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ANALYSIS OF THE BINDING OF FLUORESCENT LIGANDS TO SOLUBLE PROTEINS

USE OF SIMULTANEOUS NON-LINEAR LEAST SQUARES REGRESSION TO OBTAIN ESTIMATES OF BINDING PARAMETERS

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Abstract—The binding of three fluorescent ligands (warfarin, dansylsarcosine and 1-anilino-8-naphthalene sulphonate) to human albumin was analysed using simultaneous non-linear least squares regression analysis. Both mock and actual fluorescence data were examined and the results indicated that reliable estimates of the binding parameters as well as the molar fluorescence of bound ligand could be obtained. The advantage of this method of analysis is that it makes full use of all the experimental data and it eliminates the need for the graphical procedures usually employed to estimate the molar fluorescence of bound ligand and its binding constants. This type of analysis can be extended to other systems where some physical property of the bound ligand varies with increasing protein concentration.

The usual way of studying the interaction of a ligand with its binding site is to keep the total concentration of binding sites constant and vary the concentration of ligand. If some physical property of the bound ligand, such as its fluorescence or optical absorbance in the bound state, is being used to study binding, then the variation of this property with increasing concentration of binding sites is measured to estimate the magnitude of the physical property per mole of bound ligand. Fluorescence spectroscopy has been widely used to study protein–ligand interactions and one method which has been employed to obtain estimates of the molar fluorescence of bound ligand (F_b) from such experiments, is to plot the reciprocal of fluorescence intensity against the reciprocal of protein concentration. This method, however, has been shown to be invalid by Zierler [1] who demonstrated that double reciprocal plots develop upward curvature as the concentration of protein increases, so the desired value of F_b cannot be found by linear extrapolation [1].

This point has been re-emphasised by Panjehshahin *et al.* [2]. These authors suggested that for such experiments fluorescence should be plotted against the logarithm of protein concentration to obtain the maximum fluorescence and hence F_b. Recently, Rajkowski [3] has proposed alternative graphical methods for the estimation of F_b. Both these approaches, however, are directed towards obtaining an accurate estimate of F_b which can be subsequently used, together with data from experiments where the concentration of ligand is varied, to obtain relevant binding parameters.

In this article we show that more dependable information should be obtained by analysing simultaneously the results of experiments at constant protein concentration and at constant ligand concentration using non-linear least squares regression.

THEORY

If D_{sb}, D_{nsb} and D_u represent specifically bound, non-specifically bound and free ligand, then the total concentration of ligand in a system containing ligand and soluble protein is

$$D_T = D_{sb} + D_{nsb} + D_u. \quad (1)$$

The specific binding is assumed to be given by

$$D_{sb} = n \times P_T \times D_u/(K_d + D_u) \quad (2)$$

where K_d is the dissociation constant for the specific binding, n is the number of specific binding sites per protein molecule and P_T is the total concentration of protein. The non-specific binding will be given by

$$D_{nsb} = S \times P_T \times D_u \quad (3)$$

where S is a proportionality constant for non-specific binding. Eliminating D_u between equations (1) and (3) gives

$$D_{nsb} = (D_T - D_{sb})/(1 + 1/S \times P_T). \quad (4)$$

Rearranging equation 2 gives

$$D_u = K_d \times D_{sb}/(n \times P_T - D_{sb}). \quad (5)$$

Eliminating D_u between equation (5) and equation (1) gives

$$D_{nsb} = [(D_T - D_{sb})(n \times P_T - D_{sb}) - K_d \times D_{sb}]/[n \times P_T - D_{sb}]. \quad (6)$$

D_{nsb} can be eliminated between equations (4) and (6). The resulting equation can then be rearranged to give

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$$D^2_{sb} - D_{sb}(n \times P_T + D_T + K_d + S \times P_T \times K_d) + n \times D_T \times P_T = 0 \quad (7)$$

whence

$$D_{sb} = [b - (b^2 - 4c)^{1/2}]/(2) \quad (8a)$$

where

$$b = (n \times P_T + D_T + K_d + S \times P_T \times K_d) \text{ and}$$

$$c = n \times D_T \times P_T. \quad (8b)$$

Using equations (8a) and (8b), D_{sb} can be expressed in terms of n, P_T, D_T, K_d and S. Equation (6) then gives D_{nsb} in terms of n, P_T, D_T and D_{sb}. Equation (1) gives D_u as $(D_T - D_{sb} - D_{nsb})$. Therefore D_{sb}, D_{nsb} and D_u can each be expressed in terms of n, P_T, D_T, K_d and S.

