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A PITFALL IN THE USE OF DOUBLE-RECIPROCAL PLOTS TO ESTIMATE THE INTRINSIC MOLAR FLUORESCENCE OF LIGANDS BOUND TO ALBUMIN

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Abstract—Double-reciprocal plots of fluorescence intensity versus protein concentration are often used to obtain the intrinsic molar fluorescence (F_b) of ligands bound to acceptor molecules such as albumin. In this paper we show that these plots develop upward concave curvature as the concentration of albumin increases. Thus linear extrapolation of such plots cannot be employed to provide accurate values of F_b. It is suggested that a direct plot of fluorescence intensity versus log protein concentration should be employed to obtain F_b.

Fluorescence spectroscopy has been used to study the interaction of various fluorescent ligands with isolated cells [1], organelles such as mitochondria [2, 3], lipid micelles [4] and a number of proteins [5–7]. In order to describe these interactions in a quantitative manner it is usually necessary to measure the intrinsic molar fluorescence of bound ligand (F_b). To estimate F_b it is common practice [1–6] to vary the concentration of protein (or acceptor molecule) at a fixed ligand concentration and then to extrapolate a double-reciprocal plot of fluorescence intensity against protein concentration to infinite protein concentration. It is then assumed that all the ligand is bound so that the resultant fluorescence divided by the concentration of probe gives F_b.

Theoretical analysis of the relationship between fluorescence intensity and protein concentration has led Zierler [8] to suggest that double-reciprocal plots of fluorescence versus protein concentration become non-linear as the concentration of protein increases. Such plots develop an upward concave curvature as protein concentration increases and so, the desired value of F_b cannot be obtained by simple linear extrapolation [8]. The purpose of the present communication was three-fold: first, to report double-reciprocal plots for three fluorescent ligands commonly employed to study ligand–albumin interactions; second, to highlight what we believe is an important but little recognised, pitfall in the use of such methods to estimate F_b and third, to test Zierler's theoretical analysis [8] to see whether it provides a good description of experimental data.

MATERIALS AND METHODS

Materials. Essentially fatty acid free human albumin (Pentex, Fraction V, <0.1 mole fatty acid per mole of protein) was obtained from Miles Laboratories. The magnesium salt of 1-anilino-8-naphthalene sulphonate (ANS) was purchased from Pierce & Warriner. Dansylsarcosine and warfarin were supplied by Sigma.

Fluorescence measurements. All experiments were done at 31° using 0.1 M sodium phosphate buffer, pH 7.4. Fluorescence was measured with a Perkin Elmer MPF-3 Spectrofluorometer and the excitation/emission wavelengths for ANS, dansylsarcosine and warfarin were 400:475; 350:475 and 320:380 nm, respectively. Band width for both excitation and emission was 4 nm.

To obtain F_b, volumes (20 μl) of a solution containing 200 μM albumin and 2 μM probe were added in a cumulative manner to 2 ml of 2 μM probe. Estimates of F_b were obtained in two ways. First from a double-reciprocal plot of fluorescence intensity (F) against albumin concentration ($[P]$), and second from a direct plot of F vs log $[P]$. In the latter case, as log $[P]$ increases F approaches a plateau value (F_∞) and at plateau $F_\infty = F_b\ [D_t]$, where D_t is the total concentration of probe [8]. The values of F_b obtained from double-reciprocal plots are termed F_b (recip) and those from graphs of F vs log $[P]$ are called F_b (log).

To study the binding of probe to a fixed concentration of albumin, 20 μl aliquots of 100 μM probe and 2 μM albumin were titrated with 2 ml of 2 μM albumin. Blank titrations were done by adding 20 μl of 200 μM albumin to 2 ml of buffer and the fluorescence of unbound probe was measured after addition of 20 μl samples of 100 μM probe solution to 2 ml of buffer. All fluorescence measurements were done in triplicate and were corrected for self-absorption where necessary [9]. The concentrations of unbound and bound probe were calculated from the equations described by Maes *et al.* [5].

