Determination of Association Constants in Cyclodextrin/Drug Complexation Using the Scatchard Plot: Application to β-Cyclodextrin-Anilinonaphthalenesulfonates

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The appropriate Scatchard equation was developed for a system involving the formation of 1:1 and 1:2 substrate:cyclodextrin complexes. Simulation of this system was performed under the most common experimental conditions encountered in this type of study. The use of the equation allows for nonlinear least-squares estimation of the association constants. The interaction of the model compounds 1-anilino-8-naphthalenesulfonate (1,8-ANS) and 2(*p*-toluidinyl)-6-naphthalenesulfonate (2,6-TNS) with β-cyclodextrin (β-CD) was used to evaluate the theoretical model. Binding experiments were performed using either potentiometric titration or fluorimetric detection. The experimental data for 1,8-ANS/β-CD fit well to the 1:1 binding model, with an association constant of 87 ± 1 M^{-1}. The association constants of the 1:1 and 1:2 2,6-TNS/β-CD complexes utilizing direct potentiometry were 3737 ± 6 and 149 ± 2 M^{-1}. It is shown that fluorimetry can give biased estimates for the association constants of the complexation 2,6-TNS/β-CD, since the assumption of an equivalent quantum yield of bound species is not valid.

KEY WORDS: cyclodextrins; complexation; Scatchard plot; double-reciprocal plot; fluorescence; ion-selective electrodes; anilinonaphthalenesulfonates.

INTRODUCTION

The formation of inclusion complexes between cyclodextrins and drugs may increase drug solubility or yield a preparation with more desirable biopharmaceutical properties.

The complexation of cyclodextrins with drugs is most frequently studied using the phase-solubility technique (1) and mathematical models for the estimation of the apparent association constant(s) and complex stoichiometry. Both 1:1 and 1:1 plus 1:2 (drug:cyclodextrin) complex systems have been examined (2,3). Potentiometric, spectrophotometric, and competitive indicator spectrophotometric methods have also been used to determine the association constants of one substrate (drug) molecule to two cyclodextrin molecules (4–6). However, substantial relative errors for the association constants were found (7). Armstrong *et al.* (8) and Spino and Armstrong (7) developed a liquid chromatographic technique for the determination of stoichiometry and all relevant association constants for substrate–cyclodextrin systems. This method is based on retention equations which relate the retention of a solute to its binding constant(s) to cyclodextrin in the mobile phase.

Recently, cyclodextrin/drug complexation has been studied as a "binding phenomenon" using fluorimetric (9) and potentiometric (ion-selective electrodes; ISEs) (10–12) techniques. In this context, the Scatchard plot (10–12) and the double-reciprocal plot (9) have been utilized for calculation of the complexation constant for systems with a stoichiometric ratio of 1:1. The linearity of these plots indicates a 1:1 complexation; nonlinear double-reciprocal plots were observed (9) with species that form 1:1 as well as 1:2 complexes. However, mathematical models for the analysis of these nonlinear plots have not been described.

In the present study, the appropriate Scatchard equation is derived by using a model in which both successive equilibria (1:1 plus 1:2 complex system) are considered simultaneously. This equation was further used to analyze the interaction of the model substrates, 1-anilino-8-naphthalenesulfonate (1,8-ANS) and 2-(*p*-toluidinyl)-6-naphthalenesulfonate (2,6-TNS) with β-cyclodextrin (β-CD). These two anilinonaphthalenesulfonates exhibit 1:1 and 1:1 plus 1:2 complexation behavior, respectively (9,13). Both fluorimetric (monitoring the formed fluorescent complexes) and potentiometric assays (monitoring the free ion of the substrate using ISEs) were performed in order to evaluate the analytical power of the two techniques in analyzing complexation phenomena involving multiple equilibria. The necessary ISEs were constructed in our laboratory, the 2,6-TNS ISE being constructed for the first time for this study. In addition, experimental data on complexation between chloramine-T and β-CD, reported previously (11) as a 1:1 interaction, were reanalyzed according to the proposed model.

THEORETICAL

Derivation of the Scatchard Equation

The stepwise complexation of the substrate, S, to the ligand, L, i.e., β-cyclodextrin, is described by the equilibria:

$$S + L \overset{K_1}{\rightleftharpoons} SL \tag{1}$$

$$SL + L \overset{K_2}{\rightleftharpoons} SL_2 \tag{2}$$

where

$$K_1 = [SL]/[S]\,[L] \tag{3}$$

$$K_2 = [SL_2]/[SL]\,[L] \tag{4}$$

$[S]$ is the concentration of the free substrate, and $[L]$ is the concentration of the free ligand (β-CD).

