Biochemical Pharmacology, Vol. 45, No. 8, pp. 1715–1720, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

New procedure for analysing drug binding data, exemplified by warfarin binding to human serum albumin

(*Received* 4 *September* 1992; *accepted* 25 *January* 1993)

Abstract—Determination of binding constants for multiple binding of a ligand usually results in highly variable figures. We have found that the variations depend mainly upon cooperativity of ligand binding, and that cooperativity is generally absent on binding to human serum albumin. When this is taken into account it becomes possible to obtain binding constants with only slight variation. A computerized curve fitting procedure for analysing binding data has been established consisting of the following steps. (1) Fitting of Scatchard's equation to observed binding equilibrium data to obtain a best-fit set of Scatchard binding constants. (2) Repetition of the fitting procedure, not to obtain best fit but to generate 30 acceptable sets of Scatchard binding constants. (3) Fitting of Adair's equation to the observed points to obtain a best fit. If the sum of weighted and squared deviations is significantly smaller than the fitting of Scatchard's equation, cooperativity should be considered. If not, cooperativity cannot be demonstrated and the binding constants obtained by fitting Scatchard's equation can be accepted, with the variations found. (4) Final transformation of all Scatchard constants to Adair's. To illustrate the method warfarin data obtained by equilibrium dialysis was used.

Results of binding equilibrium studies are normally obtained as a number of points representing free and bound drug concentrations. Experimentally such measurements are straightforward. However analysing the binding data is a much more complicated task. Experimental binding data, resulting from measurement of ligand binding to macromolecular carriers, are usually described by fitting of binding constants. Determination of the constants usually results in higher variable figures, partly because cooperative solutions are included. Furthermore it is well known that cooperativity is generally absent on binding to human serum albumin. Taking this into account we propose in the present paper a new computerized curve fitting procedure giving binding constants with only slight variation. The method is illustrated with warfarin as an example because it is a well known drug and is used as a marker substance for a specific binding site on the albumin molecule [1, 2].

Analysis procedure

Data. Binding of warfarin was studied by equilibrium dialysis using the same procedure as described by Larsen *et al.* [3], 230 points were obtained. To illustrate cooperative phenomena we used the classical oxygen–haemoglobin binding data taken from Winslow *et al.* [4].

Binding equations. Binding equilibrium of a ligand with a carrier is normally described by Scatchard's equation [5]

$$v = \sum_{i=1}^{j} \frac{n_i c k_i}{1 + c k_i}$$

where v is the molar concentration of bound ligand divided by the concentration of albumin, c is the free ligand concentration, n_i and k_i represent the number of binding sites and the corresponding association constants (Scatchard constants) in the ith binding class, and j is the number of binding classes. In the following it is assumed that n_i is systematically equal to one in each class of binding sites. It is well known that even a good fit (a set of site association constants) to the experimental data does not prove that binding takes place to pre-existing independent sites [6–9]. Several writers [10–12] have proposed another model which does not assume anything about the binding mechanism. This model is a consequence of a stoichiometrical consideration and v can thereby be expressed by the Adair equation [9]:

$$v = \frac{cK_1 + 2c^2K_1K_2 + 3c^3K_1K_2K_3 + \cdots + Nc^NK_1K_2K_3 \cdots K_N}{1 + cK_1 + c^2K_1K_2 + c^3K_1K_2K_3 + \cdots + c^NK_1K_2K_3 \cdots K_N}$$

where K_1, K_2 . . . K_N are step constants (stoichiometric binding constants or Adair's constants). This equation can be used without knowledge of the actual binding mechanism if it is a homogeneous protein and only one binding ligand is present. The two binding equations are mathematically identical, when the same number of constants are used, since Adair's equation can be derived from Scatchard's by giving a common denominator to all terms [6, 7]. The stoichiometric binding constants (K_i) can be expressed by the Scatchard site association constants (k_i) in the following manner [6, 8, 13].

$$K_1 = \sum k_i$$

$$K_2 = \frac{\sum_{i<j} k_i k_j}{K_1}$$

$$\vdots$$

$$K_N = \frac{k_1 k_2 \cdots k_N}{K_1 K_2 \cdots K_{n-1}}$$

where $\Sigma_{i<j} k_i k_j$ means the sum of all combinations of $k_i k_j$ (i and j different) without repeating any combination, i.e. if there are three Scatchard constants the sum will look as follows:

$$k_1k_2 + k_1k_3 + k_2k_3.$$

Curve fitting procedure. The fitting and graphical procedures were carried out on a SUN-4/SPARC workstation. The curve fitting is in principle the same for both the Scatchard and the stoichiometric equation. In the

following the curve fitting procedure is demonstrated by using the stoichiometric equation.

