10. Hong SL, McLaughlin NJ, Tzeng C-Y and Patton G, Prostacyclin synthesis and deacylation of phospholipids in human endothelial cells: comparison of thrombin, histamine and ionophore A23187. *Thromb Res* **38**: 1–10, 1985.
11. Voss BL, Hamilton KK, Samara ENS and McKee PA, Cyclosporin suppression of endothelial prostacyclin generation: a possible mechanism for nephrotoxicity. *Transplantation* **45**: 793–796, 1988.
12. Szamel M, Berger P and Resch K, Inhibition of T lymphocyte activation by cyclosporin A: interference with the early activation of plasma membrane phospholipid metabolism. *J Immunol* **136**: 264–269, 1986.
13. Gordon JA, Hadjiagapiou C and Spector AA, Cyclosporin inhibits prostacyclin formation in cultured human endothelial cells. *Kidney Int* **31**: 457 Abstr, 1987.
14. Stahl RAK, Adler S, Baker PJ, Johnson RJ, Hen Y-P, Pritzl P and Couser WG, Cyclosporin A inhibits prostaglandin E_2 formation by rat mesangial cells in culture. *Kidney Int* **35**: 1161–1167, 1989.
15. Thomas JMF, Hullin F, Chap H and Douste-Blazy L, Phosphatidylcholine is the major phospholipid providing arachidonic acid for prostacyclin synthesis in thrombin-stimulated human endothelial cells. *Thromb Res* **34**: 117–123, 1984.

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Bovine albumin–HABA interaction: re-analysis of earlier observations indicates that ligand-induced dimerization and a competitive contaminant operate simultaneously

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The carboxylic acid 2-(4′-hydroxybenzeneazo)benzoic acid (HABA) binds to human and bovine albumin and this dye is a useful model ligand for the investigation of ligand–acceptor interactions. There are a substantial number of reports which indicate that the number of binding sites (n) and/or the apparant association constant (K_a) are dependent upon protein concentration for a variety of ligand–acceptor systems [1, 2]. Under appropriate experimental conditions the results can give a Scatchard [3] plot with a positive slope [4, 5]. This is the case for HABA [6, 7] and a variety of other systems including some containing receptors for drugs and hormones [2]. The interaction of HABA with *human* albumin showed the phenomenon of inverse dependence [6, 7] and a quantitative analysis of these observations indicated that the most likely explanation for this dependence was that the high affinity binding site on human albumin for HABA was contaminated to the extent of about 95% with a competing ligand [2]. The contaminant in this context was probably an endogenous ligand retained by the albumin molecule during its isolation and purification [2].

The binding of HABA to *bovine* albumin also exhibited anomalous binding behaviour which included an effect of protein concentration. Scatchard plots of some of the data obtained by variation of the ligand concentration appeared to exhibit a maximum near the ordinate [7]. These experiments covered a much larger range of ligand concentrations than did the earlier work with human albumin [6] and the protein concentration dependence appeared to differ from that observed with the latter. No explanation was provided by Clegg and Lindup [7] for their results with bovine albumin and HABA and so these earlier data have been subjected to quantitative analysis with the intention of understanding the molecular basis for the observed protein concentration dependence. It is hoped that this approach will be applied more generally in due course to systems, such as those involving drug and hormone receptors, which have more immediate biological importance.

Materials and methods

Binding of HABA to bovine albumin. The three sets of data which were analysed were for the binding of a range of HABA concentrations (50 to 5000 μM) to bovine albumin at each of three different concentrations (1, 2 and 4% w/v) measured by equilibrium dialysis at 37° and pH 7.4 (see Fig. 1 in Ref. 7).

