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# COMPARISON BETWEEN BINDING ANALYSES PERFORMED BY EQUILIBRIUM DIALYSIS AND PARTITIONING IN AQUEOUS TWO-PHASE SYSTEMS EXEMPLIFIED BY THE BINDING OF CIBACRON BLUE TO SERUM ALBUMIN

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## SUMMARY

Partitioning in two-phase systems was used as a separation method in binding analyses and compared with equilibrium dialysis. A phase system was chosen to give partitioning conditions with the reactants in different phases. The interaction between serum albumin and Cibacron Blue F3G-A was used as a model system. The protein partitioned to the bottom phase and the ligand to the top phase in a phase system consisting of poly(ethylene glycol) and dextran. When both albumin and Cibacron Blue were present in the phase system, the degree of binding was observed as a translocation of ligand from the top to the bottom phase. An evaluation method that compensates for the amount of free reactant in the phase with the binding protein is presented.

## INTRODUCTION

In binding assays there are two principal ways to determine the degree of binding between two reactants. If the complex formed has a property that is unique in relation to those of the participating single reactants present, this property can be used for measurement in a homogeneous solution. Otherwise, bound and free reactants must be separated in order to perform the measurement. In the second alternative, the speed and efficiency of the separation greatly influence the outcome of the experiment. If the separation is very effective, it may lead to a dissociation of the complex formed. When, *e.g.*, the binding of a ligand to a protein is studied, removal of the free ligand will displace the state of equilibrium. Further, this reaction is time dependent, which makes the time allotted for the separation procedure important[1]. These factors can evidently affect the values obtained in a binding analysis. However, they are difficult to determine and are rarely discussed in the literature. They may nevertheless explain discrepancies between different methods using different separation procedures.

In this work, partition in aqueous two-phase systems was used for the separation. This technique is simple and fast and has proved very useful in im-

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munoassays[2–5]. The points mentioned above of importance for the performance of an assay can be kept under control by choosing a phase system that gives adequate difference in partitioning of the bound and the free reactant. Partitioning has also been used in binding analyses[6,7], but up to now only with the approximation that the binding protein partitions exclusively to one of the phases. Separation in two-phase systems is not only useful when interactions between a binding protein and a ligand are studied, but also between different macromolecules[2] and between cells and proteins[3,4]. The binding reaction between albumin and the dye Cibacron Blue[8] is used as an example to demonstrate how a system is set up and how one evaluates the experimental results when the reactants do not completely partition to different phases.

## MATERIALS AND METHODS

Human serum albumin, "essentially free of fatty acids", was purchased from Sigma (St. Louis, MO, U.S.A.). Cibacron Blue F3GA was purchased as Reactive Blue from Sigma and deactivated by reaction with glucose[9]. All other chemicals used were of analytical-reagent grade.

### *Equilibrium dialysis*

Equilibrium dialysis was performed in cylindrical cells (40 × 25 mm I.D.) made of Delrin, divided by a circular dialysis membrane ("seamless cellulose tubing"; Union Carbide, New York, NY, U.S.A.).

### *Partitioning*

Albumin and the dye were incubated for 10 min in a volume of 100 μl, then 900 μl of a well mixed phase system was added to make a final concentration of 0.070 g/g of PEG 6000 (Union Carbide) and 0.100 g/g of Dextran T-70 (Pharmacia, Uppsala, Sweden). Alternatively, a phase system with a final concentration of 0.135 g/g of PEG 4000 and 0.135 g/g of magnesium sulphate heptahydrate was used. After separation (30 min), an aliquot of 200 μl was taken from each phase and diluted to 1 ml with buffer and the amount of dye was measured spectrophotometrically at 610 nm. An absorption coefficient of $1.04 \cdot 10^4$ l mol$^{-1}$ cm$^{-1}$ was used in all calculations.

### *Kjeldahl analysis*

Kjeldahl analysis of protein content was carried out using a Kjeltec System II from Tecator AB (Helsingborg, Sweden).