The total substrate concentration S_t is given by the following equation:

$$S_t = [S] + [SL] + [SL_2] = [S] + S_b \tag{5}$$

where S_b is the "total bound" substrate concentration,

$$S_b = [SL] + [SL_2] \tag{6}$$

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The total substrate concentration, S_t, can also be expressed (3) as a function of the total ligand concentration L_t:

$$S_t = \frac{1 + K_2[L]}{1 + 2K_2[L] + (1/K_1[S])} L_t + [S] \quad (7)$$

Combination of Eqs. (5) and (7) gives

$$S_b = \frac{1 + K_2[L]}{1 + 2K_2[L] + (1/K_1[S])} L_t \quad (8)$$

or

$$r = \frac{S_b}{L_t} = \frac{1 + K_2[L]}{1 + 2K_2[L] + (1/K_1[S])} \quad (9)$$

where r represents the moles of substrate bound per mole of ligand.

Substitution of $[L]$ from Eq. (3) into Eq. (9) gives

$$r = \frac{1 + (K_2[SL]/K_1[S])}{1 + 2(K_2[SL]/K_1[S]) + (1/K_1[S])} \quad (10)$$

Rearrangement of Eq. (10) gives the Scatchard equation adhering to the system of equilibria (1) and (2):

$$\frac{r}{[S]} = \frac{K_1 + K_2([SL]/[S])}{1 + 2K_2[SL]} - \frac{K_1}{1 + 2K_2[SL]} r \quad (11)$$

In Eq. (11), $[SL]$ can be expressed in terms of $[S]$ and S_t as follows.

Equation (5) may be rewritten as

$$[SL] = S_t - [S] - [SL_2] \quad (12)$$

Combination of Eqs. (3) and (4) gives

$$[SL_2] = \frac{[SL]^2}{[S](K_1/K_2)} \quad (13)$$

Combination of Eqs. (12) and (13) gives

$$[SL] = S_t - [S] - \frac{[SL]^2}{[S](K_1/K_2)} \quad (14)$$

Solution of the quadratic Eq. (14) in terms of $[SL]$ gives the only acceptable root:

$$[SL] = \left\{-1 + \sqrt{1 + \frac{4(S_t - [S])}{[S](K_1/K_2)}}\right\} \Big/ \left\{\frac{2}{[S](K_1/K_2)}\right\} \quad (15)$$

Equation (11) reveals that a plot of $r/[S]$ versus r is not linear. The corresponding equation for the double-reciprocal plot can be obtained from rearrangement of Eq. (11):

$$\frac{1}{r} = \frac{K_1}{K_1 + K_2([SL]/[S])} + \frac{1 + 2K_2[SL]}{K_1 + K_2([SL]/[S])} \frac{1}{[S]} \quad (16)$$

Again, a plot of $1/r$ versus $1/[S]$ is not linear. Both Eq. (11) and Eq. (16) collapse to the conventional linear equations for $K_2 = 0$, i.e., when a system with a simple 1:1 complex equilibrium is operating.

Simulation Studies

The proposed model was evaluated by the construction of Scatchard and double-reciprocal plots using simulated data. The curves were generated using Eqs. (11) and (16). Values were assigned for the parameters S_t, L_t, K_1, and K_2, while the value for $[S]$ was calculated with the Newton–Rhaphson iterative method using the following equation:

$$\left(\frac{K_1}{K_2} - 4\right)[S]^3 + \left[L_t\left(\frac{K_1}{K_2} - 4\right) - S_t\left(\frac{K_1}{K_2} - 8\right)\right][S]^2 + \left[L_t\left(4S_t - \frac{1}{K_2} - L_t\right) - 4S_t^2 - \frac{1}{K_1K_2}\right][S] + \frac{S_t}{K_1K_2} = 0 \quad (17)$$

Equation (17) is derived from Eq. (11) after substitution for $[SL]$ from Eq. (15) and rearrangement (see Appendix). The total bound substrate (drug) concentration, $[SL] + [SL_2]$, was calculated from Eq. (5).

MATERIALS AND METHODS

Reagents

All solutions were prepared in deionized water. 2-(*p*-Toluidinyl)-6-naphthalenesulfonate potassium salt was obtained from Sigma (St. Louis, MO). 1-Anilino-8-naphthalenesulfonate ammonium salt, β-cyclodextrin, 2-nitrophenyl octyl ether, *p*-nitrocumol, and tetraheptylammonium bromide were obtained from Fluka (Buchs, Switzerland). Polyvinyl chloride (PVC) of high molecular weight was from Jannsen Chimica (Beerse, Belgium).

Tris Buffer, 0.010 M, *pH 7.0.* This was prepared by dissolving tris(hydroxymethyl)aminomethane in water and adjusting the pH with a concentrated HCl solution.