The Adair equation is fitted to the experimental data by varying the K-values to minimize the sum, S, of weighted and squared deviations in

$$S = \sqrt{\frac{\sum_{j=1}^{N} \frac{[v_j - v_j(c_j)]^2}{w(c_j)}}{N - M}}.$$

c_j is the free ligand concentration, v_j is the observed concentration of bound ligand divided by that of the carrier, $v_j(c_j)$ is the calculated. N is the number of observations, M is the maximum number of ligand molecules bound. $w(c_j)$ is the weighting parameter calculated as

$$w(c_j) = \left(\frac{\left(\frac{c_j}{c_0}\right)^{q_1} + \left(\frac{c_j}{c_0}\right)^{q_2}}{2} \right)^2$$

where c_0 indicates a concentration of free ligand, approximately in the middle of a low variation area and q_1, q_2 are parameters chosen to allow fairly even distribution of weighted residuals throughout the range of concentrations observed. The best-fit approximation, represented by a set of K-values, is found by the iterative procedure described by Brodersen *et al.* [14]. The resulting K-values are

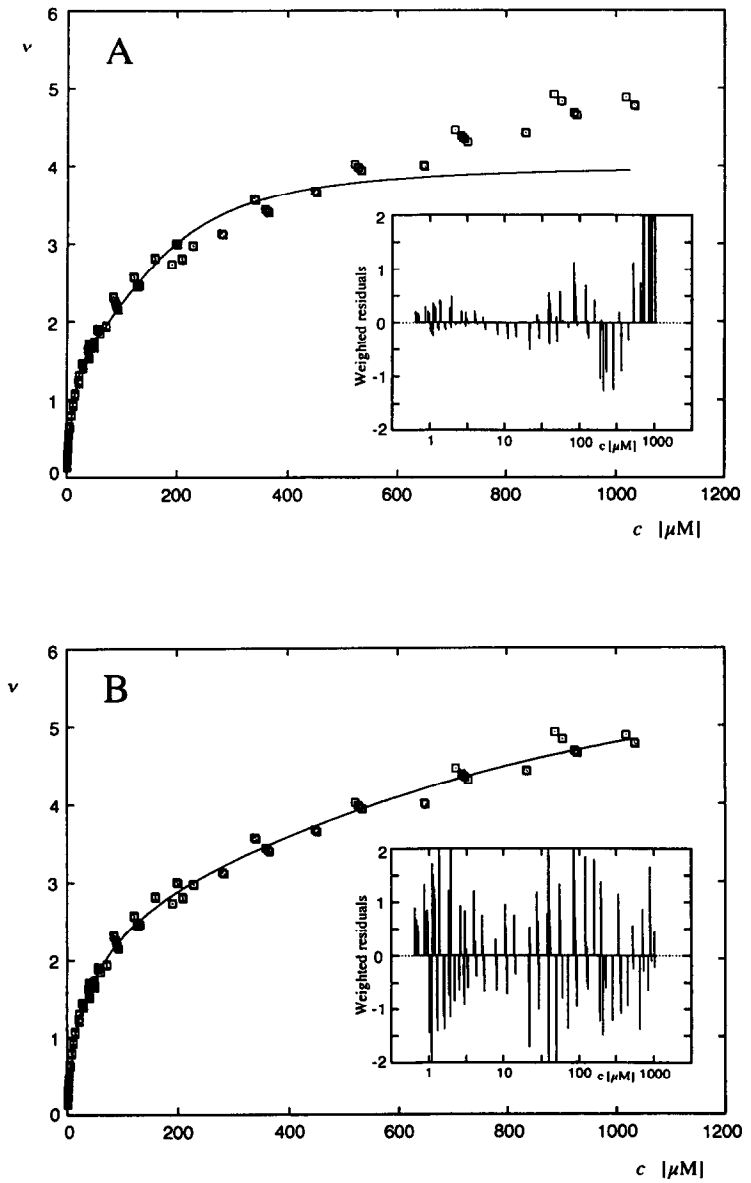

Fig. 1. Calculated warfarin binding isotherm from best-fit approximation. Observed points, v vs c, and a binding curve derived from the stepwise binding equilibrium equation (Adair equation), giving a best fit approximation of the 230 observed points. In panel A using the following weighting parameters $M = 4$, $c_0 = 1 \times 10^{-3}$, $q_1 = 0$, $q_2 = 0$. In panel B $M = 6$, $c_0 = 1 \times 10^{-5}$, $q_1 = 0.2$, $q_2 = 0.5$. Inserted figures show plots of weighted residuals vs concentration of unbound warfarin.