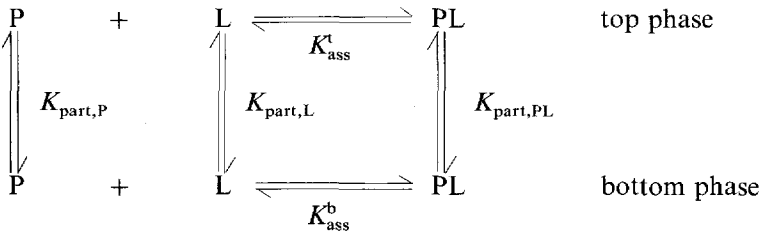
## THEORETICAL

The partitioning of a molecule in a phase system is characterized by the partitioning coefficient, $K_{part}$, defined as

$$K_{part} = C_{top}/C_{bottom} \tag{1}$$

If a ligand, L, is present in a two-phase system together with a binding protein, P, the following scheme shows the situation, where superscript t denotes the top phase and b

the bottom phase




A prerequisite for a binding assay in aqueous two-phase systems is that the reactants favour different phases, so that the degree of binding is observed as a displacement of the observed reactant, in this instance the ligand. The stoichiometry of the binding reaction is not considered at this point. In the binding analysis, the amount of ligand present in each phase after separation is determined. In each phase the ligand is present both free and bound to the binding protein. This can be expressed as in the following equations, with the symbols used here:

$$C^t_{L,tot} = C^t_L + C^t_{PL} \tag{2}$$

$$C^b_{L,tot} = C^b_L + C^b_{PL} \tag{3}$$

The total amount of complex formed, irrespective of the partitioning, has to be determined. A standard situation is that the protein, P, favours the bottom phase, whereas the ligand, L, favours the top phase. The bound ligand, PL, is then found in the bottom phase. So far, the situation is identical with the one in equilibrium dialysis. In partitioning, however, the amount of protein in the top phase must be considered. From these considerations a set of four equations can be used to describe the basic system.

The amount of free ligand in the bottom phase is obtained by rearrangement of eqn. 1 for the ligand:

$$C^b_L = C^t_L / K_{part,L} \tag{4}$$

As an initial approximation, $C^t_{L,tot}$ is used instead of $C^t_L$ in eqn. 4.

The amount of ligand bound to the protein in the bottom phase can then be expressed by

$$C^b_{PL} = C^b_{L,tot} - C^b_L \tag{5}$$

By making an analogous correction for the amount of ligand bound to protein in the top phase, eqns. 6 and 7 are obtained.

$$C^t_{PL} = C^b_{PL} \cdot K_{part,PL} \tag{6}$$

$$C^t_L = C^t_{L,tot} - C^t_{PL} \tag{7}$$

The new value for $C_L^t$ obtained in eqn. 7 can be used in eqn. 4, and the calculation should be repeated until all values are constant. Then we can use $C_L^b$ and $C_{PL}^b$, *e.g.*, for the determination of the stoichiometry and the binding constant with a Scatchard plot[10]. In the Scatchard plots $C_{PL}^b/C_P^b$ is plotted on the abscissa and $C_{PL}^b/(C_L^b \cdot C_P^b)$ on the ordinate.

If the partitioning of the reactants is studied for a system with a different partitioning behaviour of the reactants, *i.e.*, with the binding protein in the top phase and the ligand in the bottom phase, the superscripts b and t should be interchanged and the partitioning coefficients should be inverted in eqns. 4–7.

In a simple model system such as the one depicted above, eqns. 4–7 can be rearranged to give an equation system. Other systems may give more complicated equations that are more difficult or even impossible to rearrange in such a way. The method of making repeated calculations, starting from an approximation, is therefore a more general way of evaluating the experimental data.

The partition constant of the protein–ligand complex, $K_{part,PL}$, in eqn. 6 may not be known before the evaluation of an analysis. As an approximation, $K_{part,PL}$ can be replaced by $K_{part,P}$, which can be determined directly. A good estimation of $K_{part,PL}$ can be obtained indirectly in the following way. The apparent partitioning of the ligand is determined for different amounts of ligand or protein and a corresponding plot is made. For low ligand to protein ratios the curve will approach $K_{part,PL}$ asymptotically, as it can be assumed that most of the ligand is present as a protein–ligand complex under such conditions.