2,6-TNS Stock Solutions. For the potentiometric study, this solution was 0.0050 *M* with respect to 2,6-TNS in Tris buffer. For the fluorimetric study, this solution was 0.00010 *M* with respect to 2,6-TNS in Tris buffer.

1,8-ANS Stock Solution. This solution was 0.050 *M* with respect to 1,8-ANS in Tris buffer, 0.010 *M*, pH 7.0.

β-CD Solutions. For the potentiometric study this solution was 0.0020 and 0.010 *M* for 2,6-TNS and 1,8-ANS, respectively, in Tris buffer. For the fluorimetric study this solution was 0.015 *M* with respect to β-CD in the same buffer.

2,6-TNS/β-CD and 1,8-ANS/β-CD Mixed Working Solutions. The 2,6-TNS/β-CD mixed working solution was 0.0050 *M* with respect to 2,6-TNS and 0.0020 *M* with respect to β-CD in Tris buffer. The 1,8-ANS/β-CD mixed working solution was 0.050 *M* with respect to 1,8-ANS and 0.010 *M* with respect to β-CD in Tris buffer.

Electrode Construction

Electrode Assembly. Indicator electrodes of the PVC membrane type (14) were assembled in our laboratory. They consist of an electrode body [a conventional pH glass electrode (Cambridge Instrument Co.) the end of which is cut off] and an electroactive PVC membrane attached to the end of the electrode body through a small silicone tube-cap. The electroactive PVC membranes were constructed by entrap-

ping the corresponding liquid ion exchanger in a PVC matrix according to the method of Craggs *et al.* (14). The liquid ion exchanger for the 2,6-TNS ISE was its ion pair with tetraheptylammonium cation in 2-nitrophenyl octyl ether (2-NPOE) at a concentration of ≈0.01 *M* and was prepared as follows: 5 ml of a 0.010 *M* tetraheptylammonium bromide solution in 2-NPOE was shaken six times with a 0.0050 *M* 2,6-TNS solution in order to exchange Br^- with 2,6-TNS, and the aqueous phase was separated each time by centrifugation and decanted. The organic phase was then dried with anhydrous sodium sulfate to remove any water traces. The liquid ion exchanger for the 1,8-ANS ISE was its ion pair with tetraheptylammonium cation in *p*-nitrocumol at a concentration of ≈0.01 *M* and was similarly prepared as described previously (15). A small amount of these ion exchangers was used for the preparation of the PVC membranes. These membranes can be stored for at least 1 year in a closed vial.

Internal Reference Solutions. For the 2,6-TNS electrode, this solution was 0.0050 *M* with respect to 2,6-TNS in 0.00050 *M* sodium chloride, saturated with silver chloride (a higher NaCl concentration causes turbidity). For the 1,8-ANS electrode the solution was 0.010 *M* with respect to 1,8-ANS in 0.10 *M* sodium chloride, saturated with silver chloride.

After construction of the indicator electrodes, they were conditioned for 24 hr before use by immersion in a stirred solution of 0.0050 *M* 2,6-TNS or 0.0010 *M* 1,8-ANS, respectively.

Apparatuses

The system used for the measurements using ISEs consists of an Orion Model SA720 pH/ISE meter, with a readability of ±0.1 mV, connected to a Knauer Model 7334 Single Chart Recorder. The emf values were measured using a Ag/AgCl reference electrode (Orion single junction, Model 900100, filled with Orion reference electrode filling solution 90-00-01) and recorded on a Brother M-1109 printer. All measurements were carried out in a 30-ml double-walled glass cell, thermostated at a temperature of 25 ± 0.5°C with an Edmund Buhler 7400 Tubingen Type UKT30 water bath, with constant magnetic stirring of solutions. For the fluorimetric study, a Perkin Elmer 512 Double Beam Fluorescence Spectrophotometer was used and its cuvette was thermostated at 25 ± 0.5°C. For pH measurements, a Metrohm Model E-350B pH-meter with a combination glass electrode was used.

Procedures

Calibration Curve. For the 2,6-TNS ISE, 5 ml of Tris buffer was pipetted into the measurement cell, the pair of electrodes was immersed in it, and after the potential was stabilized (±0.1 mV), various aliquots of the 0.0050 *M* 2,6-TNS stock solution were added, with a 100-μl Hamilton microsyringe (concentration range, 4×10^{-6}–3×10^{-3} *M*). The emf values were recorded and measured after stabilization (±0.1 mV), following each addition. For the 1,8-ANS ISE the same procedure was used, adding aliquots from the 0.050 *M* stock solution. The potential values *E* were plotted against $-\log C$ (*pC*) to give the calibration curve using a least-squares fitting program. Corrections for the changes in volume after addition were performed by the program.