evaluated by looking at a computer-produced plot of the weighted residuals, $v_j - v_j(c_j)/\sqrt{w(c_j)}$ plotted against log c_j. Ideally the weighted residuals should then be evenly distributed above and below the zero line and should vary approximately equally throughout the concentration range. In case of a markedly uneven distribution it may be necessary to repeat the curve fitting using more suitable values of M, c_0, q_1 and q_2.

In Fig. 1 we have analysed the warfarin data using different values of M, c_0, q_1, q_2 to demonstrate the importance of these parameters. Figure 1A shows a poor fit. The inserted figure shows an obvious wave pattern of the residuals which indicate that a poor set of weighting parameters was chosen ($M = 4$, $c_0 = 1 \times 10^{-3}$, $q_1 = 0$, $q_2 = 0$). By changing the weighting parameters ($M = 6$, $c_0 = 1 \times 10^{-5}$, $q_1 = -0.2$, $q_2 = 0.5$) we obtained a significantly better fit. Figure 1B shows that the fitted curve clearly resembles the observation points. In the inserted figure the weighted deviations are evenly distributed above and below the zero line throughout the concentration range.

In order to assess the variation of the binding constants as originating from the stochastic errors of the observed data, a set of 30 acceptable solutions with slightly higher deviations than the best-fit approximation were also calculated. This was done by the previously described iterative procedure, starting with a slightly different set of K-values, generated from the best-fit constants by multiplying with a normally distributed variation factor. A probability limit of 0.75 was chosen in the present study, and a corresponding F-value was taken from the F-distribution. This is repeated until the S-value has decreased to an acceptable value, s_{acc}, fulfilling the condition

$$\left(\frac{s_{ace}}{s_0}\right)^2 < F$$

where S_0 is the value of S in the best-fit.

According to Table 1 column 2, considerable variation of binding constants is obtained, the last constants being nearly indeterminate. In spite of this, any one of the 30 sets of binding constants gives a good fit to the observed points.

Cooperativity. Our observations for binding of warfarin were successfully fitted by both binding equations, Scatchard's as well as Adair's. This is not always so. Data for binding of oxygen to haemoglobin [4] shown in Fig. 2 cannot be fitted by Scatchard's equation while an excellent fit is obtained with Adair's. Binding of oxygen to haemoglobin is cooperative. Since one equation can be derived from the other we might expect that both equations could be used equally well. However, Scatchard's equation fails to fit in cases of cooperative binding. Adair's equation can be used irrespective of interaction of bound molecules. If we could use complex values for the binding constants, composed of a real and an imaginary component, the oxygen binding data would be fitted equally well by both equations. We can utilize this to test whether cooperativity is present. Cooperativity of oxygen binding to haemoglobin is clearly demonstrated by the fact that Adair's equation fits better than Scatchard's.

Binding analysis. Determination of binding constants for multiple binding of a ligand usually results in higher variable figures. The variation is partly due to the fact that cooperative solutions are included in the analysis. It appears to be a general feature of ligand binding to albumin that cooperativity is absent. In the literature only a few cases have been described. An exception is binding of protons, which is clearly cooperative in a certain range of pH values [15]. In the following we describe a method for analysing binding data by which it becomes possible to obtain binding constants with comparatively slight variation.

First the computer generated the binding constants until a minimum of weighted and squared deviations of the

Table 1. Association constants for binding of warfarin to human serum albumin at pH 7.4 and 37°

	Scatchard constants (M^{-1})		Adair constants (M^{-1})		Transformed Scatchard to Adair constants (M^{-1})	
k_i or K_i	Best fit	Range of 30 solutions (probability limit 0.75)	Best fit	Range of 30 solutions (probability limit 0.75)	Best fit	Range of 30 solutions (probability limit 0.75)
1	187,083	166,290–209,920	218,025	200,100–239,130	215,137	198,750–234,270
2	19,519	11,414–25,138	23,934	19,645–30,820	25,281	22,592–28,185
3	4435	2310–10,011	7479	3962–11,393	6637	5939–7609
4	2161	863–4434	2408	369–5887	2560	2326–2816
5	854	379–1670	578	0.02–8910	1139	987–1255
6	495	108–1457	2253	9.09–4,648,500	561	462–680