Analysis of results. For dansylsarcosine and warfarin estimates of n, the number of binding sites, and K_d, the dissociation constant, and their standard errors were obtained by nonlinear least squares regression analysis [10] using the following relationship:

$$r = \frac{n\,[D_u]}{K_d + [D_u]} \qquad (1)$$

where r is the molar ratio of bound ligand to albumin

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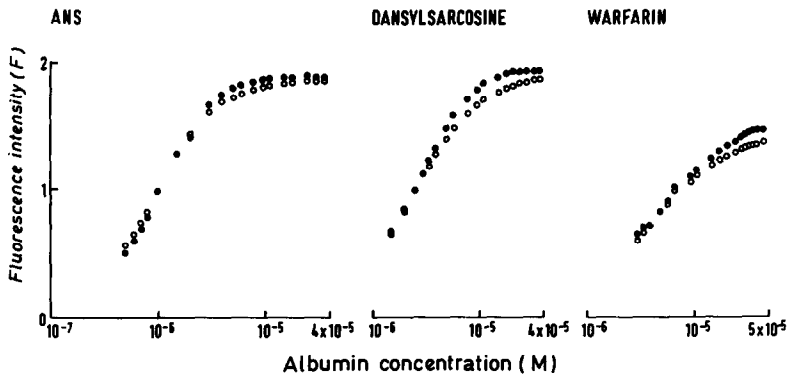

Fig. 1. Fluorescence intensity (F) plotted against log human albumin concentration (log [P]). The concentration of each ligand was 2 μM. Filled circles (●) represent measured values of F and are the mean of three experiments. The standard errors of these data are too small to be shown. Open circles (○) are theoretical values of F (F_{calc}) calculated from equation (3).

and $[D_u]$ is the concentration of unbound ligand. Equation (1) did not satisfactorily fit the data for ANS, so it was assumed that ANS adsorbed to two independent classes of sites: the first class saturable and the second non-saturable over the concentration range of ligand used. For ANS estimates of n and K_d were obtained by regression analysis [10] using the following modification of equation (1):

$$r = \frac{n[D_u]}{K_d + [D_u]} + C[D_u] \quad (2)$$

where C is a constant that represents the quotient of n divided by K_d for the non-saturable sites. Binding data were plotted according to the method of Scatchard [11].

Zierler [8] has shown that the relationship between F and ligand binding is of the form:

$$F = 0.5\,F_b[([D_t] + K_d + n[P]) - (([D_t] + K_d + n[P])^2 - (4n[D_t][P]))^{\frac{1}{2}}] \quad (3)$$

where $[D_t]$ is the total concentration of ligand. In order to test how well equation (3) described the results, values of F_b (log) and n and K_d derived from F_b (log), were used to calculated theoretical values of F (F_{calc}) for each value of $[P]$ from the above relationship. Equation (3) was derived by Zierler [8] to describe binding to a single class of site and its use to calculate F_{calc} for ANS is therefore questionable. However, we have used this equation to fit the ANS data because calculations, using equation (2), suggest that at most 9% of ANS would be bound to non-saturable sites under the experimental conditions used to study the dependence of F on $[P]$.

RESULTS

Graphs of fluorescence intensity (F) vs log albumin concentration (log[P]) are shown in Fig. 1. It can be seen that as log [P] increases F approaches a maximum value, F_∞, from which F_b (log) can be readily calculated for all three ligands. The values of F_b (log) obtained from such plots are listed in Table 1. Figure 2 illustrates the same data when plotted as $1/F$ against $1/[P]$. Initially all graphs were linear, but as the concentration of albumin increased, non-linearity became evident in the form of upward concave curvature. The extent of curvature was greatest for ANS and least for warfarin (Fig. 2). Regression analysis of the linear portions of the graphs displayed in Fig. 2 was used to estimate $1/F_\infty$ from which F_b (recip) was calculated. Values of F_b (recip) are given in Table 1 and it is clear from this table that F_b (recip) is greater than F_b (log) for all three probes. In addition, the differences between the two estimates of F_b increase as curvature of the double-reciprocal plots becomes more marked.