If F_u, F_{sb} and F_{nsb} are the molar fluorescences of unbound, specifically bound and non-specifically bound ligand respectively then the total fluorescence may be written as

$$F_T = F_u \times D_u + F_{sb} \times D_{sb} + F_{nsb} \times D_{nsb}. \quad (9)$$

In this case F_T can be expressed as a function of n, P_T, D_T, K_d, S, F_u, F_{sb} and F_{nsb} by combining equations (6), (8a), (8b) and (9). If the model fits the experimental data then the resulting equations should be valid whether P_T is kept constant and D_T is varied or vice versa. Non-linear least squares regression can be used to fit the measured experimental quantity to the corresponding theoretical equation derived from the model. The usual method of doing this is to insert into the equations approximate initial values for the parameters to be estimated so that the curve-fitting program can modify these values until an optimum fit is obtained.

An extension of the principle of non-linear least squares regression allows more than one set of data to be fitted simultaneously to an appropriate equation. Published examples are the programs ALLFIT [4] and the related program LIGAND [5] which are capable of fitting a theoretical expression to several sets of data simultaneously, with various parameters being fixed or variable, or forced to be the same for particular sets of data. The overall usefulness of ALLFIT can be greatly increased by using numerical differentiation of the expression to be fitted to the data [6]. The modifications required in order to use ALLFIT to fit equation (9) (with D_{sb}, D_{nsb} and D_u given by equations (8a) with (8b), (6) and (1) respectively) to fluorescence data obtained in ligand binding studies are available on request from Dr D. Mackay.

MATERIALS AND METHODS

Generation of mock data. Artificial data were produced in what will be called 'paired data-sets'. In the case of mock data, each paired data-set consists of one set of data calculated at constant total ligand concentration and a second set calculated at constant total protein concentration. The values chosen for K_d, n, F_{sb}, S, F_{nsb} and F_u were (in most cases) 1.20 μM, 1.00, 0.90 μM^{-1}, 0.05 μM^{-1} (equivalent to 50.0 mM^{-1}), 1.50 μM^{-1} and 0.10 μM^{-1}, respectively. Theoretical values of total fluorescence F_T were calculated at twenty total protein concentrations (in the range 1 to 45 μM) at a total ligand concentration of 2 μM and at thirteen ligand concentrations (in the range 1 to 13 μM) at a total protein concentration of 2 μM. In all cases an error term was generated (from a normal population with a mean zero and a standard deviation related to that found in the real experiments) and superimposed on the calculated theoretical value for the fluorescence. The absolute standard deviation was taken to be constant since replicate measurements from the real experiments showed no significant dependence of the standard deviation on the magnitude of the fluorescence, over the range of intensities measured. The distribution and numbers of data points, and the magnitudes of the various fluorescence and binding parameters were chosen to be essentially similar to those of real experiments [2].

Experimental data. Experimental results were obtained using methods already described by Panjehshahin *et al.* [2]. The variation of fluorescence with concentration of defatted human albumin was studied at pH 7.4 and 31° for the three ligands warfarin, dansylsarcosine and 1-anilino-8-naphthalene sulphonate (ANS). In these experiments the total concentration of each ligand (D_T) was kept constant at 2 μM and the concentration of human albumin (P_T) varied from 1 to 45 μM. The variation of fluorescence with concentration of each ligand was also studied, under the same experimental conditions, keeping P_T constant at 2 μM whilst D_T ranged from 1 to 13 μM. The excitation:emission wavelengths for dansylsarcosine, warfarin and ANS were respectively 350:475, 320:380 and 400:475 nm. The bandwidth for both excitation and emission was 4 nm. Only in the case of warfarin was the molar fluorescence of unbound ligand, F_u, significantly greater than zero.

