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A method to estimate binding constants at variable protein concentrations

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The association constants of the binding of chlorpromazine and imipramine to serum albumin at low saturation of the protein were determined by a new experimental approach with the protein concentration rather than the ligand concentration being varied. This approach is suitable for estimating binding constants in systems with one class of binding sites. In addition, the method is proposed to complement conventional binding studies of systems with two classes of binding constant with higher accuracy.

Studies of the binding of small molecules to macromolecules, e.g. drugs to proteins, are of great importance in pharmacology and biochemistry. In conventional binding studies, the concentration of the drug is varied, the free drug concentrations are measured, .and the association constants and binding capacities of the system are determined by graphical or computerized methods (Baulieu & Raynaud 1970; Blondeau & Robel 1975; De Meyts & Roth 1975; Fletcher & Spector, 1968, 1977; Klotz & Hunston 1975; Ohnishi et al 1972; Perrin et al 1974; Rosenthal 1967; Scatchard 1949; Thompson & Klotz 1971; Weder et al 1974; Woosley & Muldoon 1976). Most of these methods are based on a model of a stepwise equilibrium (Baulieu & Raynaud 1970; Fletcher & Spector 1968, 1977; Klotz & Hunston 1975; Ohnishi et al 1972; Perrin et al 1974; Scatchard 1949; Thompson & Klotz 1971). With all of these methods, the binder concentration is kept constant and the concentration of the ligand is varied. Accurate calculation of all binding data, especially of systems with two classes of binding sites or with specific sites in the presence of non-specific sites, is hampered by considerable experimental errors. Even though some of the methods are known to be more accurate than others (Weder et al 1974; Atkins & Nimmo 1975; Feldman 1972; Nimmo et al 1977; Vallner et al 1976; Woosley & Muldoon 1977), all of them have serious drawbacks.

The number of classes of binding sites determined is often unreliable due to an inadequate theoretical and experimental basis (Baulieu & Raynaud 1970; Klotz & Hunston 1975; Ohnishi et al 1972; Feldman 1972).

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Negative cooperativity is not distinguishable from binding to more than one class of binding sites (De Meyts & Roth 1975; Klotz & Hunston 1975; Perrin et al 1974; Thompson & Klotz 1971). The distinction between the contribution of specific and non-specific binding in one system is difficult (Winkler & Hübner 1977).

Even if the correct model for the binding system is chosen, experimental errors may still be too large for binding parameters to be accurately estimated (Weder et al 1974; Vallner et al 1976).

Variation of the ligand concentration over a wide range is required. The data at low protein saturation are of particular importance for an accurate determination of binding parameters (Weder et al 1974; Vallner et al 1976). Because of experimental and statistical reasons, errors in most binding models become large in this range. If binding constants of different classes of binding sites differ only little it is almost impossible to calculate them accurately (Weder et al 1974; Vallner et al 1976).

In this study an experimental approach has been developed which allows the accurate determination of the binding constant of a ligand to a protein at low saturation of the protein. This approach may avoid serious disadvantages in estimating binding parameters that arise because of lack of accuracy of data at low protein saturation.

Binding of chlorpromazine and imipramine to serum albumin by the equilibrium dialysis technique illustrates the validity of the proposed approach.

MATERIALS AND METHODS

Experimental procedure

Chlorpromazine (Bayer, GFR), imipramine (Ciba-Geigy, Switzerland), fraction V bovine serum albumin (Miles Lab., USA), and buffer reagents

(analytical grade) were used without further purification. [^{14}C] Imipramine was purchased from The Radiochemical Centre, Amersham (U.K.), [^{14}C] chlorpromazine from Applied Science Labs (U.S.A.).

Dialysis experiments were carried out according to Weder & Bickel (1970a, b); Weder et al (1971) with a Dianorm apparatus. Visking cellulose membranes (Union Carbide, USA) of 25 μm thickness and an exclusion limit of 10 000–20 000 daltons were used. Dialysis experiments were performed for 2½ h at 37° C, pH 7·4.

The concentration of imipramine was determined by counting the radioactivity of [^{14}C] imipramine in both dialysis chambers with a Packard Tri-Carb liquid scintillation counter. Chlorpromazine was measured colorimetrically (Auterhoff & Kühn 1973; Cimbura 1972) or by counting the radioactivity of [^{14}C] chlorpromazine.

Calculations were carried out with a Hewlett-Packard 9800 programmable calculator.

