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# GRAPHICAL ANALYSIS OF COMPETITIVE BINDING OF COMPARABLE CONCENTRATIONS OF LIGAND, INHIBITOR AND PROTEIN

## LIGAND BINDING TO SERUM ALBUMIN

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**Abstract**—In contrast to analysis for competitive binding in enzyme kinetics, no linear plot for analysing competitive binding of two ligands to a protein, where the concentrations of the three reactants are comparable, seems to exist. In the present communication it is shown that in this situation a linear plot can be obtained by the use of the simple equation $\bar{\nu}_A/\bar{\nu}_B = K_A/K_B \times [A_f]/[B_f]$, where $\bar{\nu}_A$ and $\bar{\nu}_B$ are the average number of moles of ligand A and ligand B bound per mole of protein, respectively; $[A_f]$ and $[B_f]$ are the concentrations of free ligand A and free ligand B, respectively; and $K_A$ and $K_B$ are the corresponding association constants. The plot is commented on both theoretically and experimentally using ligand binding to human serum albumin as an example.

A major part of biological events involves interaction between small molecules and macromolecules, e.g. binding of ligand to transport protein, interaction between hormone and hormone receptor, and association of substrate to enzyme. Very often such interactions are influenced by the presence of other ligands. In order to gain insight into the nature of an inhibitory effect, investigations can be carried out in an attempt to determine whether the effect is the result of competition or is caused by more indirect means. In the case of binding of substrate and inhibitor to an enzyme, an analysis of kinetic data by, for example, the Lineweaver–Burk plot [1] can be used to distinguish between these possibilities. A necessary assumption in the calculations carried out is that the concentration of the free form of inhibitor is equal to that of total inhibitor. Ordinarily this approximation introduces only a minimal error because the concentration of inhibitor and substrate usually is very high compared with that of the enzyme. By contrast, no appropriate graphical procedure specifically constructed for that purpose seems to exist in the case of binding of ligand and displacer to a binding protein. Usually one resorts to the double-reciprocal plot based on the formulation of Klotz [2] or a plot analogous to the Scatchard plot [3]. When using these plots it is often assumed, as in the analysis of enzyme kinetics, that the concentration of the free form of the inhibitor can be considered equal to that of total inhibitor. However, when studying binding of ligand and inhibitor to a binding protein, the concentrations of the three reactants usually are comparable in order to secure an acceptable experimental accuracy of the binding data. This implies that the concentration of total inhibitor cannot be used in the analysis and that the concentration of free inhibitor has to be determined and used in the calculations.

Another, and relatively simple, graphical procedure to analyse whether or not two ligands of comparable concentrations bind competitively to a protein is proposed in the present communication. The plot includes the concentrations of both free ligand and free displacer and operates with straight lines. The slopes of the lines can be controlled by independent experiments. The use of the alternative graphical procedure has been illustrated by analysing binding of two ligands to human serum albumin.

### THEORY

The equations which follow deal with the binding of two ligands, A and B, to one site on a protein. If the binding of the ligands conforms to a competitive scheme, the following equations are obeyed:

$$\bar{\nu}_A = \frac{K_A[A_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (1)$$

$$\bar{\nu}_B = \frac{K_B[B_f]}{1 + K_B[B_f] + K_A[A_f]} \quad (2)$$

where $\bar{\nu}_A$ and $\bar{\nu}_B$ are the average number of moles of A and B, respectively, bound per mole of protein; $[A_f]$ and $[B_f]$ are the concentrations of the free forms of A and B, respectively; and $K_A$ and $K_B$ are the corresponding association constants. From equations (1) and (2) the following relationship can be obtained:

$$\frac{1}{\bar{\nu}_A} = \frac{1}{[A_f]}\left(\frac{1 + K_B[B_f]}{K_A}\right) + 1 \quad (3)$$

If experiments are carried out with a great surplus of B, the inhibitor, putting the concentration of total B equal to that of free B may introduce a minimal error. Only in that case can the term in parentheses in equation (3) be regarded as a constant, and plotting $1/\bar{\nu}_A$ as a function of $1/[A_f]$ will result in a straight