Binding Experiments. For the potentiometric studies, 5 ml of a solution of β-CD (0.0020 *M* for 2,6-TNS experiments and 0.010 *M* for 1,8-ANS experiments) was pipetted into the measurement cell, and the pair of the electrodes was immersed in it. After the potential was stabilized (±0.1 mV), small amounts of either the mixed working 2,6-TNS/β-CD solution or the 1,8-ANS/β-CD solution were added with a 100-μl Hamilton microsyringe. The emf values were recorded to check stabilization and measured after each addition. For fluorimetric studies, a set of 40 mixed 2,6-TNS/β-CD solutions was prepared in Tris buffer. Each of these solutions had a concentration of 1×10^{-5} *M* for 2,6-TNS, while the β-CD concentration ranged from 0 to 0.0105 *M*. The excitation wavelength was 324 nm and the emission wavelength was 488 nm.

Data Analysis

Whenever a nonlinear least-squares method for the analysis of experimental data was required, the Statgraphics Statistical Graphics System was applied on an IBM-compatible computer.

RESULTS AND DISCUSSION

Electrode Characteristics

The 2,6-TNS electrode shows a near-Nernstian response in the range of 1×10^{-5}–3×10^{-3} *M* 2,6-TNS concentrations. The slope at 25°C was 55–57 mV/decade ($r > 0.9997$) and the detection limit was 9×10^{-6} *M*.

The attached electrode membrane was found to have an operative life of about 1 month, after which a new one was attached to the silicone tube-cap. The slope of the electrode remained relatively constant and the potential for the same aqueous solution varied ±2 mV during its operative life. The response time was very short (the potential was stabilized in about 1–2 sec after each addition). The 1,8-ANS electrode characteristics have been described previously (15).

Simulation Studies

Scatchard plots generated using Eq. (11) of $r/[S]$ versus r for 1:1 and 1:2 substrate to β-CD complexes are shown in Fig. 1. The simulations were performed either keeping L_t constant and varying S_t (Fig. 1A) or the opposite (Fig. 1B), mimicking the most commonly used procedures in cyclodextrin/drug binding studies. In both cases the curvature of the Scatchard plots was decreased by decreasing the ratio of association constants K_1/K_2. The different experimental procedures used for the simulation are reflected in the different shapes of the Scatchard plots (Figs. 1A and B). In addition, the numerical values of the x axes in Figs. 1A and B reveal that the curves in Fig. 1A cover a much wider range for the variable r than the corresponding curves in Fig. 1B. It can be concluded, therefore, that the Scatchard plots obtained by keeping L_t constant and varying S_t are more complete than those obtained following the opposite procedure.

Double-reciprocal plots (not shown) generated by Eq. (16) of $1/r$ versus $1/[S]$ for 1:1 and 1:2 substrate to β-CD