Theory

In a system with one class of binding sites the binding equilibrium

$$nL + B \leftrightharpoons BL_n$$

can be described by the binding equation

$$r = \frac{n\,K\,L_f}{1 + K\,L_f} \quad .. \quad .. \quad .. \quad (1)$$

where r represents the binding degree (moles of ligand bound per mole of macromolecule), n the number of binding sites, L_f the concentration of unbound ligand and K the association constant. Because

$$r = \frac{L_b}{B_o} \quad .. \quad .. \quad .. \quad (2)$$

(L_b: concentration of bound ligand, B_o: total binder concentration) and

$$n = \frac{L_{b\,max}}{B_o} \quad .. \quad .. \quad .. \quad (3)$$

($L_{b\,max}$: maximum concentration of ligand bound) equation 1 can be rewritten as

$$L_b = \frac{L_{b\,max}\,L_f\,K}{1 + L_f\,K} \quad .. \quad .. \quad (4)$$

If the experimental conditions are chosen in the way that the total ligand concentration L_o is very small compared with the binder concentration B_o, i.e.

$$L_o << B_o \quad .. \quad .. \quad .. \quad (5)$$

only few binder molecules can bind a ligand molecule, the concentration of binder associated with ligand B_b becomes negligible relative to the total binder concentration B_o, and the free binder concentration B_f approximately equals the total binder concentration B_o:

$$B_f \approx B_o \quad .. \quad .. \quad .. \quad (6)$$

If in this low saturation range each binder molecule binds one ligand molecule at the most (in a non-cooperative process), then the number of moles ligand bound equals the number of occupied binder molecules:

$$B_b = L_b \quad .. \quad .. \quad .. \quad (7)$$

$$B_{b\,max} = L_{b\,max} \quad .. \quad .. \quad (7a)$$

If the protein concentration is varied and the ligand concentration is kept constant, the protein can formally be considered as the ligand and the drug as the binder at the described low saturation range. Because of the validity of equations 6, 7, and 7a in this range, equation 4 can then be transformed:

$$L_b = \frac{L_{b\,max}\,B_o\,K}{1 + B_o\,K} \quad .. \quad .. \quad (8)$$

Equation 8 corresponds to the Michaelis-Menton equation. Analogue transformations of these kinetic equations can be made to obtain linear graphs in order to calculate binding parameters:

$$\frac{1}{L_b} = \frac{1}{L_{b\,max}\,K}\,\frac{1}{B_o} + \frac{1}{L_{b\,max}} \quad .. \quad (9)$$

according to Lineweaver & Burk (1934).

$$\frac{L_b}{B_o} = K\,L_{b\,max} - K\,L_b \quad .. \quad .. \quad (10)$$

according to Scatchard (1949)

$$L_{b\,max} = L_b + \frac{L_b}{B_o}\,\frac{1}{K} \quad .. \quad .. \quad (11)$$

according to Eisenthal & Cornish-Bowden (1974). Each of these three equations allows the determination of binding data graphically or by computer. Plots according to Scatchard and to Lineweaver and Burk were fitted by linear regression. The direct linear plot, equation 11, was solved for each pair of data points (B_o, L_b) and (B_o', L_b') as follows (Woosley & Muldoon 1977):

$$L_b' = \frac{L_{b\,max}\, B_o'}{\frac{1}{K} + B_o'} \quad \ldots \quad (12)$$

Equation 11 for (B_o, L_b) in equation 12:

$$L_b' = \frac{L_b + \frac{\frac{1}{K} L_b}{B_o}}{1 + \frac{1}{K\, B_o}} \quad \ldots \quad (13)$$

Solved for K

$$K = \frac{\frac{L_b}{B_o} - \frac{L_b'}{B_o'}}{L_b' - L_b} \quad \ldots \quad (14)$$

For m experimental points $\frac{1}{2}$ m (m − 1) estimates for K are obtained. The median rather than the mean of these $\frac{1}{2}$ m (m − 1) values is chosen as the best estimate for the binding constant (Cornish-Bowden & Eisenthal 1974).

RESULTS

Figs 1–4 show results of binding experiments with chlorpromazine or imipramine and bovine serum albumin at very low protein saturation. The concentration of the drugs was 5 μM and the protein concentration was varied from 75 to 1810 μM (5 to 120 mg ml^{-1}). The protein to ligand ratio B_o/L_o was 25 to 360. The results are presented according to Lineweaver & Burk (1934), to Scatchard (1949), and to Eisenthal & Cornish-Bowden (1974).