Analysis of data. The program ALLFIT was modified so that it could fit equation (9) (combined with equations (8a), (8b), (6) and (1)) to the real or mock fluorescence data. The resulting program will be referred to as ALLPTDT. The Y-variable was the experimentally measured fluorescence and the X-variable was D_T or P_T. There were seven parameters; namely, the constant value of P_T or D_T as appropriate, the dissociation constant, K_d, for specific binding, the number, n, of specific binding sites per protein molecule, the molar fluorescence of the ligand when specifically bound, F_{sb}, the proportionality constant for non-specific binding, S, the molar fluorescence of the non-specifically bound ligand, F_{nsb}, and the molar fluorescence of unbound ligand, F_u. The latter was considered to be a constant accurately measured in a separate experiment.

In the present series of analyses ALLPTDT was employed to fit the theoretical equations to each paired data-set using a microcomputer (16-bit microprocessor; 640 KB base memory). Any of the curve-fitting parameters could be set equal to zero or to any other fixed numerical value if desired. The values of the parameters could be allowed to optimise with the restriction that the value of one or more of the parameters be common for the two sets of data. However the program can be used to fit more than two curves simultaneously if required.

For each paired data-set the statistical significance of any forced change in a parameter was assessed

by testing the significance of the resulting change in goodness of fit as judged by the mean sum of squared deviations of the experimental points about the fitted curve. For example, to test if the data support the idea that the molar fluorescence due to specific binding differs from that due to non-specific binding the data can first be fitted with F_{sb} forced to equal F_{nsb} (Fit 1) and then with F_{sb} allowed to differ from F_{nsb} (Fit 2). If the sums of squared deviations for the first and second fits are SS_1 and SS_2 and the corresponding numbers of degrees of freedom are DF_1 and DF_2 then the statistical significance of the difference in fits is assessed by calculating the variance ratio

$$F = \frac{(SS_1 - SS_2)/(DF_1 - DF_2)}{SS_2/DF_2}.$$

The greater sum of squares SS_1 corresponds to the smaller number of adjustable parameters and therefore to the greater number of degrees of freedom DF_1. A statistically significant value for F indicates that the fit with the larger number of adjustable parameters (or lower number of degrees of freedom) is significantly better. In the above example a significantly better fit might be obtained when the values of F_{sb} and F_{nsb} are allowed to be different.

In the case of real experiments only one paired data-set was available for each of the three ligands. In these circumstances the standard errors of the various parameters, estimated using ALLPTDT, are based on the spread of the experimental points about the theoretical curves, and are very approximate. By contrast, when ALLPTDT has been used repeatedly to estimate parameters from five or more individual paired data-sets, as was done with the mock data, the parameter values quoted are the means of all the individual values in the group and the standard errors of the parameters are based on the spread of these individual values about the mean.

RESULTS

Analysis of mock experiments

The aim of analysing mock experiments was to test whether the analytical technique could give reasonably accurate unbiased estimates of K_d, n, S, F_{sb} and F_{nsb} first from highly accurate data and then from data with errors similar in magnitude to those likely to be present in real experiments.

Each mock experiment consisted of one set of data generated at constant P_T and another set at constant D_T. Each such paired data-set was analysed using ALLPTDT, first forcing K_d, n, F_{sb}, S, F_{nsb} to be the same for both curves but allowing F_{sb} and F_{nsb} to differ from each other. Appropriate constant values were entered for P_T, D_T, and F_u as required. The analysis was then repeated with the additional condition that F_{sb} should be equal to F_{nsb} for both curves.