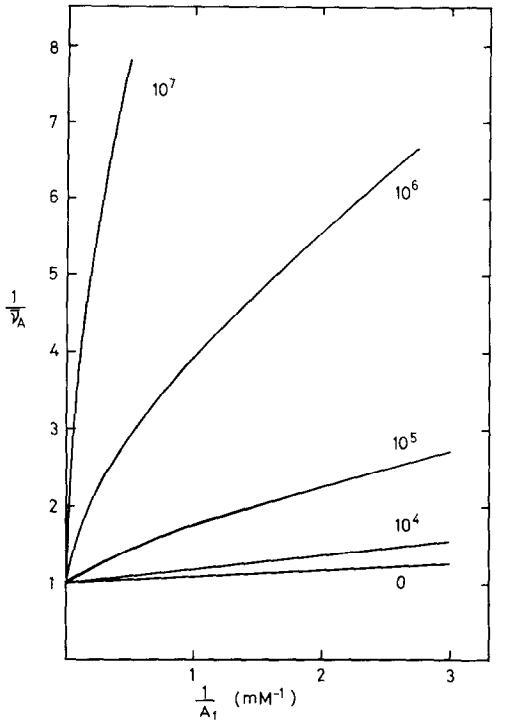


Fig. 1. Theoretical examples of competitive binding of two ligands, A and B, to one site on a protein. The concentrations of total B and protein were constant ($10^{-4}$ M), whereas the concentration of total A varied. The association constant of A was the same in all the examples given ($10^4$ M$^{-1}$), whereas that of B varied as indicated in the figure. Zero denotes binding of A in the absence of B; $\bar{\nu}_A$and $A_f$ represent the average number of moles of A bound per mole of protein and the concentration of free A, respectively. The curves were constructed on the basis of equation (3).

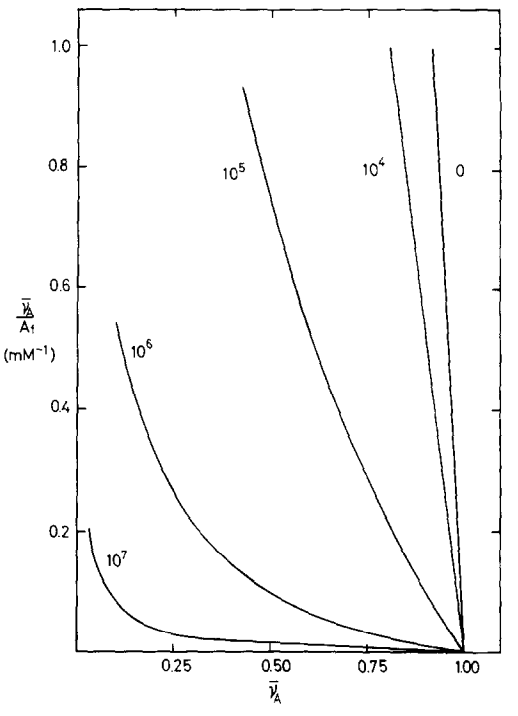


Fig. 2. Theoretical examples of competitive binding of two ligands, A and B, to one site on a protein. The curves were constructed using the same data as in Fig. 1 but plotted according to equation (4).

line intercepting the ordinate axis at 1. By contrast, if the concentrations of the three reactants A, B and protein are comparable, which usually is the case when studying ligand binding to serum albumin, the error introduced by the same assumption invalidates the analysis, because in this case the term in parentheses in equation (3) is not a constant.

The use of comparable concentrations of A, B and protein has two consequences. First, both the concentration of free A and free B has to be determined. Second, plotting $1/\bar{\nu}_A$ as a function of $1/[A_f]$ does not result in straight lines but in hyperbolic curves. This is apparent from Fig. 1, which shows theoretical examples of competitive binding of ligands A and B. Furthermore, it is apparent from Fig. 1 that the course of the curves are dependent on the order of magnitude of the association constants. Using the concentration of total inhibitor and not that of free inhibitor in the calculations influences not only the form but also the position of the curves (not shown). Therefore, an estimation of the inhibitor's association constant from such a graphical analysis will be incorrect if this erroneous approximation is used. The course of the curves is also dependent on the amount of B added (not shown).

Rearranging equation (3) to a form analogous to the Scatchard equation gives the following relationship:

$$\frac{\bar{\nu}_A}{[A_f]} = 1 - \bar{\nu}_A \left( \frac{K_A}{1 + K_B[B_f]} \right) \qquad (4)$$

The theoretical examples given in Fig. 1 are presented in Fig. 2, calculated according to equation (4). It is seen that also in this plot the results do not describe straight lines but hyperbolic curves, the position and course of which are dependent on the association constants. It is also seen from Fig. 2 that the possibility of misinterpretation exists if the study does not include experiments carried out at low $\bar{\nu}_A/[A_f]$ values. In that case it could wrongly be concluded that $\bar{\nu}_A$ is reduced by the presence of ligand B. Thus, using this plot it is essential to carry out experiments at relatively high ligand–protein molar ratios. This can complicate the analysis if the protein possesses more binding sites for the ligand. Schary *et al.* [4] and Aarons *et al.* [5] have calculated that variations in the concentration of total B also result in hyperbolic curves with principally the same form as the curves in Fig. 2.