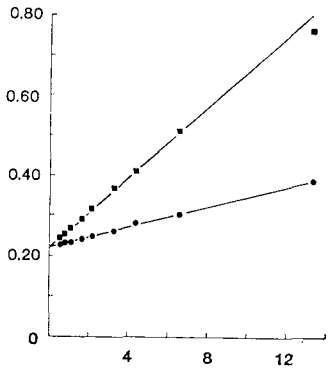

FIG. 1. Binding of chlorpromazine and imipramine to bovine serum albumin plotted according to Lineweaver and Burk. Protein concentration varied from 75 to 1800 μM, 37°C, pH 7·4. ● chlorpromazine 5 μM (correlation coefficient 0·9995) ■ imipramine 5 μM (correlation coefficient 0·9989). Each point is the median of four experiments. Ordinate: $1/L_b$ [μM^{-1}]. Abscissa: $1/B_o$ [mM^{-1}].

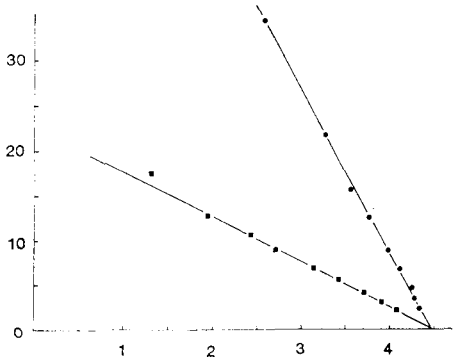

FIG. 2. Binding of chlorpromazine and imipramine to bovine serum albumin plotted according to Scatchard. Protein concentration varied from 75 to 1800 μM, 37°C, pH 7·4. ● chlorpromazine 5 μM (correlation coefficient 0·9989) ■ imipramine 5 μM (correlation coefficient 0·9968). Each point is the median of four experiments. Ordinate: $L_b/B_o \times 10^3$. Abscissa: L_b [μM].

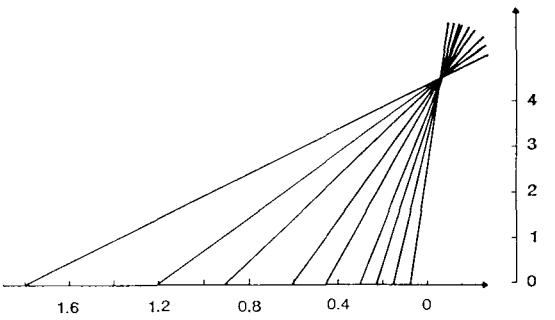

FIG. 3. Direct linear plot of the binding of chlorpromazine to bovine serum albumin. Protein concentration varied from 75 to 1800 μM, chlorpromazine 5 μM, 37°C, pH 7·4. Each line is the median of four experiments. Ordinate: L_b max [μM]. Abscissa: 1/K [mM].

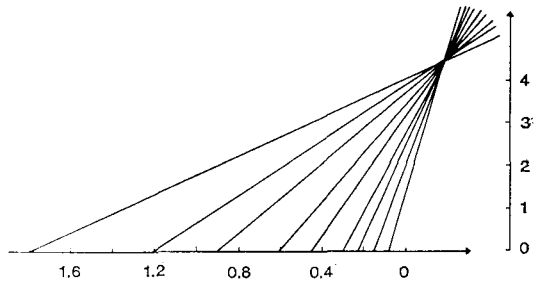

FIG. 4. Direct linear plot of the binding of imipramine to bovine serum albumin. Protein concentration varied from 75 to 1800 μM, 37°C, pH 7·4, chlorpromazine 5 μM. Each line is the median of four experiments. Ordinate: L_b max [μM]. Abscissa: 1/K [mM].

Results obtained at lower protein to drug ratios B_o/L_o (0·015 to 0·6) are shown in Fig. 5. The ratio of protein to bound drug B_o/L_b varied from about 1 to 2 with imipramine and 0·5 to 1 with chlorpromazine in these experiments.

In Table 1, the results obtained in this study and analysed by different methods are compiled and compared with selected data of the literature.

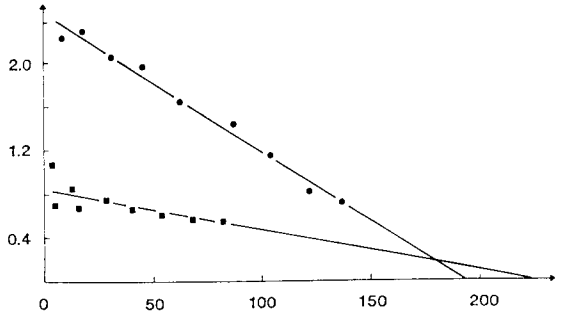

FIG. 5. Binding of chlorpromazine and imipramine to bovine serum albumin plotted according to Scatchard. Protein concentration varied from 4 to 150 μM, 37°C, pH 7·4, ● chlorpromazine 250 μM (correlation coefficient 0·9938) ■ imipramine 250 μM (correlation coefficient 0·7835). Each point is the median of four experiments. Ordinate: L_b/B_o. Abscissa: L_b [μM].