Analysis of experimental data. Each set of data was fitted separately to two model equations, by a non-linear least squares regression method. The Adair equation (no. 5 in Ref. 8) was applied because it is the most general description of the binding of a single ligand to a non-interacting protein. Polysteric effects are not included but given this restriction it covers all binding models for a single ligand and the best one can be derived from the Adair constants β_j [8]. The Adair constants β_j where j goes from one to the total number of sites needed in the description are the equilibrium constants for the j-th overall reaction of j ligands X with the protein P.

$$P + jX \rightleftarrows PX_j\ (\beta_j).$$

It is the Adair constants that are estimated by the fitting procedure, but the results are often given more conveniently as the stepwise (or consecutive) association constants K_j which are the equilibrium constants for the reactions:

$$PX_{j-1} + X_j \rightleftarrows PX_j\ (K_j).$$

These two equivalent sets of equilibrium constants are related by

$$\beta_j = K_1.K_2 \ldots . K_j.$$

The Adair equation does not contain the protein concentration however and so cannot explain the effect of this on binding. The success or failure of the Adair equation to describe the experimental data, the shape of the binding curve and the dependence of the Adair constants upon protein concentration can nevertheless all provide information or clues about the molecular binding mechanism.

The data were also fitted to the competitive contaminant model [1] since this model provided a quantitative explanation for the protein concentration dependence observed in the interaction of HABA with human albumin [2]. This model is based on the proposition that some of the binding sites for ligand X on protein P are contaminated by another ligand Y. The competition for these sites is assumed to be

mutually exclusive, i.e. only one of the two ligands can be bound to the same site at any one time. The contaminant Y is present in the system at a fixed ratio to P because it is associated with the protein. This concentration ratio of Y to P is called the contaminated fraction (β), so the uncontaminated fraction $\alpha = 1 - \beta$ [1].

Results and discussion

The three sets of data and the fits to the Adair equation are displayed (Fig. 1) as the Scatchard plots (i.e. r/x versus r where x is the concentration of unbound ligand and r is the average number of ligand molecules bound per protein molecule) and the binding parameters obtained are given in Table 1. It can be seen that the overall behaviour of all three sets of data were described well by the Adair equation. The positive slope of the binding isotherm which the experimental points manifest for the two highest concentrations of albumin was also represented well by the fit.

The data corresponding to the lowest albumin concentration (1%) did not show a positive slope or maximum but the fit nevertheless did have a region with a positive slope (Fig. 1). The sigmoidal character of the fit was, however, smaller than for the higher albumin concentrations. Apparently it was necessary for the Adair curve to have this downward curvature for $r \rightarrow 0$ in order to describe the general behaviour of the data points. In a statistical sense only the point corresponding to the smallest value of r was represented poorly by the fit and this point had the lowest accuracy. Thus the fit is statistically acceptable and this is illustrated by the small r.m.s. value (Table 1) which was similar to those for the other fits.

The most pronounced behaviour predicted by the competitive contaminant model [1, 2] is a steep rise near the ordinate. Since the results obtained at low concentrations of ligand do appear to have a clear tendency towards a steep rise (Fig. 1) for low r-values the data were also fitted to the competitive contaminant model. The best fit to the contaminant model is shown in Fig. 2. The steep slope for $r \rightarrow 0$ is represented well and we intuitively accepted this as a better fit than that based on the Adair equation, a conclusion that is also supported statistically since the r.m.s. value of the contamination fit is significantly lower than that of the Adair fit (see Table 1). The data for the two highest albumin concentrations (2 and 4%) could not be fitted to the competitive contaminant model which was not surprising because the data have a downward curvature for $r \rightarrow 0$ while the model predicts the opposite.

The most noticeable feature for the Adair fit was that all curves intersected at the same point on the r/x axis (Fig. 1). Furthermore the sigmoid nature of the curves increases with increasing protein concentration. These characteristics are in agreement with a ligand-induced dimerization model discussed by Nichol and Winzor [9], Cann and Hinman [10] and Cann [11]. This model proposes that the ligand X and the protein P interact to form a PX complex which dimerizes according to the reaction schemes

$$P + X \rightleftarrows PX \; (K_1) \qquad (1)$$

$$2\,PX \rightleftarrows P_2X_2 \; (K_d) \qquad (2)$$

where the equilibrium constants are given in parentheses and K_1 is the association constant and K_d the dimerization constant.