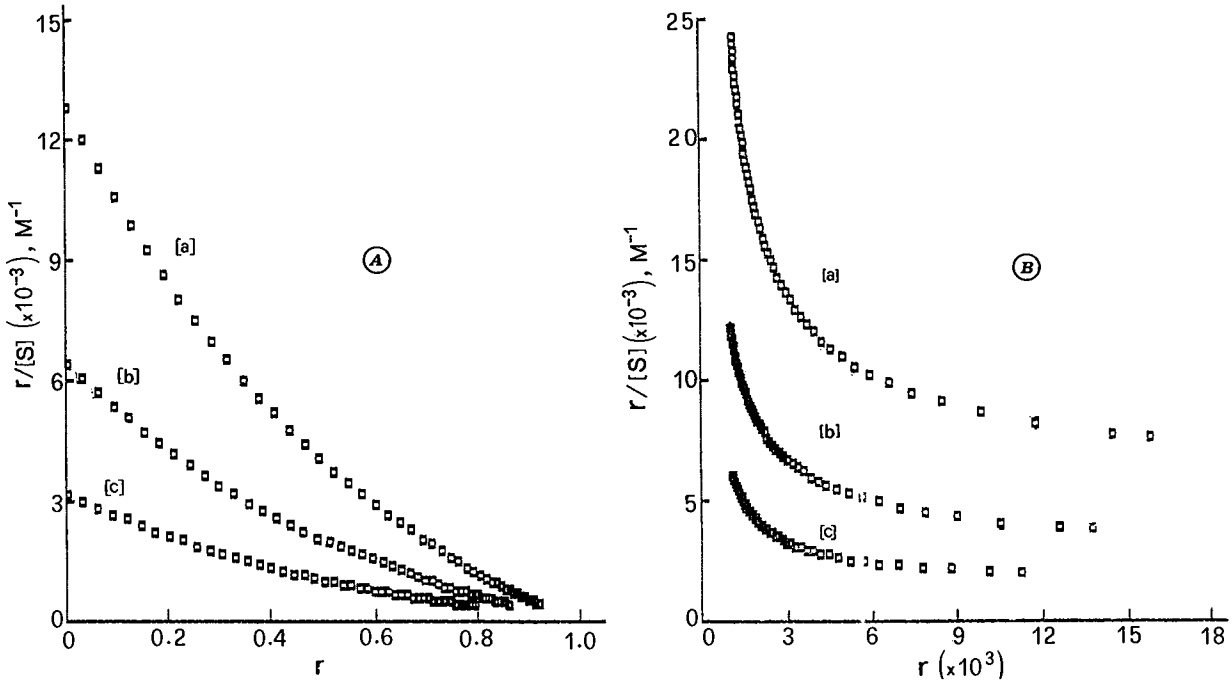

Fig. 1. Simulated Scatchard plots constructed according to Eq. (11) for 1:1 plus 1:2 complexation formation: (A) keeping L_t constant ($L_t = 3 \times 10^{-3}$ *M*) and varying S_t ($S_t = 5 \times 10^{-6}$–5×10^{-3} *M*); (B) keeping S_t constant ($S_t = 1 \times 10^{-5}$ *M*) and varying L_t ($L_t = 5 \times 10^{-4}$–1×10^{-2} *M*). [a] $K_1 = 8000\ M^{-1}$, $K_2 = 200\ M^{-1}$; [b] $K_1 = 4000\ M^{-1}$, $K_2 = 200\ M^{-1}$; [c] $K_1 = 2000\ M^{-1}$, $K_2 = 200\ M^{-1}$.

complexes were also constructed. Both simulations, keeping L_t or S_t constant, resulted in nonlinear plots. However, nonlinearity was found to be more apparent in the simulations with a constant total substrate concentration, S_t. Plots of the concentration of the various substrate species versus the total substrate concentration S_t or the total ligand concentration L_t were also constructed for the two experimental procedures simulated (Figs. 2A and B). Although the concentration profiles of the two complexes SL and SL_2 are different, both bound species are present throughout the experiments simulated (Figs. 2A and B).

Binding Studies

The two experimental procedures simulated are commonly applied in fluorimetric and potentiometric (using ISEs) studies. The former technique utilizes a constant total substrate concentration S_t; the latter utilizes a constant total ligand concentration L_t. We felt that the two anilinonaphthalenesulfonates, 1,8-ANS and 2,6-TNS, are ideal models for this study since they exhibit different complexation behavior (9,16,17), while both techniques, i.e., fluorimetry and direct potentiometry can be applied.

1,8-ANS/β-CD Interaction. The Scatchard plot for the binding of 1,8-ANS to β-CD using potentiometry is shown in Fig. 3A. The plot is indicative of 1:1 stoichiometry, and the association (complexation) constant determined is given in Table I, along with the values estimated in fluorimetric studies, and reported in the literature (9,13). All studies support a 1:1 complex formation for the 1,8-ANS/β-CD interaction and a low binding affinity with an association constant ≈100 M^{-1} (Table I).

2,6-TNS/β-CD Interaction. Figure 3B shows the Scatchard plot obtained with direct potentiometry for 2,6-TNS complexed to β-CD. The nonlinear character of Fig. 3B is indicative of 1:1 plus 1:2 complexation. The good fit of Eq. (11) to the experimental data in Fig. 3B suggests that the complexation follows the proposed model of the equilibria (1) and (2). This observation is in agreement with earlier reports on the 2,6-TNS/β-CD interaction (9,16,18). However, the values of association constants estimated by nonlinear regression analysis of the potentiometric data do not agree with the corresponding values obtained with fluorimetry (9) (Table I). The large differences in K_1 and K_2 values observed prompted us to perform a detailed fluorimetric study for this interaction. The results obtained for a wide β-CD concentration range are shown in Fig. 4A as a double-reciprocal plot (9). The nonlinear character of this plot,

Table I. Estimates of Association Constants for 1,8-ANS/β-CD and 2,6-TNS/β-CD Complexation[a]

	1,8-ANS	2,6-TNS	
Method	K_1, M^{-1}	K_1, M^{-1}	K_2, M^{-1}
Fluorescence	70.9[b]		
	110 (4)[c]	1980 (84)[c]	600 (95)[c]
Ion Selective Electrodes	86.9 (1.2)	3737 (6)	149 (2)

[a] Calculated at 25°C; standard deviations of estimates in parentheses.
[b] From Ref. 13.
[c] From Ref. 9.