Five such mock experiments were generated with the standard deviation of the fluorescence measurement set equal to 0.0017, which is about one tenth of the error found from replicate measurements in the experimental fluorescence studies. The values used for the various parameters were: $K_d = 1.2\,\mu M$, $n = 1.00$, $F_{sb} = 0.90\,\mu M^{-1}$, $S = 50.0\,mM^{-1}$, $F_{nsb} = 1.5\,\mu M^{-1}$ and $F_u = 0.10\,\mu M^{-1}$. For each such mock experiment the fit obtained by forcing F_{nsb} to be equal to F_{sb} was significantly worse ($P < 0.05$) than the fit obtained when the two fluorescences were allowed to have different values.

In order to see if increasing the number of experiments could counteract an increase in experimental error, 30 mock experiments were also generated using these same parameter values but with a more realistic standard deviation of 0.017 for the fluorescence measurements. This deviation is similar in magnitude to that estimated from replicate fluorescence measurements in the real experiments. In only two out of these 30 mock experiments was the fit obtained by allowing F_{nsb} to differ from F_{sb} significantly better than when these fluorescences were forced to be equal.

Ten other mock experiments were also generated using these same parameters except that the amount of non-specific binding was increased by raising the proportionality constant S five-fold to $250\,mM^{-1}$. In all ten experiments the fit obtained when F_{sb} was allowed to differ from F_{nsb} was significantly better than when the molar fluorescences were forced to be equal.

The means and standard errors of the various parameters obtained by applying ALLPTDT to these groups of mock experiments are summarised in Tables 1–3. Medians and ranges are also given to provide an approximate check on the symmetry of the groups of parameter estimates calculated from these mock experiments.

Analysis of real data

Results obtained using ALLPTDT to fit equation (9) (with equations (8a), (8b), (6) and (1)) to the experimental results obtained with warfarin, dansylsarcosine and ANS are shown in Table 4. Only one paired data-set was available for each ligand. Of the three compounds only warfarin had a non-zero value for intrinsic fluorescence in the unbound form ($F_u = 0.058\,\mu M^{-1}$). For each probe the non-specific binding was first assumed to be negligible, so S and F_{nsb} were set equal to zero for the data obtained at constant D_T and at constant P_T. The program was then allowed to fit equation (9) to the two sets of data (Table 4). This procedure was then repeated, but with non-zero values of S and F_{nsb} and with F_{sb} forced to be equal to F_{nsb} (Table 4), to test for the presence of a significant amount of non-specific binding. Finally in those cases where significant amounts of non-specific binding were detected equation (9) was fitted to the data sets allowing F_{sb} to differ from F_{nsb} (Table 4).

Figures 1 and 2 illustrate the differences in the goodness of the fits obtained using an earlier method [2] and using ALLPTDT to analyse results obtained with warfarin. In the earlier method the molar fluorescence of bound warfarin was estimated from the limiting fluorescence taken from a plot of fluorescence against the logarithm of the albumin concentration. This molar fluorescence was subsequently used to estimate the values of D_u and D_{sb} by solving the simultaneous equations $D_T = D_u + D_{sb}$

Table 1. Analysis by ALLPTDT of mock data with a low experimental error

No. of paired sets	Comments	K_d (μM)	n	F_{sb} (μM^{-1})	S (mM^{-1})	F_{nsb} (μM^{-1})
5	$F_{sb} \neq F_{nsb}$	1.199 ± 0.005*	0.997 ± 0.004	0.904 ± 0.003	53.7 ± 2.9	1.425 ± 0.066
	Minimum†	1.188	0.986	0.897	46.0	1.258
	Median	1.206	1.000	0.906	56.5	1.453
	Maximum	1.209	1.007	0.911	61.7	1.608
	Same data but with					
5	$F_{sb} = F_{nsb}$	1.164 ± 0.004	0.954 ± 0.001	0.936 ± 0.000	89.8 ± 0.15	0.936 ± 0.000
	Minimum	1.155	0.950	0.935	89.6	0.935
	Median	1.165	0.954	0.936	89.8	0.936
	Maximum	1.175	0.956	0.936	90.0	0.936
True values		1.200	1.000	0.900	50.0	1.500

* Values are mean ± SE mean, based on five individual values.
† Minimum, median and maximum values of each parameter.
In these experiments, experimental error of the fluorescence values was set very low corresponding to a standard deviation of 0.0017, which is about one tenth of that observed in replicate measurements in the real experiments.