The dimerization model predicts that the curves for different protein concentrations do not intersect but approach the same point on the r/x axis as $r \rightarrow 0$ with a value equal to K_1 [9]. The fitted curves (Fig. 1) do indeed intersect the r/x axis at a value equal to K_1. Another prediction is that the sigmoid nature of the curve increases with the value of K_dC_p where C_p is the total protein concentration and the fitted curves also showed this behaviour. A value of 1 for K_dC_p produced a slightly sigmoid curve, cf. Fig. 1 of Nichol and Winzor [9], while a value of 10 resulted in a rise from the intersection with the r/x axis to a point with about double that value. Comparison of our Fig. 1 with Fig. 1 of Nichol and Winzor [9] shows that at a rough estimate $K_dC_p \sim 5$ for the 4% (600 μM) bovine albumin solution. This corresponds to a dimerization constant (K_d) of the order of $10^4\,M^{-1}$.

The apparent absence of sigmoid character of the experimental data at 1% can be explained in terms of the effect of a competitive contaminant. The effect of the contaminant is in the opposite direction to dimerization and gives rise to an increase, rather than a decrease, of the slope of the Scatchard plot as r approaches zero. The combination of these effects results in the shoulder which is seen near the

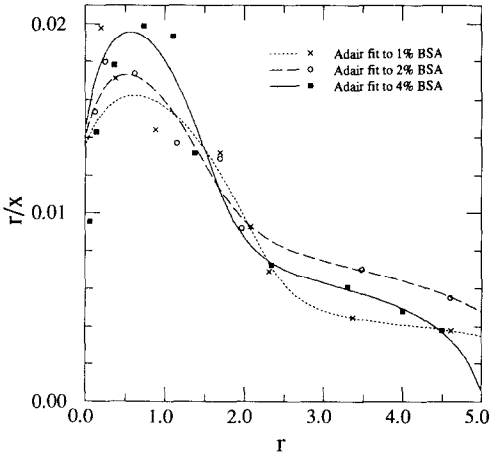

Fig. 1. Experimental binding data for the binding of HABA to three concentrations of bovine albumin as indicated together with the theoretical fits to the Adair equation. See Table 1 for the corresponding estimates of the binding parameters.

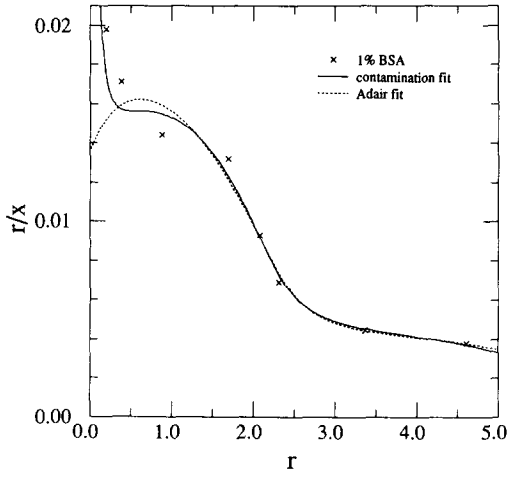

Fig. 2. Experimental binding data for the binding of HABA to 1% bovine albumin together with the theoretical fit to the competitive contaminant model. The fit to the Adair equation is included for comparison. See Table 1 for the estimates of the binding parameters by both models.