Figure 3 shows the influence values of F_b have on the Scatchard plots for dansylsarcosine and warfarin. Plots calculated from either F_b (log) or F_b (recip) were essentially linear in shape, but those graphs calculated from F_b (recip) were displaced downwards by comparison to those derived from F_b (log) (Fig. 3). The displacement for warfarin was small and there was little difference in either n or K_d for data derived from F_b (recip) or F_b (log) (Table 1). By contrast, the Scatchard plot for dansylsarcosine when calculated from F_b (recip) was displaced to a marked degree (Fig. 3). Estimates of K_d were similar, but n obtained from F_b (recip) was about 50% smaller than its corresponding value when F_b (log) was used to calculate binding data (Table 1). Figure 4 shows that for ANS the Scatchard plot was curvilinear when derived from F_b (log) and almost linear when F_b (recip) was used. Data calculated from F_b (log) were analysed assuming the presence of two independent classes of binding sites. It can be seen from Table 1 that n and K_d vary considerably depending upon whether F_b (recip) or F_b (log) was used to derive binding data.

Using equation (3), that relates F to $[P]$, and estimates of n and K_d obtained from data calculated from F_b (log), theoretical values of F (F_{calc}) were obtained. For each probe, these values are shown as open circles in Fig. 1. It is clear that the correspondence between F and F_{calc} is very good for ANS over the whole range of protein concentration.

Table 1. Intrinsic molar fluorescence of bound ligand (F_b) and binding parameters estimated from either a double-reciprocal plot of fluorescence intensity ($1/F$) vs human albumin concentration ($1/[P]$) or a direct plot of fluorescence intensity (F) against log albumin concentration (log $[P]$)

	Double-reciprocal plot ($1/F$ vs $1/[P]$)			Direct plot (F vs log $[P]$)		
Probe	F_b* (recip) (μM^{-1})	n	K_d ($\times 10^7$ M)	F_b (log) (μM^{-1})	n	K_d ($\times 10^7$ M)
Warfarin	0.82	0.91 ± 0.03†	31 ± 3	0.73	1.01 ± 0.03	31 ± 2
Dansylsarcosine	2.3	0.38 ± 0.004	16 ± 1	0.97	0.83 ± 0.02	10 ± 1
1-Anilino-8-naphthalene sulphonate (ANS)	18	0.15 ± 0.002	53 ± 6	0.94	1.6 ± 0.07	5.2 ± 0.6

* Obtained by regression analysis of the linear portions of the graphs illustrated in Fig. 2.
† Standard error of estimate.

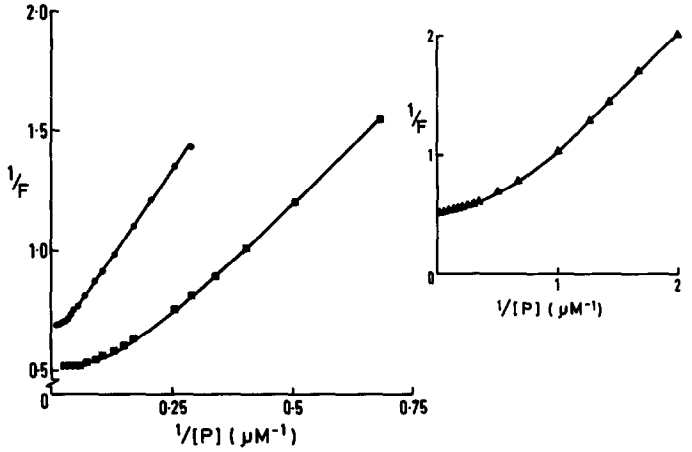

Fig. 2. Double-reciprocal plots of fluorescence intensity (F) against human albumin concentration ($[P]$) for ANS (insert, ▲), dansylsarcosine (■) and warfarin (●). The concentration of each ligand was 2 μM. Points represent mean of three experiments and standard errors are too small to be shown.