A listing of a computer program in Fortran IV or Basic, which performs the above calculations is available from the authors on request.

## RESULTS AND DISCUSSION

The interaction between human serum albumin and Cibacron Blue was studied with equilibrium dialysis and partition affinity ligand assay (PALA). In both instances different amounts of the dye were added to a constant amount of albumin. The outcome of the equilibrium dialysis analysis is presented as a Scatchard plot in Fig. 1.

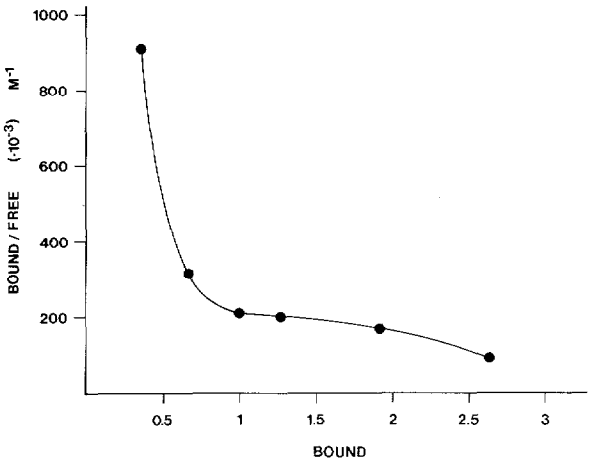



Fig. 1. Scatchard plot for the binding reaction between human serum albumin and Cibacron Blue using equilibrium dialysis to separate free and bound ligand.

The result might be interpreted as a binding situation with several heterogeneous sites with different binding properties. The dye, however, adsorbed to the dialysis membrane, as do many other ligands of a hydrophobic nature. In this way a competitive binding situation arose where both the membrane and the protein competed for binding the ligand. The free ligand concentration used in the Scatchard equation should then be corrected for the amount of ligand adsorbed to the membrane. The association constant given by the Scatchard plot would, however, still be erroneous because the adsorption displaces the equilibrium of the protein–ligand interaction. When the result was recalculated with the free ligand concentration in the Scatchard equation replaced by the total amount of ligand added, the plot in Fig. 2 was obtained. As expected[8], two molecules of Cibracon Blue could bind to one albumin molecule. The association constant was found to be $2.3 \cdot 10^4$ l mol$^{-1}$ ($\pm 0.1 \cdot 10^4$, 2 × S.D.) which corresponds well with published values obtained by frontal analysis chromatography ($0.1 \cdot 10^4$–$4.7 \cdot 10^4$ l mol$^{-1}$)[11] and affinity electrophoresis ($0.56 \cdot 10^4$ l mol$^{-1}$)[12]. Fig. 2 thus gives a better description of the binding reaction than does Fig. 1, although a rather rough approximation is made. It is, however, difficult to make a strict comparison of association constants, as the dye is available only in a low grade of purity and the results may vary between different batches.