Table 1. Binding parameters obtained by fitting data for the interaction of HABA with bovine albumin to the Adair equation and the competitive contaminant model*

BSA concn. (%)	Adair model† K_1	K_2	K_3	Minimum no. of sites	r.m.s.‡
1	0.014	0.013	0.003	6(8)§	0.047
2	0.014	0.016	0.001	7	0.048
4	0.014	0.022	0.000	5	0.047
	Contaminant model				
	α‖	k¶	IC_p^{**}	K_1	
1	0.05 of 1	0.62	600	0.004	0.041

* All binding constants are given in units of $10^6\,M^{-1}$.

† Only the first three stepwise association constants are given.

‡ The root mean square value which is the SD of r from the best least-squares fit to the data.

§ The data for 1% albumin also gave a satisfactory fit to the competitive contaminant model and the binding constants obtained are shown in the lower part of the table. Two data points with r-values of 5.6 and 7.4, which had a large experimental error, were not included in the analysis with 6 sites. If these two points were included a minimum of 8 sites had to be used and the estimated values of K changed slightly to 0.015, 0.011 and 0.003.

‖ The uncontaminated fraction of the high affinity site.

¶ The "true" association constant for the high affinity site.

** The product of the association constant of the contaminant for the high affinity site multiplied by the total concentration of protein.

ordinate in the Scatchard plot of the data (Fig. 2). The apparent absence of the effect of the contaminant with the data for the two higher albumin concentrations (2 and 4%) is in agreement with the contaminant model [1] which predicts that the observable effect decreases with increasing albumin concentration. Note, however, that the effect of the contaminant in masking the true high affinity binding increases with increasing protein concentration.

Our interpretation of the binding of HABA to bovine albumin is that there are two opposing effects, neither of which is normally considered to operate. Both of these mechanisms are needed in combination to provide a good mechanistic explanation for the binding curves. The two effects which appear to operate are ligand-induced dimerization of the albumin, which is more apparent at the two higher protein concentrations, and the presence of a competitive contaminant which is apparent at the lowest albumin concentration.

The effect of dimerization is manifested both as a peak in the Scatchard plot (near $r \sim 0.3$) which increases with albumin concentration and also as the common intercept on the r/x axis of the binding isotherms for each of the protein concentrations. It is noteworthy that although the peak could be seen with the data from the two highest albumin concentrations the common intercept on the r/x axis of the binding isotherms could only be observed after the data were fitted to the Adair equation. Even the fit to the data at the lowest albumin concentration showed this behaviour despite the fact that the data for the three lowest r-values showed an increase in r/x as $r \to 0$, rather than the decrease produced by the Adair fit. Fitting the data to the Adair equation was therefore of prime importance for the analysis.

It should be noted that the analysis can distinguish between several conceivable dimerization schemes. The ligand-induced dimerization describing the present data is a dimerization of a ligand–acceptor complex as described by Eqns 1 and 2 and not, for example, a cross-linking of the acceptor by a bivalent ligand. The latter interaction would have given rise to a very different behaviour of the binding curves as shown by Nichol and Winzor [9].

The observable effect of a competitive contaminant, which decreases with the increasing protein concentration as discussed above, was detectable at only the lowest protein concentration, where the characteristic effect was a rise in the Scatchard plot for $r \to 0$. A fit of these data to the competitive contaminant model [1], neglecting any effect of dimerization, gave values of the parameters (Table 1) which were similar to those obtained previously for human albumin [2]. It must be noted however that the present data for low r values were sparse, unlike the situation with human albumin where all the values of r were in the range of 0.05 to 1. Given this limitation, the agreement between the results for the two albumin preparations is satisfactory and provides further confidence in the correctness of the interpretation and analysis of the data.