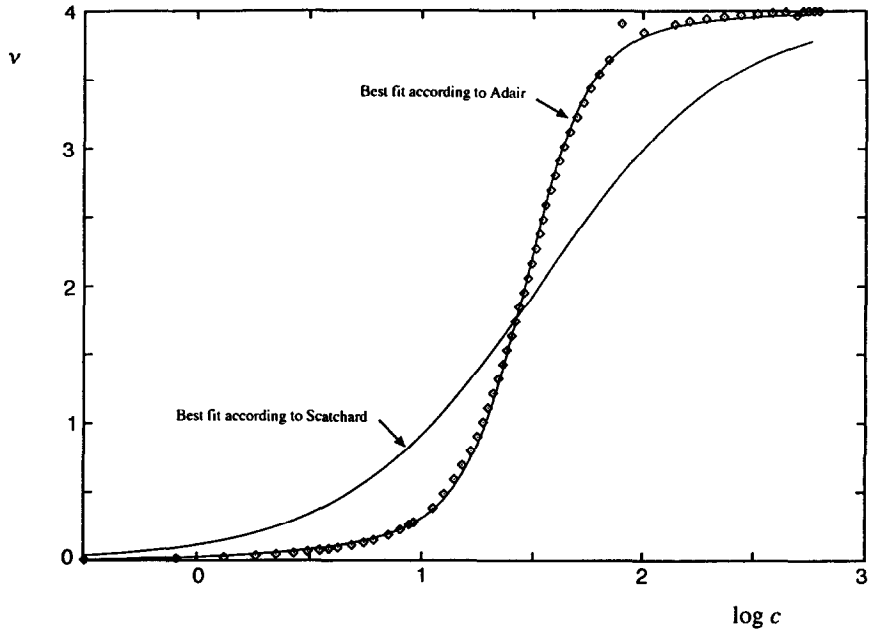

Fig. 2. Best-fit curves obtained from Scatchard's and Adair's equations, fitted to the oxygen–haemoglobin equilibrium data of Winslow *et al.* [4]. Semilogarithmic plot of concentrations of bound vs free oxygen, plotted as v vs log c.

points from the curve was obtained and then generated 30 acceptable variations. In this way we obtained one best-fit set of Scatchard binding constants and 30 acceptable variant sets, and likewise one set of best-fit Adair's constants and 30 variants of those (see Table 1 columns 1 and 2). If the sum of weighted and squared deviations is significantly smaller than by fitting Scatchard's equation, cooperativity should be considered. If not, cooperativity cannot be demonstrated and the binding constants obtained by fitting Scatchard's equation can be accepted as final, with the variations found.

The calculated isotherms for warfarin (not shown) according to the Scatchard's and Adair's equations clearly demonstrates that the two equations can be fitted equally well to the data. Furthermore, the sum of weighted and squared deviations ($s_{Scat} = 0.037507$, $s_{Adair} = 0.037195$) are statistically the same. This demonstrates that cooperativity in the case of warfarin is absent. This is further supported by looking at a Scatchard plot (c/v vs v) (not shown) where no upward bend is seen.

As we have excluded cooperativity we can transform Scatchard site association constants into stoichiometric binding constants (see Table 1 column 3). Comparing columns 2 and 3 we see that the Adair constants in column 3 (transformed Scatchard constants) show a lesser variation, especially K_7–K_6, compared to the Adair constants found in column 2. This is perhaps better illustrated in Klotz's affinity profiles (iK_i vs i) [16] shown in Fig. 3. The binding constants in Fig. 3A compared to 3B show less variation because cooperative solutions are not included.

Discussion

The Scatchard equation has been used by several authors [17, 18] to analyse serum albumin binding data. However, the Scatchard equation fails if cooperative binding is present, which is why we have chosen to eliminate cooperative binding before using the Scatchard equation. We believe it is best to report stoichiometric constants because the presence of actual sites or of independent binding cannot be concluded from equilibrium measurements while a traditional report of Scatchard constants implies that a number of independent sites with known binding constants exist in the albumin molecule. There has been much discussion of the Scatchard versus the Adair equations [6, 8, 9, 19].

The aim of the present study is to present a method for analysing multiple binding. If cooperativity is absent it becomes possible to obtain binding constants with only slight variation. For this purpose we needed a curve fitting procedure. Different authors have presented methods for non-linear least squared parameter estimations, suitable for analysis of ligand binding equilibrium data [20–23]. We found that the iterative curve fitting procedure, presented by Brodersen *et al.* [14] served our purposes well.