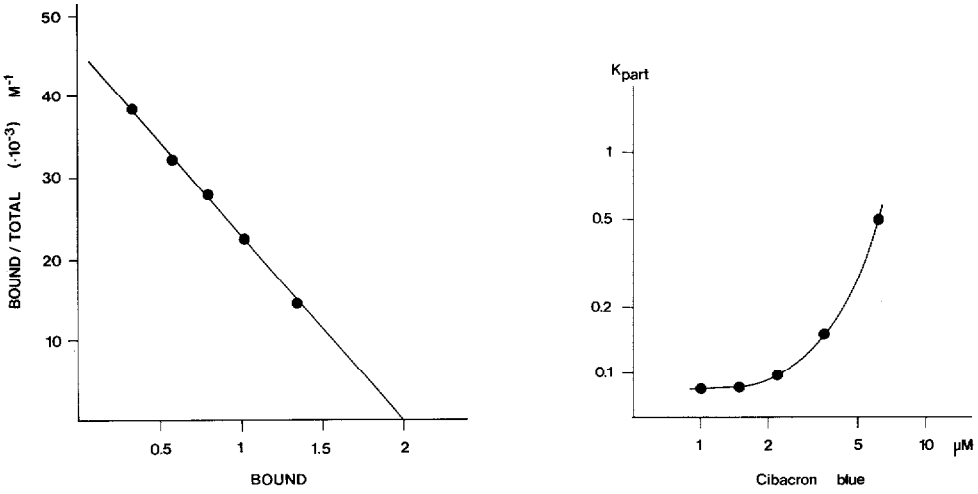



Fig. 2. Scatchard plot for the same system as in Fig. 1, but with the concentration of free ligand in the Scatchard equation replaced by the total concentration of ligand.

Fig. 3. The observed partitioning of Cibacron Blue in two-phase systems when different amounts of dye were mixed with a constant amount of human serum albumin. The concentration of albumin in the lower phase was 76 n*M*.

The same binding assay was studied in a two-phase system consisting of PEG and dextran. First the partitioning properties of the reactants were determined. Albumin had a partition coefficient of 0.137 and Cibacron Blue 7.79. The partitioning of the dye–albumin complex was determined by plotting the partitioning of the dye in the assay as a function of the dye concentration (Fig. 3). When the dye concentration is decreased the measured partitioning approaches that of the protein–ligand complex. In order to confirm the determination of the partition coefficient, the pro-

tein content in each phase was measured in a Kjeldahl analysis. Because of the comparatively low sensitivity, phase systems of 20 ml were used. Protein concentration determinations based on spectrophotometric measurements could not be used, owing to possible interference from the dye. The partitioning coefficients of the dye and the dye–albumin complex were used in the evaluation of the binding analysis as described under Theoretical. An example of the calculations using eqns. 4–7 is given in Table I and the Scatchard plot obtained is shown in Fig. 4. It can clearly be seen that the plot is similar to that in Fig. 2; however, the association constant ($2.6 \cdot 10^4$ l mol$^{-1}$; $\pm 0.1 \cdot 10^4$, 2 × S.D.) obtained is different. The discrepancies between the results obtained by the two methods may be explained by the fact, that the values for equilibrium dialysis (Fig. 2) are calculated using an approximation that does not account for the fact that the adsorption phenomenon may affect the association constant.

TABLE I

CALCULATION OF THE CONCENTRATION OF FREE AND BOUND LIGAND FOR ONE OF THE POINTS IN FIG. 3, USING EQNS. 4–7

| *Iteration number** | *Free ligand (M)* | *Bound ligand (M)* | *Scatchard* | |
|---|---|---|---|---|
| | | | "*Y*" | "*Y*"/*free* (*l mol*$^{-1}$) |
| 1 | $5.76 \cdot 10^{-6}$ | $1.30 \cdot 10^{-4}$ | 1.715 | $2.98 \cdot 10^5$ |
| 2 | $3.87 \cdot 10^{-6}$ | $1.32 \cdot 10^{-4}$ | 1.740 | $4.49 \cdot 10^5$ |
| 3 | $3.84 \cdot 10^{-6}$ | $1.32 \cdot 10^{-4}$ | 1.740 | $4.52 \cdot 10^5$ |
| 4 | $3.84 \cdot 10^{-6}$ | $1.32 \cdot 10^{-4}$ | 1.740 | $4.52 \cdot 10^5$ |

* The iterations were initiated with the following values:
Partition coefficient of Cibacron Blue: 7.79
Partition coefficient of complex: 0.085
Concentration of albumin: $7.60 \cdot 10^{-5}$ *M*
Concentration of ligand in bottom phase: $1.36 \cdot 10^{-4}$ *M*
Concentration of ligand in top phase: $4.49 \cdot 10^{-5}$ *M*

Another phase system, consisting of PEG and magnesium sulphate, was also used for the separation of bound and free ligand. This phase system gave a more efficient separation; $K_{part}$ for albumin was 0.025 and for Cibacron Blue 11.4, and consequently the system caused some dissociation of the protein–dye complex. A binding analysis was made and a Scatchard plot similar to that obtained with the PEG–dextran system was found. However, measurements with low concentrations of the dye were difficult to perform and subject to significantly increased experimental errors.