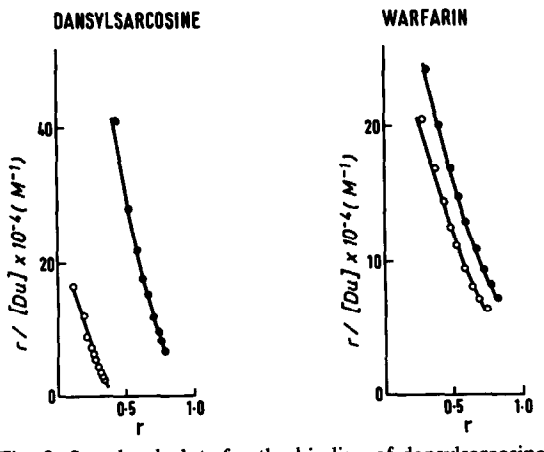

Fig. 3. Scatchard plots for the binding of dansylsarcosine (1.0–13 μM) and warfarin (1.0–13 μM) to 2 μM human albumin at 31°. Filled circles (●) represent points calculated from F_b (log) and open circles (○) were derived from F_b (recip).

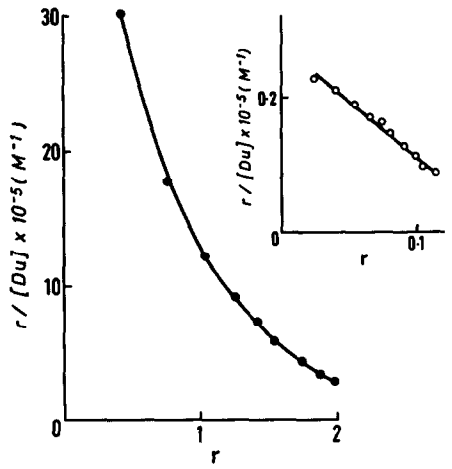

Fig. 4. Scatchard plot for the binding of ANS (1.0–13 μM) to 2 μM human albumin at 31°. Filled circles (●) represent points calculated from F_b (log) and open circles (○), insert, were obtained from F_b (recip).

For dansylsarcosine and warfarin, F and F_{calc} are in close agreement at low values of $[P]$. However, as $[P]$ increases there is a tendency for F_{calc} to be less than F for both probes.

DISCUSSION

The results of this study confirm the prediction of Zierler [8] that plots of $1/F$ vs $1/[P]$ become non-linear as $[P]$ increases. It is also clear that linear extrapolation of such graphs will not provide reliable estimates of F_b. Indeed these estimates are likely to be greater than those obtained from a direct plot of F against log $[P]$. Similar non-linear relationships have been reported in more complicated experimental systems where submitochondrial particles [3, 12] and phosphatidylcholine micelles [4] were used as the acceptor molecules together with ANS [3, 4, 12] and 12-(9-anthroxyloxy)-stearic acid [4] as the ligands. These studies and the present one emphasise the need to study the dependence of F on $[P]$ over the wide range of protein or acceptor molecule concentrations, otherwise double-reciprocal plots may appear linear. The results also indicate that using F_b (recip) to calculate binding data will result in estimates of n and K_d that may be very different from those obtained using F_b (log). In addition, the actual shape of the Scatchard plot may be influenced by whether F_b (recip) or F_b (log) has been used in its construction. Clearly this could affect interpretation of these plots.