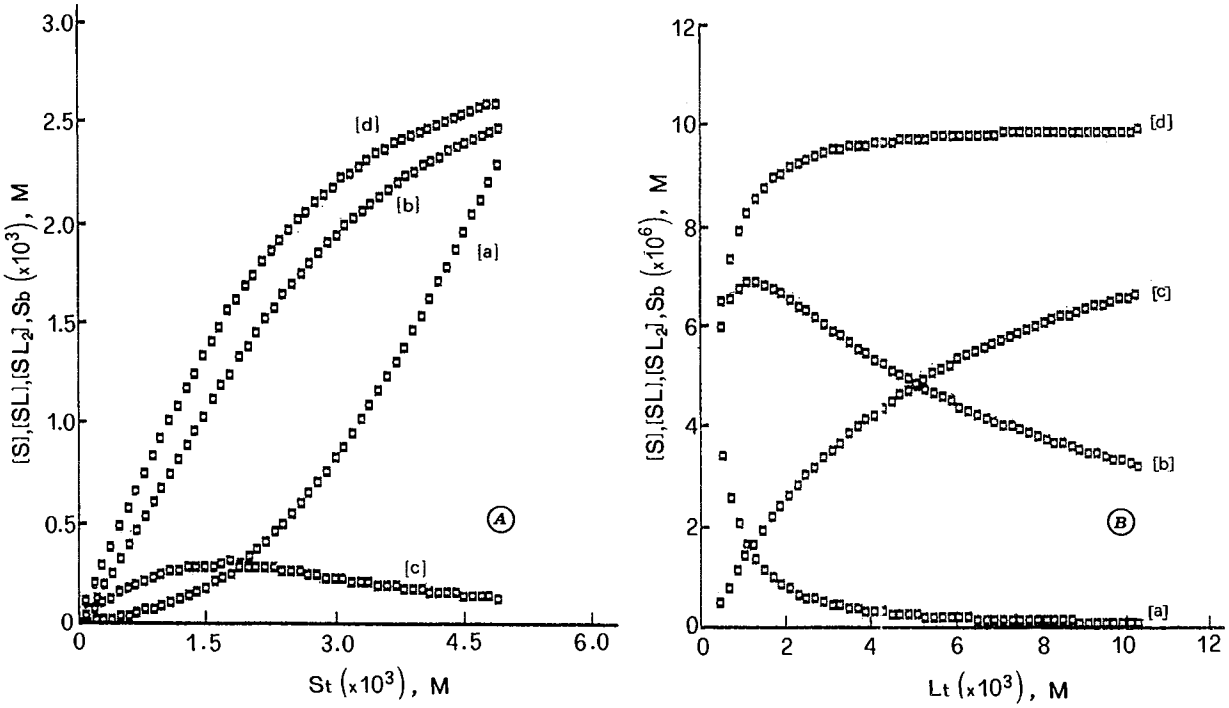

Fig. 2. Simulated plots of the various substrate species concentrations during complexation experiments, constructed according to the 1:1 plus 1:2 model, using $K_1 = 4000\ M^{-1}$ and $K_2 = 200\ M^{-1}$; (A) keeping L_t constant ($L_t = 3 \times 10^{-3}\ M$) and varying S_t ($S_t = 6 \times 10^{-6}$–$6 \times 10^{-3}\ M$); (B) keeping S_t constant ($S_t = 1 \times 10^{-5}\ M$) and varying L_t ($L_t = 5 \times 10^{-4}$–$1 \times 10^{-2}\ M$). [a] Free substrate [S]; [b] 1:1 substrate:cyclodextrin complex [SL]; [c] 1:2 substrate:cyclodextrin complex [SL_2]; [d] total bound substrate S_b.

clearly indicated in Figs. 4B and C, implies that the calculation of K_1 and K_2 from discrete linear regions (9) could be subject to error. This observation is substantiated by the results of the simulation study in Fig. 2, which shows that