DISCUSSION

In Figs 1–4 results obtained at very low saturation of the protein are presented in three frequently used plots. As stated by several authors (Atkins & Nimmo 1975; Nimmo et al 1977; Woosley & Muldoon 1977), the direct linear plot is the method of choice for estimating an association constant of a ligand binding to one class of binding sites in experiments with low statistical variability. If experimental errors become larger, a linearized graph (Scatchard, Lineweaver-Burk) may be more suitable. In the direct linear plot one cannot distinguish between large variation and binding to more than one class of binding sites (Kolassa & Turnheim 1976). Because of the well-known drawbacks of the presentation according to Lineweaver and Burk (1934) (Atkins & Nimmo 1975; Nimmo et al 1977; Woosley & Muldoon 1977) data with large experimental errors were presented according to Scatchard (1949).

The direct linear plot (Figs 3 and 4) illustrates the usefulness of the experimental approach presented in this study: a very small experimental error occurred when the protein concentration rather than the ligand concentration was varied. This allowed accurate estimation of the association constant. The calculated constants were within a range of constants determined by other authors (Table 1).

The determination of the first association constant K_1 in models with two classes of binding sites is crucial in the characterization of the interactions of small molecules with macromolecules. Several authors showed how experimental errors affect the estimation of K_1 (Weder et al 1974; Vallner et al 1976). K_1-values determined by commonly used methods (Beaulieu & Raynaud 1970; Blondeau & Robel 1975; Ohnishi et al 1972; Perrin et al 1974; Scatchard, 1949) with variation of L_o are usually inaccurate, especially when the two association constants K_1 and K_2 differ by a factor of less than 10 (Weder et al 1974; Vallner et al 1976). The

Table 1. Association constants of chlorpromazine (CPZ) and imipramine (IP) binding to bovine serum albumin (BSA).

Drug	(BSA)	Exper. method	pH	Ionic strength	°C	Analyt. method	K (M^{-1})	Ref.
CPZ 5 μM	variable	ed	7·4	0·20	37	LB	$1·79 \times 10^4$	this study
						S	$1·79 \times 10^4$	this study
						DL	$1·78 \times 10^4$	this study
CPZ 250 μM	variable	ed	7·4	0·20	37	S	$1·14 \times 10^4$	this study
CPZ variable	1%	gf	7·4	0·20	22	S	$2·1 \times 10^4$	Krieglstein 1972
	2%	ed	7·0	0·07	10	LB	$6·17 \times 10^3$	Nambu 1972
			6·0	0·07	10	LB	$4·39 \times 10^3$	Nambu 1972
			5·2	0·07	10	LB	$2·17 \times 10^3$	Nambu 1972
IP 5 μM	variable	ed	7·4	0·20	37	LB	$5·55 \times 10^3$	this study
						S	$5·29 \times 10^3$	this study
						DL	$4·99 \times 10^3$	this study
IP 250 μM	variable	ed	7·4	0·20	37	S	$4·59 \times 10^3$	this study
IP variable	1%	gf	7·4	0·20	22	S	5×10^3	Glasser 1970
IP variable	2×10^{-5} M	ed	7·4	0·19	20	S	$6·00 \times 10^3$	Weder 1970b
					4	S	$10·60 \times 10^3$	Weder 1970b
	1×10^{-5} M	uc	7·4	0·04	21	S	$7·81 \times 10^3$	Weder 1970b

Experimental methods: ed equilibrium dialysis, gf gel filtration, uc ultracentrifugation. Analytical methods: LB according to Lineweaver and Burk, S according to Scatchard, DL direct linear plot.

experimental points obtained at low protein saturation which are important in order to calculate the whole set of binding parameters (Weder et al 1974; Vallner et al 1976), are seriously biased by experimental errors: the lower the concentration of a ligand, the less accurate will its determination be. Mathematical treatments such as the plotting of the interdependant values L_f and L_b against each other, magnify the experimental errors (Weder et al 1974; Vallner et al 1976). The approach presented in this study avoids this disadvantage: only one measured quantity, L_b, is plotted against B_0 or a function of it. Since B_0 is prefixed rather than measured with experimental error, statistical errors become smaller.