Binding measurements and analysis provide the essential data for the evaluation of ligand–carrier affinities [24]. With the wealth of drug and ligand binding data, combined with current advances in recombinant technology, and with a more complete understanding of albumin structure [25] and function, researchers should soon be in a position to understand and predict ligand displacement interactions for a variety of endogenous and exogenous ligands.

Acknowledgements—We wish to thank Prof. R. Brodersen, Institute of Medical Biochemistry, University of Aarhus, for introducing us to the use of mathematical procedures in modern biochemistry. The authors gratefully acknowledge Dr Vigo H. Nielsen for critical comments during the study.

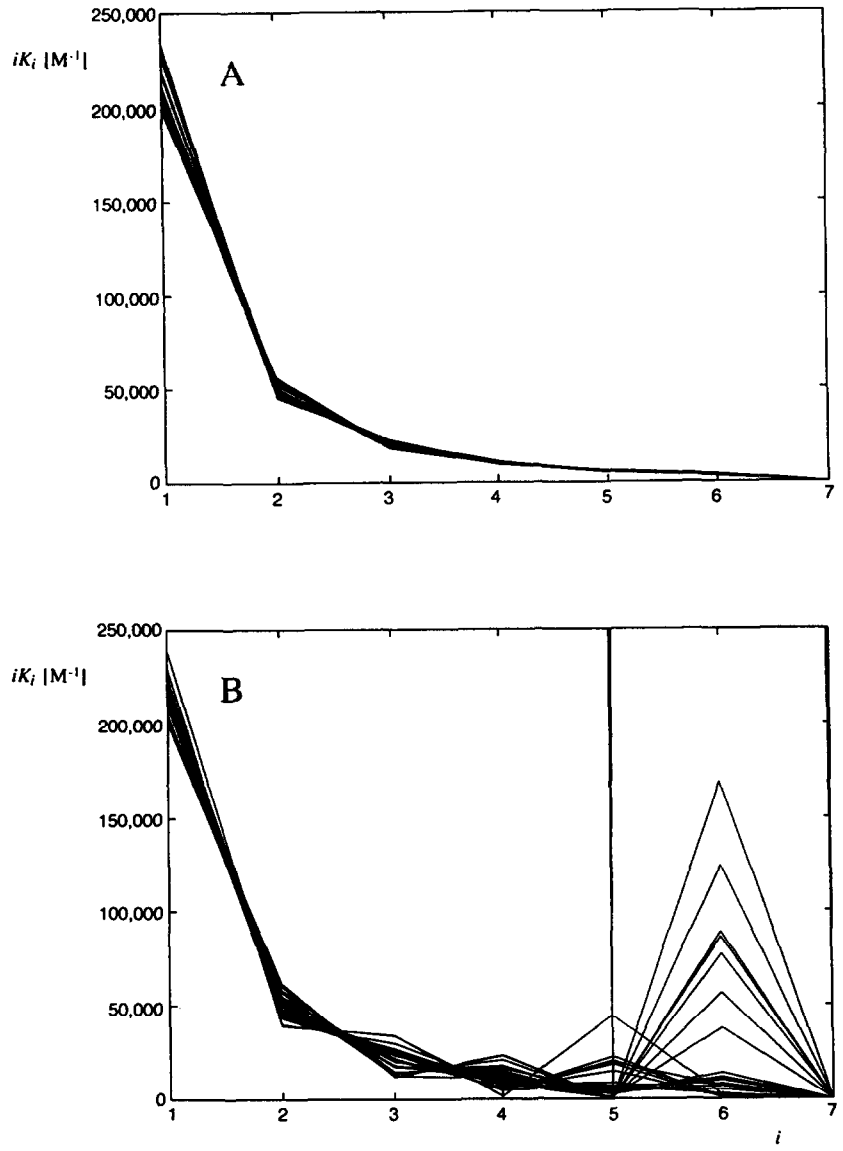

Fig. 3. Klotz's affinity profiles for binding of warfarin to human serum albumin. The observed data were fitted by Scatchard's equation in panel A, 30 acceptable variants, followed by transformation of Scatchard binding constants into Adair's. Panel B shows Adair constants obtained by fitting of Adair's equation.