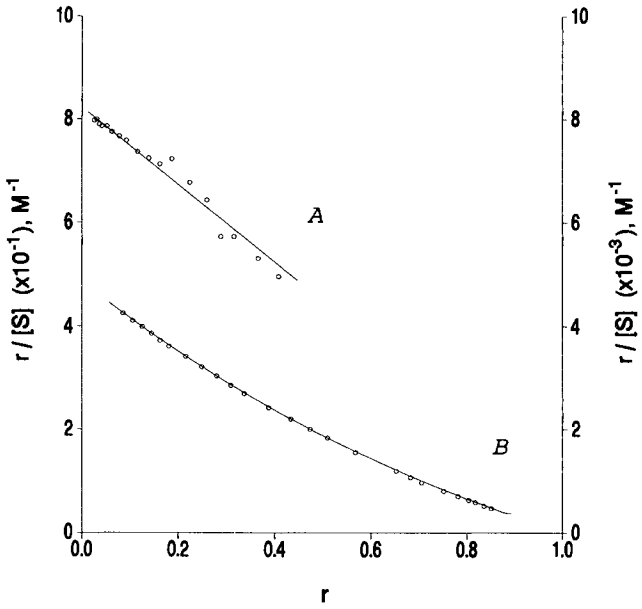

Fig. 3. Scatchard plots for the 1,8-ANS/β-CD association (A and left-hand ordinate) and 2,6-TNS/β-CD association (B and right-hand ordinate). The fitted lines (A, 1:1 binding model, $R^2 = 0.981$; B, 1:1 plus 1:2 binding model, $R^2 = 0.9999$) are drawn over the experimental points.

both bound species, SL and SL_2, are simultaneously present in solution throughout the experiment with varying L_t (fluorescence experiment). Therefore, the validity of the calculation of K_1 and K_2 from the terminal (Fig. 4B) and the initial (Fig. 4C) portions, respectively, of the nonlinear fluorescence diagram (Fig. 4A) is questionable because of the coexistence of the two species, SL and SL_2, observed in the simulation experiment (Fig. 2B).

In fluorescence studies it is commonly assumed that the fluorescence intensity, F, is proportional to the total concentration of the bound species (19). In the present study this proportionality can be expressed by Eq. (18):

$$F = fS_b = f([SL] + [SL_2]) \tag{18}$$

where f is a fluorescent coefficient. However, because of the dissimilarities in chemical structure (9) of SL and SL_2, it is inappropriate to assume that the bound species exhibit equivalency in their quantum yield. A more valid assumption for the fluorescence intensity contribution of the two species can be expressed mathematically by the equation

$$F = f_1\,[SL] + f_2\,[SL_2] \tag{19}$$

where f_1 and f_2 are the individual fluorescence coefficients for the species SL and SL_2, respectively. A relevant treatment has been recently addressed in the literature (17). In order to validate this hypothesis, an attempt was made to fit Eq. (19) to the experimental data and estimate the coefficients f_1 and f_2. Initially, the concentrations of the bound species, [SL] and [SL_2], for the fluorescence experiments