The importance of including the contaminant model in an analysis of protein binding data was emphasized in a previous theoretical article [1] and in analyses of gold [12] and HABA [2] binding to human albumin. The apparent association constant found if the presence of a competing ligand was ignored could be smaller, by an order of magnitude or more, than the true binding constants. This is also illustrated by the results obtained here for the 1% bovine albumin. The apparent association constant for the binding of one ligand molecule to one protein molecule, K_1, was $1.4 \times 10^4\,M^{-1}$ for the present data when analysed by the Adair equation, while the association constant (k_0) for the high affinity binding site obtained with the contaminant model was $6 \times 10^5\,M^{-1}$. It should be noted that only the 1% data could be used to determine the "true" association constant for the high affinity site. The data obtained with higher concentrations of albumin only revealed the apparent binding constant and this illustrates the importance of observing the binding to a range of protein concentrations.

A few words concerning an "apparent" versus a "true" high affinity binding constant may be helpful. The true binding constant for the high affinity site is a measure of the affinity of the ligand in the absence of any competition from another ligand, contaminant or otherwise. If the protein is highly contaminated as found here and previously [2, 12] then only for very low concentrations and r values can this "true" binding be observed. For almost all ligand concentrations (except the lowest) the observed binding will appear as binding to contaminated sites, where the ligand has to compete with the contaminant in order to bind, with the consequence that apparent affinity is lower.

This work has shown that the protein concentration dependence of the binding of HABA to bovine albumin can be explained by the simultaneous occurrence of both HABA-induced dimerization of the HABA–albumin complex and the presence of an unknown competitive contaminant. The more pronounced effect (for high protein concentration) was attributed to HABA-induced dimerization of the HABA–albumin complex, with a dimerization constant of the order of $10^4\,M^{-1}$. The effect of a competitive contaminant was discernible at the lowest albumin concentration and the binding parameters were similar to those obtained previously with human albumin [2], where only the latter mechanism was observed. The approach used here can be applied equally well to other ligand–acceptor combinations, including drugs and

hormones for example, to provide further insight into the molecular basis of the binding mechanism and such questions as receptor heterogeneity.

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REFERENCES

1. Pedersen JB and Pedersen SM, Effect of a contaminating competitive ligand on ligand-binding curves. Inverse protein concentration dependence. *Biophys Chem* **32**: 79–87, 1988.
2. Pedersen JB, Pedersen SM and Lindup WE, Contamination by a competitive ligand as an explanation for the inverse dependence of HABA binding parameters upon the protein concentration. *Biochem Pharmacol* **38**: 3485–3490, 1989.
3. Scatchard G, The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
4. Bowmer CJ and Lindup WE, Binding of phenytoin, L-tryptophan and *o*-methyl red to albumin. Unexpected effect of albumin concentration on the binding of phenytoin and L-tryptophan. *Biochem Pharmacol* **27**: 937–942, 1978.
5. Bowmer CJ and Lindup WE, Scatchard plots with a positive slope and role of albumin concentration. *J Pharm Sci* **67**: 1193–1195, 1978.
6. Bowmer CJ and Lindup WE, Inverse dependence of binding constants upon albumin concentration. Results for L-tryptophan and three anionic dyes. *Biochim Biophys Acta* **624**: 260–270, 1980.
7. Clegg LS and Lindup WE, Scatchard plots with a positive slope: dependence upon ligand concentration. *J Pharm Pharmacol* **36**: 776–779, 1984.
8. Pedersen JB and Pedersen SM, A general method of deriving the best binding site model consistent with experimental binding data. *Biophys Chem* **22**: 255–262, 1985.
9. Nichol LW and Winzor DJ, Ligand-induced polymerization. *Biochemistry* **15**: 3015–3019, 1976.
10. Cann JR and Hinman ND, Hummel-Dreyer gel chromatographic procedure as applied to ligand-mediated association. *Biochemistry* **15**: 4614–4622, 1976.
11. Cann JR, Ligand-binding by associating systems. In: *Methods in Enzymology* (Eds. Hirs CHW and Timasheff SN), Vol XLVIII pp. 299–307. Academic Press, New York, 1978.
12. Pedersen SM, Effect of protein concentration on the binding of gold (I) to human serum albumin. *Biochem Pharmacol* **36**: 2661–2666, 1987.

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