Taking the ratio of equations (1) and (2), the following equation is obtained:

$$\frac{\bar{\nu}_A}{\bar{\nu}_B} = \frac{K_A}{K_B} \times \frac{[A_f]}{[B_f]} \qquad (5)$$

From equation (5) it is seen that plotting $\bar{\nu}_A/\bar{\nu}_B$ as a function of $[A_f]/[B_f]$ results in a straight line for any

pair of ligands bound to a common site on a protein. The slope of the line is equal to the ratio of $K_A$ and $K_B$. This value can be checked by determining the ratio of the binding constants of A and B, respectively, without the presence of the other ligand. In the following this alternative plot is used to analyse simultaneous binding of L-tryptophan and diazepam to human serum albumin.

## MATERIALS AND METHODS

Binding of L-tryptophan and diazepam to defatted human serum albumin was determined by ultrafiltration. The experimental data are taken from a recent publication from this laboratory [6].

## RESULTS AND DISCUSSION

In order to evaluate the use of equation (5), binding of a pair of ligands to human serum albumin was studied. The simplest way of testing the applicability of the graphical procedure would be to study binding of ligands to a protein which possesses only one binding site for the ligands. Binding of ligands to serum albumin is usually characterized by the existence of more than one binding site. Most often the binding can be described in terms of one to two high-affinity binding sites and a greater number of binding sites with lower association constants [7]. As an approximation to the simpler situation, binding of ligands with only one high-affinity binding site [6] was used in the present context. Furthermore, $\bar{\nu}$ was kept below 0.4 in order to minimize ligand binding to secondary sites.

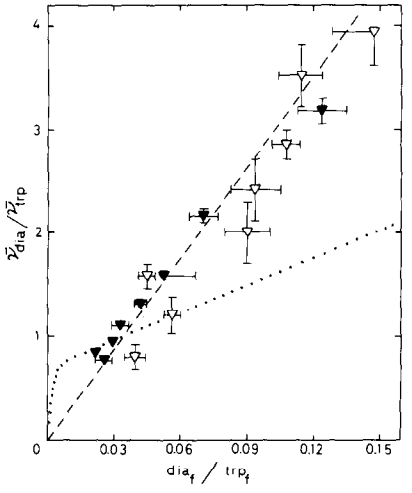


Fig. 3. Binding of L-tryptophan and diazepam to human serum albumin. Binding of L-tryptophan (0.025–0.110 mM) to albumin (▼) in the presence of diazepam (0.067 mM), and binding of diazepam (0.034–0.153 mM) to albumin (▽) in the presence of L-tryptophan (0.050 mM). The concentration of albumin was 0.38 mM, and the media contained 33 mM sodium phosphate buffer, pH 7.0, 20°. Each point represents the average of three duplicate experiments. The broken line is a theoretical curve for binding of the two ligands, assuming competition for a common high-affinity binding site on albumin, calculated according to equation (5). The dotted curve was calculated assuming independent binding of the two ligands to albumin. $\bar{\nu}_{trp}$ and $\bar{\nu}_{dia}$ represent the average number of moles of L-tryptophan and of diazepam bound per mole of albumin, respectively, and $trp_f$ and $dia_f$ are abbreviations of the concentration of free L-tryptophan and free diazepam, respectively.

Figure 3 shows the data for binding of L-tryptophan and diazepam to albumin. The broken regression line is a theoretical curve drawn through the origin of the axes of abscissa and ordinate with the slope $K_D/K_T$, i.e. assuming competitive binding of the two ligands to a common high-affinity binding site on the protein. $K_D$ and $K_T$ are the association constants for diazepam ($4.7 \times 10^5\ M^{-1}$) and L-tryptophan ($1.6 \times 10^4\ M^{-1}$), respectively [6]. The dotted binding curve was calculated assuming independent ligand binding. It is seen from the figure that the two curves are very different. The experimental results follow the broken line and not the dotted curve, indicative of competitive binding of L-tryptophan and diazepam to a common high-affinity binding site on albumin.

The possibility of competitive ligand binding to albumin can also be analysed by a non-linear graphical procedure. With the aid of equations (1) and (2) and the following relationship

$$[B_t] = [B_f] + \bar{\nu}_B [P] \qquad (6)$$

where $[B_t]$ and [P] represent the concentrations of total B and total protein, respectively, a binding curve for ligand A in the presence of a constant concentration of total B can be calculated. In an analogous way a binding curve for ligand B in the presence of a constant concentration of total A can be constructed [6]. If the experimental results follow the hereby calculated binding curves, competitive binding of the two ligands is probable.

The last mentioned procedure is able to single out the effect of ligand A on the binding of ligand B from the effect of ligand B on the binding of ligand A. In contrast, the procedure based on equation (5) collects all experimental results in one analysis. This plot operates with straight lines, which facilitate an objective evaluation of the results. Another aspect in the use of the plot is that it is based on the ratios of experimentally determined data. In this way the effect of experimental errors is reduced.

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