Table 2. Analysis by ALLPTDT of mock data with a random error close to that observed in real experiments

No. of paired sets	Comments	K_d (μM)	n	F_{sb} (μM^{-1})	S (mM^{-1})	F_{nsb} (μM^{-1})
30	$F_{sb} \neq F_{nsb}$	1.160 ± 0.019*	0.960 ± 0.014	0.923 ± 0.009	76.4 ± 10.0	2.017 ± 0.328
	Minimum†	0.950	0.809	0.861	8.5	0.546
	Median	1.172	0.979	0.912	71.0	1.368
	Maximum	1.321	1.078	1.047	207.9	7.78
	Same data but with					
30	$F_{sb} = F_{nsb}$	1.144 ± 0.016	0.940 ± 0.006	0.936 ± 0.001	91.2 ± 0.8	0.936 ± 0.001
	Minimum	0.977	0.874	0.929	83.9	0.929
	Median	1.152	0.937	0.935	91.1	0.935
	Maximum	1.330	0.998	0.945	100.4	0.945
True values		1.200	1.000	0.900	50.0	1.500

* Values are mean ± SE mean, based on 30 individual values.
† Minimum, median and maximum values of each parameter.
The random error of the fluorescence values corresponding to a standard deviation of 0.017 which is close to that observed for replicate measurements in real experiments.

Table 3. Analysis by ALLPTDT of mock data with a random error close to that observed in real experiments. The proportionality constant (S) for non-specific binding was increased five-fold to 250 mM^{-1} in this analysis

No. of paired sets	Comments	K_d (μM)	n	F_{sb} (μM^{-1})	S (mM^{-1})	F_{nsb} (μM^{-1})
10	$F_{sb} \neq F_{nsb}$	1.294 ± 0.062*	1.085 ± 0.047	0.893 ± 0.008	229.2 ± 17.1	1.623 ± 0.075
	Minimum†	0.919	0.799	0.864	170.2	1.301
	Median	1.303	1.118	0.897	232.1	1.723
	Maximum	1.659	1.354	0.939	316.7	1.938
10	Same data but with $F_{sb} = F_{nsb}$	1.101 ± 0.076	0.724 ± 0.033	1.044 ± 0.013	486.6 ± 5.0	1.044 ± 0.013
	Minimum	0.681	0.534	1.041	456.1	1.041
	Median	1.080	0.735	1.043	486.8	1.043
	Maximum	1.524	0.890	1.053	513.1	1.053
True values		1.200	1.000	0.900	250.0	1.500

* Values are mean ± SE mean, based on 10 individual values.

† Minimum, median, maximum values of each parameter.

The random error of the fluorescence values corresponded to a standard deviation of 0.017 which is close to that observed for replicate measurements in real experiments.

Table 4. Analysis of real fluorescence data using ALLPTDT

Probe	Comments	K_d (μM)	n	F_{sb} (μM^{-1})	S (mM^{-1})	F_{nsb} (μM^{-1})
Warfarin	Fit 1, assuming no nsb	3.962 ± 0.157*	1.006 ± 0.017	0.7911 ± 0.005	—	—
	Fit 2, with nsb, and $F_{sb} = F_{nsb}$	3.825 ± 0.492	0.973 ± 0.114	0.790 ± 0.006	2 ± 6	0.790 ± 0.006
Fit 2 was not significantly better than Fit 1 ($P = 0.75$), indicating that there is no significant amount of non-specific binding (nsb)						
Dansylsarcosine	Fit 1, assuming no nsb	0.987 ± 0.053*	0.776 ± 0.011	1.032 ± 0.008	—	—
	Fit 2, with nsb, and $F_{sb} = F_{nsb}$	0.666 ± 0.051	0.643 ± 0.020	1.018 ± 0.006	14 ± 2	1.018 ± 0.006
	Fit 3, with nsb, but $F_{sb} \neq F_{nsb}$	0.681 ± 0.096	0.652 ± 0.053	1.003 ± 0.060	8 ± 55	17.7 ± 1240
Fit 2 was better than Fit 1 ($P < 0.01$) indicating a significant amount of non-specific binding (nsb). Fit 3 was better than Fit 2, but not significantly so ($P = 0.24$)						
ANS	Fit 1, assuming no nsb	1.281 ± 0.086*	2.196 ± 0.035	0.996 ± 0.011	—	—
	Fit 2, with nsb, and $F_{sb} = F_{nsb}$	0.740 ± 0.079	1.697 ± 0.067	0.980 ± 0.008	51 ± 8	0.980 ± 0.008
	Fit 3, with nsb, but $F_{sb} \neq F_{nsb}$	0.733 ± 0.074	1.697 ± 0.067	0.960 ± 0.041	5 ± 95	10.6 ± 215
Fit 2 was better than Fit 1 ($P < 0.01$), indicating a significant amout of non-specific binding (nsb). Fit 3 was better than Fit 2 but not significantly so ($P = 0.10$).						