Haigh and Sawyer [4] have demonstrated by numerical simulation that the extent to which graphs of $1/F$ vs $1/[P]$ are non-linear appears to depend on the value of K_d. Their simulations showed that non-linearity was first evident when K_d was about 3×10^{-6} M and became more pronounced as K_d decreased. The results presented here support these calculations since curvature was least for warfarin ($K_d = 3.1 \times 10^{-6}$ M) and most marked for ANS ($K_d = 5.2 \times 10^{-7}$ M). However, the absolute value of K_d is unimportant since it is its ratio to $[P]$ that determines curvature. This is well illustrated in Fig. 2 where curvature of the double-reciprocal plot for ANS is present at much lower values of $[P]$ than that for dansylsarcosine or warfarin. Zierler [8] has also shown that the degree to which double-reciprocal plots are curves depends, in part, on the relative sizes of K_d and $[D_t]$, the total concentration of ligand. As $[D_t]$ becomes small with respect to K_d, the plot of $1/F$ vs $1/[P]$ develops a more linear form and in this case F_b (recip) will be in closer agreement with F_b (log). To determine F_b it is usual to use a total ligand concentration of between 2 and 5 μM [2–6]. Under these circumstances, and with albumin concentrations used in this study, non-linearity of double-reciprocal plots is only likely to be seen when $K_d < 10^{-5}$ M, and for those ligands with a $K_d > 10^{-5}$ M it is probable that there will be little difference between F_b (recip) and F_b (log). However, as Zierler [8] has stated, a discrepancy between F_b (recip) and F_b (log) will always exist even if graphs of $1/F$ vs $1/[P]$ approximate to a straight line.

For ligands with a single set of binding sites, Zierler [8] has shown that $1/F$ is related to $1/[P]$ by the equation

$$\frac{1}{F} = \frac{1}{F_\infty} + \left(\frac{K_d}{F_\infty[n - r]}\right)\frac{1}{[P]}.$$

If plots of $1/F$ vs $1/[P]$ are to be linear, then $(K_d/F_\infty[n - r])$ must remain constant. As $[P]$ increases r will also increase so $(K_d/F_\infty[n - r])$ cannot be constant, hence graphs of $1/F$ against $1/[P]$ will not be linear over a wide range of $[P]$ values. The above equation can be altered to give the relationship between F and the independent variables $[D_t]$ and $[P]$, and this is shown in equation (3). We have used equation (3) to ascertain whether or not Zierler's theoretical analysis provides a good description of the dependence of F on $[P]$. To do this theoretical values of fluorescence (F_{calc}) were obtained from equation (3) using n and K_d determined from F_b (log) and these values were then compared to the actual fluorescence intensity (F). The overall agreement between F and F_{calc} was good for all three ligands and this implies that for albumin much of the curvature seen when $1/F$ is plotted against $1/[P]$ is implicit in the mathematical relationship between F and $[P]$. There was, however, a negative deviation of F_{calc} from F for dansylsarcosine and warfarin at higher protein concentrations. The reason for such a deviation is not clear, but physico-chemical factors such as self-association of albumin molecules, light scattering and inadequate compensation for self-absorption may play a role here.

From a practical point of view, the present work has a number of important implications. First, plots of $1/F$ vs $1/[P]$ do not give reliable estimates of F_b, especially when the ligand is highly bound, and when such estimates are used to calculate binding data erroneous values of n and K_d may be obtained. Second, it is preferable to obtain F_b from direct plots of F vs log $[P]$. This method is simple, but relies on the plateau value of fluorescence (F_∞) being achieved before $[P]$ is large enough to interfere with fluorescence measurement. For ligands with a low affinity and/or large number of binding sites, F_∞ may not be reached. In these cases Zierler [8] has suggested that F_b can be estimated from the slopes of graphs of F vs $[P]$ and F against $[D_t]$ as both $[P]$ and $[D_t]$, respectively, tend toward zero. Third, it is relevant to note that double-reciprocal plots are also used to obtain the molar extinction coefficient of bound ligand when difference spectroscopy is employed to quantitate binding. As the relation between change of absorbance and $[P]$ is similar to that between F and $[P]$, it is likely that such plots will give unreliable estimates of the molar extinction coefficient when highly bound ligands are studied.

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