Institute of Medical Biochemistry
University of Aarhus
Ole Worms Allé Bldg
170, DK-8000 Aarhus C
†Department of Cardiology
Holstebro Hospital
DK-7500 Holstebro
‡Computer Science Department
University of Aarhus
DK-8000 Aarhus C
Denmark

HENRIK VORUM*
DORTE BANG†
KIM FISKER‡

* Corresponding author.

REFERENCES

1. Sjöholm IB, Kober A, Lungstedt-Påhlman I, Seiving B and Sjödin T, Binding of drugs to human serum albumin XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. *Mol Pharmacol* **16**: 767–777, 1979.
2. Müller WE and Wollert U, Human serum albumin as a "silent receptor" for drugs and endogenous substances. *Pharmacology* **19**: 59–67, 1979.
3. Larsen FG, Larsen CG, Jacobsen P and Brodersen R, Interaction of warfarin with human serum albumin—a stoichiometric description. *Mol Pharmacol* **27**: 263–270, 1985.
4. Winslow HM, Swenberg M-L, Berger RL, Shrager, RI, Luzzanna M, Samaja, M and Rossi-Bernadi LJ,

Oxygene equilibrium curve of normal human blood and its evaluation by Adair's equation. *J Biol Chem* **252**: 2331–2337, 1977.
5. Scatchard G, The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
6. Fletcher JE, Spector AA and Ashbrook JD, Analysis of macromolecule-ligand binding by determination of stepwise equilibrium constants. *Biochemistry* **9**: 4580–4587, 1970.
7. Fletcher JE, Ashbrook JD and Spector AA, Computer analysis of drug-protein binding data. *Ann NY Acad Sci* **226**: 69–81, 1973.
8. Klotz IM and Hunston DL, Protein interactions with small molecules. Relationships between stoichiometric binding constants, and empirical binding parameters. *J Biol Chem* **250**: 3001–3009, 1975.
9. Klotz IM and Hunston DL, Protein affinities for small molecules: Conceptions and misconceptions. *Arch Biochem Biophys* **193**: 314–328, 1979.
10. Adair GS, The hemoglobin system. (Part VI). The oxygen dissociation curve of hemoglobin. *J Biol Chem* **63**: 529–545, 1925.
11. Klotz IM, The application of the law of mass action to binding be proteins. Interactions with calcium. *Arch Biochem* **9**: 109–117, 1945.
12. Brodersen R, Honoré B, Pedersen AO and Klotz IM, Binding constants for ligand-carrier complexes. *Trends Pharmacol Sci* **9**: 252–257, 1988.
13. Muralt von AL, The titration constants of multivalent substances. *J Am Chem Soc* **52**: 3518–3523, 1930.
14. Brodersen R, Nielsen F, Christiansen JC and Andersen K, Characterization of binding equilibrium data by a variety of fitted isotherms. *Eur J Biochem* **169**: 487–495, 1987.
15. Honoré B and Brodersen R, Ionization of tyrosine residues in human serum albumin and in its complexes with bilirubin and laurate. *Int J Peptide Protein Res* **39**: 24–28, 1992.
16. Klotz IM, Protein interactions with small molecules. *Acc Chem Res* **7**: 162–168, 1974.
17. Kragh-Hansen U, Molecular aspects of ligand binding to serum albumin. *Pharmacol Rev* **33**: 17–53, 1981.
18. Peters T Jr, Serum albumin. *Adv Protein Chem* **37**: 161–245, 1985.
19. Weber G and Anderson S, Multiplicity of binding. Range of validity and practical test of Adair's equation. *Biochemistry* **4**: 1942–1947, 1965.
20. Garfinkel L, Kohn MC and Garfinkel D, System analysis in enzyme kinetics. *Crit Rev Bioeng* **2**: 329–361, 1977.
21. Reich JG and Zinkel I, Analysis of kinetic and binding measurements IV. Redundancy of model parameters. *Stud Biophys* **43**: 91–107, 1974.
22. Johnson ML, The analysis of ligand-binding data with experimental uncertainties in the independent variables. *Anal Biochem* **148**: 471–478, 1985.
23. Reich JG, Wangermann G, Falck M and Rhode K, A general strategy for parameter estimation from isosteric and allosteric-kinetic data and binding measurements. *Eur J Biochem* **26**: 358–379, 1972.
24. Klotz IM, Ligand-protein binding affinities. In: *Protein Function—a Practical Approach* (Ed. Creighton TE), pp. 25–54. IRL Press, Oxford, 1989.
25. He MH and Carter DC, Atomic structure and chemistry of human serum albumin. *Nature* **358**: 209–215, 1992.