* Values are mean ± SE mean, the latter based on the spread of experimental points about the theoretical curve.

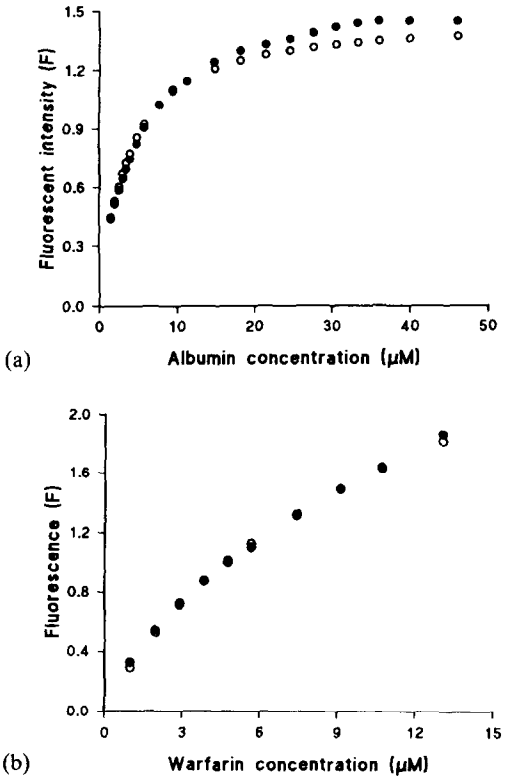

Fig. 1. (a) Fluorescence intensity (F) plotted against human albumin concentration (P_T). The concentration of warfarin was 2 μM. (b) Fluorescence intensity (F) plotted against the concentration of warfarin. The concentration of human albumin was 2 μM. For both figures closed circles (●) represent measured values of F and are the mean of three experiments. The standard errors of the data are too small to be shown. Open circles (○) are theoretical fluorescences calculated using the values 0.058 μM^{-1}, 0.730 μM^{-1}, 3.1 μM and 1.01, respectively, for F_u, F_{sb}, K_d and n. F_{sb} was determined from a plot of F versus log albumin concentration [2].

