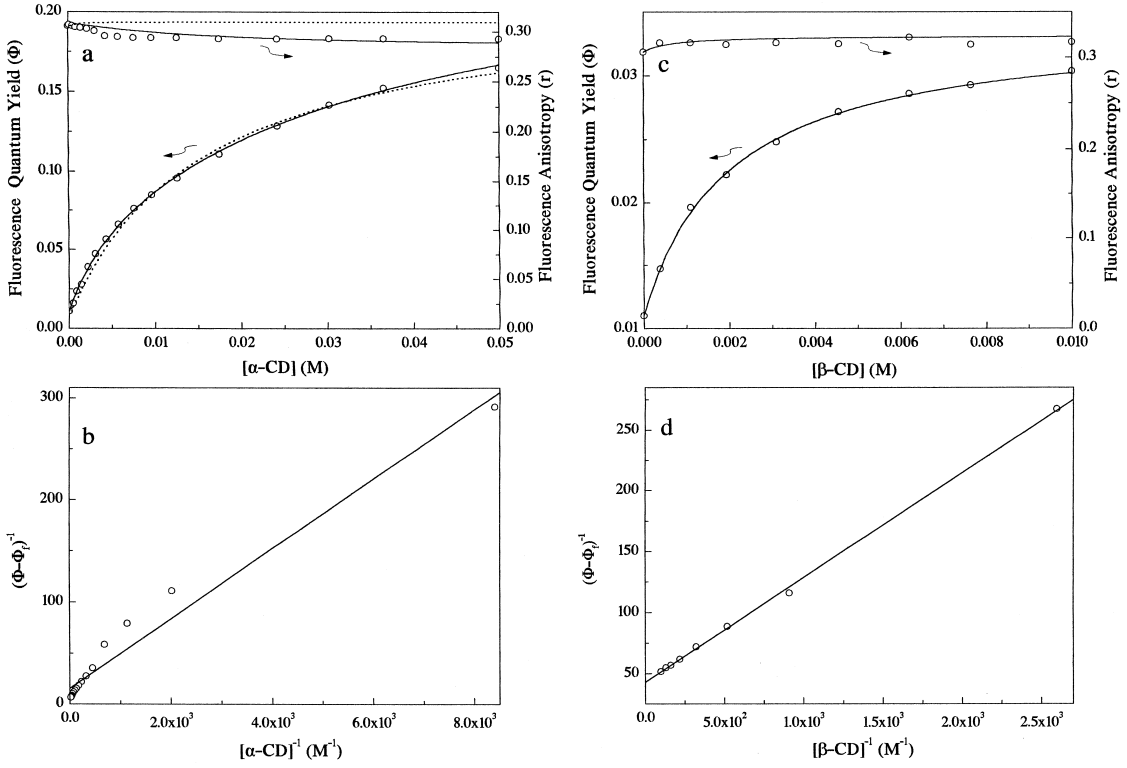

Fig. 3. Left: DPB/α-CD system. (a) Non-linear fits of Eqs. (2) and (4) to the experimental data; dotted line 1/1 stoichiometry, i.e., Eq. (2); solid line 2/1 stoichiometry, i.e., Eq. (4). Upper part: Plot of r versus [CD]; simulations according to Eq. (8) with parameters taken from Table 1, solid line for 2/1 stoichiometry, dotted line for 1/1. (b) Double reciprocal plot according to Eq. (6). Right: DPB/β-CD system. (c) Non-linear fits of Eqs. (2) and (4) to the experimental data, the two fits coincide. Upper part: open circles, experimental r values of DPB versus [β-CD]; solid line, simulated curve of r versus [β-CD] according to Eq. (8) with parameters taken from Table 1. (d) Double reciprocal plot according to Eq. (6).

system was further confirmed by the fluorescence decay curve analysis, which gave three different lifetimes, viz. $\tau_f = 0.08$ ns for the free DPB, $\tau_1 = 0.21$ ns for the complex (PMβ-CD)–DPB and $\tau_2 = 0.64$ ns for $(PM\beta\text{-CD})_2$–DPB. Finally, corroborative evidence favoring the 2/1 stoichiometry was obtained, as described previously, from the simulation of the r versus [CD] curve which is shown in the upper part of Fig. 3a. Note that the r versus [CD] variation simulated on the basis of 1/1 stoichiometry (dotted line) does not agree at all with the experimental data, further confirming the 2/1 assignment. Finally, the experimental data for DPB/α-CD, shown in Table 1, clearly demonstrate that Eq. (5) is not at all satisfied in this case. Indeed, while the left-hand side of Eq. (5) has the experimental value $K_1/K_2 = 10$, the right-hand side has the value $R = 45$.

3.3. DPB / β-CD

In this case, both Eqs. (2) and (4) gave good and identical non-linear fits as shown in Fig. 3c, while the double reciprocal plot according to Eq. (5) (see Fig. 3d) was found to be linear ($R^2 = 0.9996$). In view of our previous discussion, it was impossible to determine the stoichiometry with only this information; additional evidence was required. It was provided again by the analysis of the fluorescence decay, which gave only two different lifetimes, one for the free DPB $\tau_f = 0.08$ ns and one for the (β-CD)–DPB complex, $\tau_1 = 0.19$ ns, across the entire cyclodextrin concentration range, thus suggesting a

simple 1/1 stoichiometry. Furthermore, using the equilibrium parameters obtained from the 1/1 nonlinear fit, the lifetimes and the corresponding rotational correlation times we have confirmed the 1/1 stoichiometry by means of the r versus [β-CD] simulations shown in the upper part of Fig. 3c. Note that in this case Eq. (5) is meaningless, since the parameters K_2 and Φ_2 do not exist.

4. Conclusions

The important conclusion of this work is that whenever Eq. (5) is satisfied, computer fits to the experimental data of the equations corresponding to the 1/1 and 2/1 stoichiometries, viz. Eqs. (2) and (4), do not always suggest the correct stoichiometry of weak complexation reactions or inclusion compound formations. The same is true also for double reciprocal plots according to Eq. (6), where the linearity of the ensuing curve does not constitute unequivocal proof of 1/1 stoichiometry. Unfortunately, the validity of Eq. (5) cannot be examined unless the stoichiometry of the interaction and the equilibrium parameters are known. Therefore, in practice, one must fit Eqs. (2) and (4) to the data, and if only one of these fits is acceptable is the correct stoichiometry the one corresponding to this good fit. Alternatively, one can plot the data in the double reciprocal plot fashion, viz. according to Eq. (6), and if this plot is not linear it is concluded that the stoichiometry is other than 1/1. Otherwise, i.e., if either the plot of the data according to Eq. (6) is linear or both Eqs. (2) and (4) give good fits to the experimental points, then the stoichiometry cannot be determined by computer fitting alone and other direct experimental evidence is required.

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