The binding analysis described above could be utilized as a basic set-up in different assays. In a direct binding assay, the partitioning of a constant amount of Cibacron Blue was measured and plotted as a function of the amount of serum albumin present (Fig. 5). The binding of various ligands could be measured in competitive binding assays, where the ligand and Cibacron Blue competed for the binding to albumin as exemplified in Fig. 6.

From the practical point of view, the difference between equilibrium dialysis

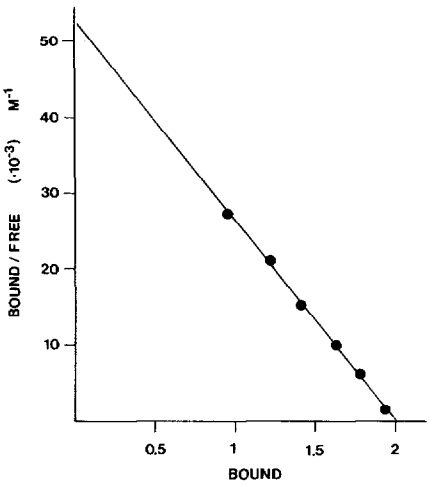



Fig. 4. Scatchard plot for the binding reaction between human serum albumin and Cibacron Blue. Free and bound ligand were separated by partitioning in two-phase systems consisting of PEG and dextran. The concentration of albumin in the lower phase was 76 n*M*.

and PALA is obvious. The former procedure required 24 h to reach equilibrium and one dialysis cell is required for each determination. The PALA procedure required 1 h, including the time for evaluation, and the binding and separation take place in standard test-tubes. Further, PALA has potential for the analysis of interactions between macromolecules and cells and other reactant pairs that are difficult to handle by conventional methods.

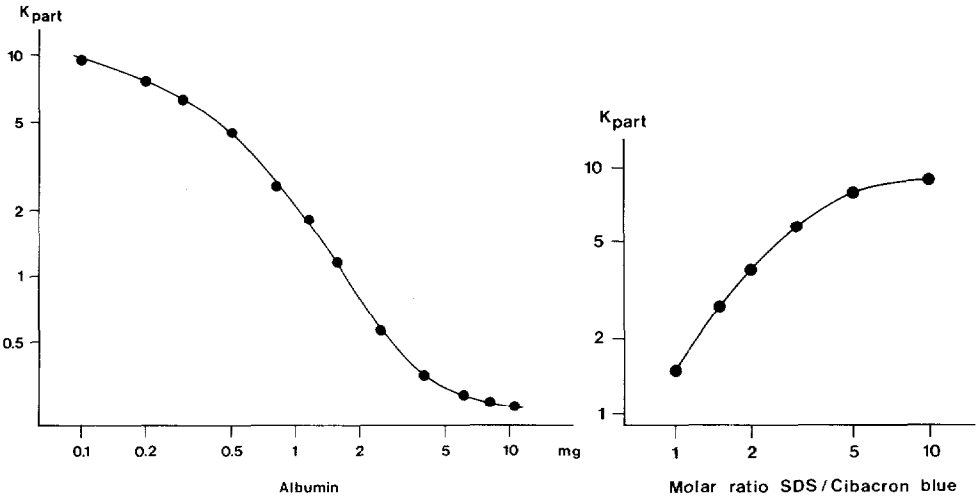



Fig. 5. Direct binding assay of human serum albumin. The partitioning of a constant amount of Cibacron Blue was measured as a function of the amount of albumin present in the system.

Fig. 6. Competitive binding assay of sodium dodecyl sulphate (SDS). SDS and Cibacron Blue competed for the binding to human serum albumin. The partitioning of a constant amount of Cibacron Blue was measured as a function of the amount of SDS present in the system.

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