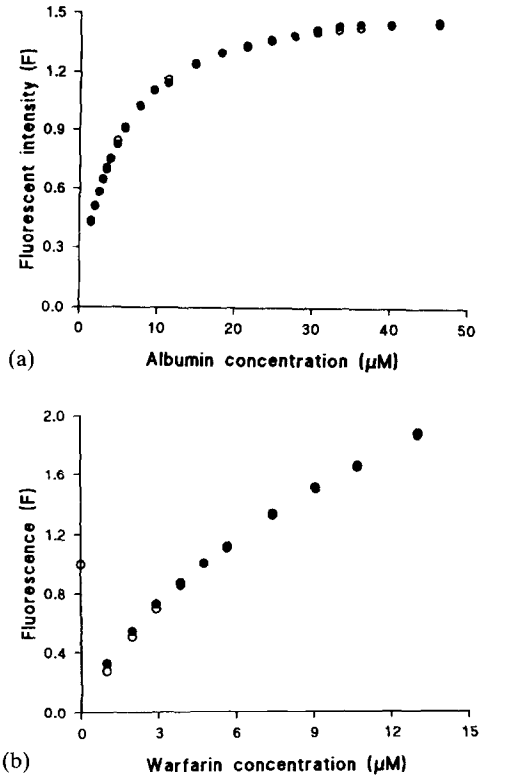

Fig. 2. (a) Fluorescence intensity (F) plotted against human albumin concentration (P_T). The concentration of warfarin was 2 μM. (b) Fluorescence intensity (F) plotted against the concentration of warfarin. The concentration of human albumin was 2 μM. For both figures closed circles (●) represent measured values of F and are the mean of three experiments. The standard errors of the data are too small to be shown. Open circles (○) are theoretical fluorescences calculated using the values 0.058 μM^{-1}, 0.791 μM^{-1}, 3.962 μM and 1.006 for F_u, F_{sb}, K_d and n respectively, obtained using ALLPTDT (see Table 4).

and $F_T = F_u \times D_u + F_{sb} \times D_{sb}$. These values in turn were used to obtain estimates of the binding parameters, n and K_d, from the equation $D_{sb}/P_T = n \times D_u/(K_d + D_u)$, which was fitted by non-linear least squares regression. The values of n, K_d, F_{sb} and F_u can be used to calculate expected fluorescences at chosen values of P_T and D_T. Plots of the observed and calculated fluorescence values obtained by this method, are shown in Fig. 1a and b. When the program ALLPTDT was applied to the same experimental data the binding parameters and molar fluorescence were fitted simultaneously, and were used to obtain the calculated fluorescence values shown in Fig. 2a and b. These figures show that the goodness of fit obtained using ALLPTDT is better than that given by graphical estimation of F_{sb}. [2] Equally good fits were obtained using ALLPTDT to analyse data for dansylsarcosine and ANS.

DISCUSSION

Analysis of five mock experiments, with an assumed error about one tenth of that observed in the real experiments, shows that with such accurate data use of ALLPTDT provides estimates of K_d, n, F_{sb}, S and F_{nsb} which are all in good agreement with the true values (Table 1). When the program was used to analyse the very same data but with F_{sb} forced to have the same value as F_{nsb}, in every case the latter fit was significantly worse than when F_{sb} and F_{nsb} were allowed to have different values. Even more important is the fact that in these examples forcing F_{sb} and F_{nsb} to be equal produced values for K_d, n, F_{sb}, S and F_{nsb} which were incorrect in spite of low apparent standard errors. The discrepancy between the mean estimate and the true value was greatest for F_{nsb} and S (Table 1). This shows that if non-specific binding is present in a system but F_{sb} is not in fact equal to F_{nsb}, then the equation with F_{sb} set equal to F_{nsb} may still fit the experimental data reasonably well but the exact numerical values of S and F_{nsb} are likely to be incorrect.

When the magnitude of experimental error in the fluorescence measurements was increased to a value close to that observed in real experiments, analysis

of thirty paired data-sets using ALLPTDT gave mean estimates of K_d, n, F_{sb}, S and F_{nsb} which were still close to the true values (Table 2). However only two out of these thirty mock experiments gave significantly better fits when F_{nsb} was allowed to differ from F_{sb} instead of being forced to have the same value. Except in the case of F_{nsb}, the true values of the various parameters tended to be at one or other end of the observed distribution of individual measured values, suggesting that some bias is produced by the least squares fitting method as a result of the increased experimental error (Table 2). Forcing the values of F_{sb} and F_{nsb} to be equal, again gave mean estimates of K_d, n, F_{sb}, S and F_{nsb} which had lower standard errors but which, especially for S and F_{nsb}, were clearly incorrect.