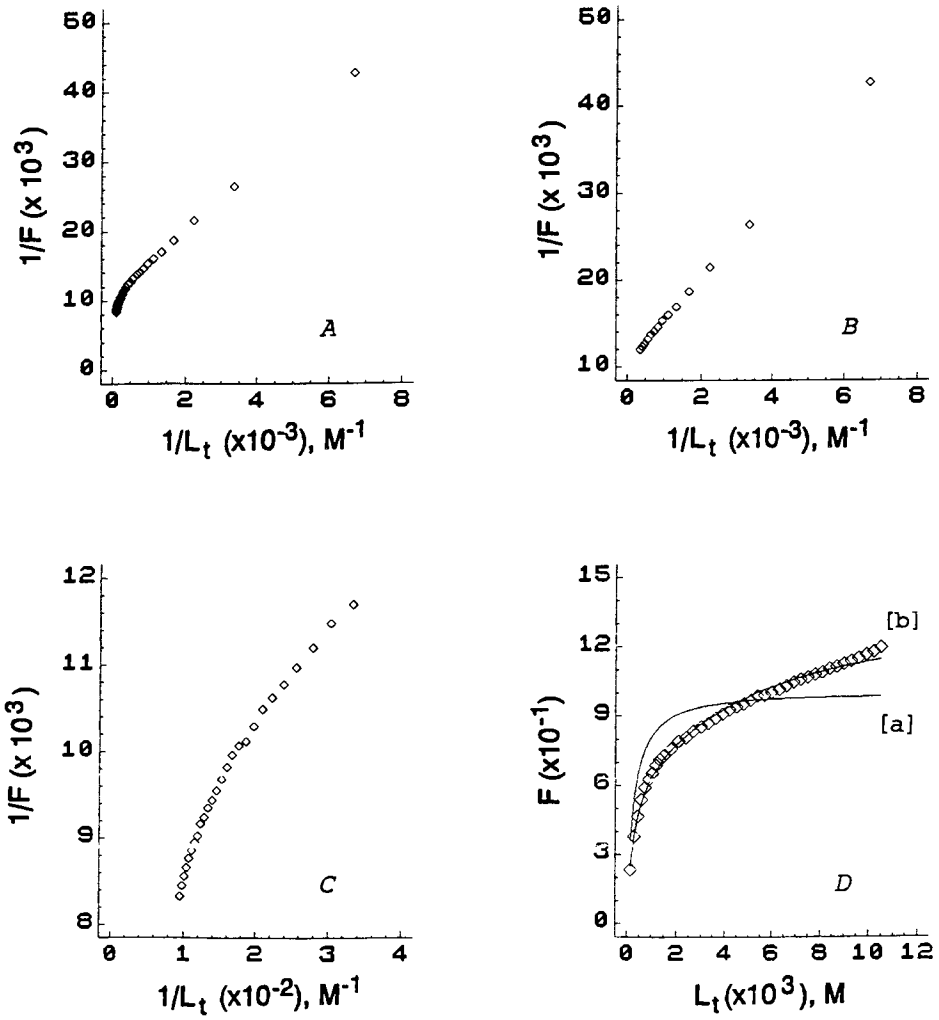

Fig. 4. Fluorescence diagrams for the 2,6-TNS/β-CD association. (A) Double-reciprocal plot of fluorescence data according to Catena and Bright (9). (B) Region for the calculation of K_1. (C) Region for the calculation of K_2. (D) Fitting of Eqs. (18) and (19) to the fluorescence data for calculation of the fluorescence coefficients, using the values of the association constants estimated with ISEs and reported in Table I: [a] Eq. (18), $R^2 = 0.72$; [b] Eq. (19), $R^2 = 0.99$.

were calculated from the mole-fraction equations (20) and (21), respectively (20):

$$[SL] = \frac{K_1 [L]}{1 + K_1 [L] + K_1K_2 [L]^2} S_t \quad (20)$$

$$[SL_2] = \frac{K_1K_2 [L]^2}{1 + K_1 [L] + K_1K_2 [L]^2} S_t \quad (21)$$

using the values of K_1 and K_2 estimated in the study with ISEs (Table I). The concentration of free ligand in Eqs. (20) and (21) was set equal to L_t since β-CD was in large excess. Subsequently, Eqs. (18) and (19) were fitted to the experimental data and the results are shown in Fig. 4D. Indeed, the observed fluorescence intensity values are well predicted by the model described by Eq. (19). The estimates of f_1 and f_2 were found to be 6.7 (±0.085) × 10^6 and 1.5 (±0.01) × 10^7 for the species SL and SL_2, respectively. These values are also in accord with the chemical structure of the two species (9); the complex SL_2 is more rigid than SL, and this is reflected in the greater value of f_2 than f_1 (21). In contrast, Eq. (18) does not describe the data adequately (Fig. 4D), thus ruling out the validity of the equivalent contribution of the species SL and SL_2.

Two important conclusions can be drawn from these observations. First, the values of K_1 and K_2 derived from the direct potentiometry study are valid estimates of the real association constants. Second, caution should be exercised whenever fluorimetry is applied in complexation studies involving multiple equilibria, and the assumption of an equivalent quantum yield of bound species is questionable. Similar concerns have been addressed previously for protein and cyclodextrin binding studies, relying on the assumption of the identical quantum yield of the fluorescence probe 1,8-ANS at each site of the binder (15,22).

Chloramine-T/β-CD Interaction. In a previous article (11) we described the chloramine-T/β-CD interaction as a 1:1 complexation. In this study, we reanalyzed the data by fitting Eq. (11) to the experimental data. The good fit (R^2 =

0.999) is conclusive evidence for the 1:1 plus 1:2 complexation. The estimates of association constants K_1 and K_2 were found to be 844 ± 12 and 52 ± 4 M^{-1}, respectively.

In conclusion, the appropriate Scatchard equation for the formation of 1:1 and 1:2 substrate to cyclodextrin complexes was used to estimate the association constants, K_1 and K_2. This method requires only valid data for the concentration of free or bound substrate species. The technique of ion-selective electrodes is particularly suitable for this type of analysis since it provides measurement of the free ion in the presence of bound ion.

APPENDIX

Rearranging Eq. (11) and substituting SL from Eq. (15) gives

$$\frac{2r - K_1[S]}{K_1[S](1 - 2r)} = \sqrt{1 + \frac{4(S_t - [S])}{[S](K_1/K_2)}} \qquad \text{(A1)}$$

Squaring Eq. (A1) and rearranging the resulting equation, we obtain

$$[1 - K_1^2[S]^2 - 4(S_t - [S])K_1K_2[S]]r^2 + [K_1^2[S]^2 - K_1[S] + 4(S_t - [S])K_1K_2[S]]r - (S_t - [S])K_1K_2[S] = 0 \qquad \text{(A2)}$$

With the notation employed above, r can be expressed by the equation

$$r = \frac{S_t - [S]}{L_t} \qquad \text{(A3)}$$

Substituting Eq. (A3) in Eq. (A2), simplifying the resulting equation by dividing it by $S_t - [S]$, and rearranging, Eq. (17) is obtained.

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