For a model with two classes of binding sites

$$r = \frac{n_1 K_1 L_f}{1 + K_1 L_f} + \frac{n_2 K_2 L_f}{1 + K_2 L_f} \quad .. \quad .. (15)$$

n_1 is an integer number in a non-cooperative binding process (Klotz & Hunston 1975; Scatchard 1949) which can easily be estimated even if considerable experimental errors occur. The parameters of the second class of binding sites can be estimated by graphical (Rosenthal 1967; Thompson & Klotz 1971; Feldman 1972; Vallner et al 1976) or numerical (Baulieu & Raynaud 1970; Blondeau & Robel 1975; Fletcher & Spector 1968, 1977; Ohnishi et al 1972; Perrin et al 1974; Weder et al 1974; Vallner et al 1976) parameter-fitting, when K_1 and n_1 are determined by the proposed alternative method.

The results obtained at higher saturation of the protein (Fig 5, Table 1) were not only less accurate, but can lead to wrong conclusions. Since the theoretical approximations ($B_f = B_0$, $B_b = L_b$) on which the model was based are no longer valid. The apparent constants obtained by linearization of the data in Fig 5 should not be interpreted as true binding constants.

The advantages of the method proposed in this paper can be summarized as follows:

The association constant of a system with one class of binding sites (e.g. receptors) can be determined with high accuracy.

In a system with two classes of binding sites, inaccuracy in estimating K_1 with conventional methods can be avoided. Experimental treatment of this binding system can be complemented by the presented approach for low protein saturation.

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REFERENCES

Atkins, G. L., Nimmo, I. A. (1975) Biochem. J. 149: 775–777

Auterhoff, H., Kühn, J. (1973) Arch. Pharmaz. 306: 241–248

Baulieu, E. E., Raynaud, J. P. (1970) Eur. J. Biochem. 13: 293–304

Blondeau, J. P., Robel, P. (1975) Ibid. 55: 375–384

Cimbura, G. (1972) J. Chromatogr. Sci. 10: 287–293

Cornish-Bowden, A., Eisenthal, R. (1974) Biochem. J. 139: 721–730

De Meyts, P., Roth, J. (1975) Biochem. Biophys. Res. Commun. 66: 1118–1126

Eisenthal, R., Cornish-Bowden, A. (1974) Biochem. J. 139: 721–730

Feldman, H. A. (1972) Anal. Biochem. 48: 317–338

Fletcher, J. E., Spector, A. A. (1968) Computers Biomed. Res. 2: 164–175

Fletcher, J. E., Spector, A. A. (1977) Mol. Pharmacol. 13: 387–399

Glasser, H., Krieglstein, J. (1970) Naunyn-Schmiedeberg's Arch. Pharmacol. 265: 321–334

Klotz, I. M., Hunston, D. L. (1975) J. Biol. Chem. 250: 3001–3009

Kolassa, N., Turnheim, K. (1976) Experientia 32: 1355–1356

Krieglstein, J., Meiler, W., Staab, J. (1972) Biochem. Pharmacol. 21: 985–997

Lineweaver, H., Burk, D. (1934) J. Am. Chem. Soc. 56: 658–666

Nambu, N., Nagai, T. (1972) Chem. Pharm. Bull. 20: 2463–2470

Nimmo, I. A., Atkins, G. L., Strange, R. C., Percy-Robb, I. W. (1977) Biochem. J. 165: 107–110

Ohnishi, T., Masoro, E. L., Bertrand, H. A., Yu, B. P. (1972) Biophys. J. 12: 1251–1265

Perrin, J. H., Vallner, J. J., Wold, S. (1974) Biochim. Biophys. Acta 371: 482–490

Rosenthal, H. (1967) Anal. Biochem. 20: 525–532

Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51: 660–672

Thompson, C. J., Klotz, I. M. (1971) Arch. Biochem. Biophys. 147: 178–185

Vallner, J. J., Perrin, J. H., Wold, S. (1976) J. Pharm. Sci. 65: 1182–1187

Weder, H. J., Bickel, M. H. (1970a) Z. Anal. Chem. 252: 253–255

Weder, H. J., Bickel, M. H. (1970b) J. Pharm. Sci. 59: 1563–1569

Weder, H. G., Schildknecht, J., Kesselring, P. (1971) Am. Lab. (10): 15–21

Weder, H. G., Schildknecht, J., Lutz, R. A. Kesselring, P. (1974) Eur. J. Biochem. 42: 475–481.

Winkler, E., Hübner, G. (1977) Stud. Biophys. 66: 211–216

Woosley, J. T., Muldoon, T. G. (1977) J. Steroid Biochem. 8: 625–629

Woosley, J. T., Muldoon, T. G. (1976) Biochem. Biophys. Res. Commun. 71: 155–160