Using ALLPTDT to analyse ten paired sets of mock data in which the amount of non-specific binding had been increased five-fold (Table 3) gave mean estimates for K_d, n, F_{sb}, S and F_{nsb} which were in good agreement with the true values. The increased amount of non-specific binding resulted in a clearer distinction between F_{sb} and F_{nsb}, since in all ten of these experiments the fit allowing F_{nsb} to differ from F_{sb} was significantly better than the fit obtained when they were forced to be equal. Again forcing F_{sb} to be equal to F_{nsb} gave values of S and of F_{nsb} which had low standard errors but were incorrect.

When ALLPTDT was used to analyse the results of experiments with warfarin (comprising one paired data-set) it was found that inclusion of non-specific binding (with F_{sb} set equal to F_{nsb}) did not produce a statistically significant improvement in the fit of the equation to the experimental results (Table 4). This suggests that in these experiments the binding of warfarin when measured by fluorescence is almost entirely to high affinity sites.

When ALLPTDT was used to analyse results obtained with dansylsarcosine (again consisting of only one paired dataset), inclusion of non-specific binding (with $F_{sb} = F_{nsb}$) did significantly improve the fit. Allowing F_{sb} to differ from F_{nsb} further improved the fit of the theoretical equation to the data but not to a statistically significant extent. It is noticeable that allowing F_{sb} to differ from F_{nsb} resulted in a large increase in the standard error estimates for S and for F_{nsb}. A possible explanation for this finding is set out below. The common value of F_{sb} and F_{nsb} obtained for dansylsarcosine in Fit 2 (Table 4) is largely dependent on the high affinity binding component. Suppose that the non-specific binding is responsible for a small proportion of the total fluorescence. Under these conditions, with only a small uncertainty in F_{nsb} (since it is constrained to be equal to F_{sb}) there will be only a small uncertainty in the magnitude of S. However when F_{nsb} is freed from this constraint, that part of the fluorescence due to low affinity binding could result from a very small amount of non-specific binding with a high value of F_{nsb}, or vice versa, or to non-specific binding with intermediate values of S and F_{nsb}. The uncertainties in S and F_{nsb} are therefore greatly increased. From the results of the analyses for dansylsarcosine shown in Table 4, it will be seen that for Fit 2 the value of the product $S \times F_{nsb}$ is $0.0143\,\mu M^{-2}$ while for Fit 3 the product is $0.0142\,\mu M^{-2}$. The fact that the values of $S \times F_{nsb}$ are so similar for the two fits is consistent with the interpretation discussed above. So is the fact that the values of K_d and n are very similar for Fits 2 and 3. The overall conclusion is that for dansylsarcosine some of the measured fluorescence is due to non-specific binding but the individual values obtained for S and F_{nsb} should be treated with caution. The values of the various parameters obtained from Fit 2 can be regarded as adequately fitting the experimental data.

In the case of ANS the inclusion of non-specific binding (with F_{sb} set equal to F_{nsb}) again significantly improved the goodness of fit. Allowing F_{nsb} to differ from F_{sb} further improved the fit but not to a statistically significant extent. The comments made above concerning the high error estimates for S and for F_{nsb}, when the latter was allowed to differ from F_{sb}, apply equally well to the analyses of the ANS data. The values of the various parameters for ANS from Fit 2 (Table 4) give an acceptable fit of the theoretical equations to the paired data-set.

In summary, we have shown that reliable values of the desired parameters K_d, n, and S, as well as the molar fluorescences F_{sb} and F_{nsb}, can be obtained from simultaneous least squares regression analysis of sufficiently accurate fluorescence data. Provided that any error in the fluorescence measurements is random then increased error can be largely counteracted by increasing the number of experiments. This method of analysis has the advantage that it makes full use of all the experimental data and eliminates the need for the graphical methods currently employed to estimate first the molar fluorescence of bound ligand and then the binding parameters. It also permits the investigator to choose the most appropriate model to fit directly the fluorescence data. This method of analysis can be applied to other systems in which some physical property of the ligand differs quantitatively according to whether the ligand is specifically bound, non-specifically bound or in the unbound state. Although it would be possible to devise more elaborate models than those considered here, ultimately the number of parameters which can be evaluated with reasonable accuracy is limited by the amount of data available and by the magnitude of the experimental errors.

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