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CONTAMINATION BY A COMPETITIVE LIGAND AS AN EXPLANATION FOR THE INVERSE DEPENDENCE OF HABA BINDING PARAMETERS UPON THE PROTEIN CONCENTRATION

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Abstract—There is evidence that the apparent association constant (K) and/or the number of binding sites (n) are inversely dependent upon protein concentration for a number and variety of ligands with no obvious structure–activity relationships. A model recently shown to explain this effect with an inorganic ligand has now been applied to 2-(4′-hydroxybenzeneazo) benzoic acid (HABA) and also explains the inverse protein dependence of the binding of this compound to human albumin. The model explains this inverse dependence on the basis of a highly bound contaminant which competes with the ligand for the high affinity site. HABA was found to interact with human albumin at three or more sites, the high affinity site which was about 95% contaminated had an association constant of 2×10^5/M, an order of magnitude higher than that found previously when the effect of a contaminant was not considered. The association constant of the competitive contaminant was estimated to be about 5×10^6/M. Since the model accounts for the phenomenon in terms of a property of the protein, rather than of the ligand, it could provide a general explanation for this effect with other ligand–acceptor combinations including a wide variety of drug and hormone receptor preparations.

It is now known that apparent binding constants are not independent of protein concentration for a number of ligand-acceptor combinations and there is now a considerable amount of both direct and indirect evidence [1–25]. In general there is an inverse relationship with the apparent association constant (K) and/or the number of binding sites (n) apparently decreasing as the protein concentration increases. In an experiment with a single ligand concentration and a range of protein concentrations the results obtained would typically lead to a Scatchard plot [26] with a positive slope, see e.g. [20]. This phenomenon has been most commonly observed with either human or bovine albumin but there is evidence for its occurrence with other proteins as well [12, 21, 27, 28]. The increase in the use of receptor binding assays *in vitro* means that this phenomenon is likely to become more widely encountered, particularly if the protein concentration is made an experimental variable.

A theoretical binding model which can explain the inverse dependence of binding constants upon protein concentration has recently been proposed [25, 29]. This model explains the inverse dependence upon protein concentration in terms of a highly bound competitive contaminant which binds to the same site as the ligand of interest. Albumin is available in a relatively pure form, certainly much more so than any receptor preparation, but a typical sample may nevertheless contain several contaminating substances with the potential for interaction. Although small amounts of other proteins such as the globulins may be contaminants the major substances likely to be relevant are other small organic molecules of natural origin such as the fatty acids, bilirubin, thiol compounds and carboxylic acids such as those which accumulate in uraemia [30–33]. Other substances such as *N*-acetyltryptophan and octanoic (caprylic) acid may be added as stabilisers for the pasteurization of albumin [32] and inorganic ions such as chloride may also be present. Although these contaminants, from whatever source, may not be present individually in sufficient concentration their effects may become apparent when several share the same site as the ligand of interest.

The contaminant model has been applied successfully to the inorganic drug aurothiosulphate [25, 29] which binds to the free sulphydryl group on human albumin, a site which was known to be contaminated. Since albumin is a typical carrier for organic acids and because the binding constants of the azo dye 2-(4′-hydroxybenzeneazo) benzoic acid vary inversely with the protein concentration [4] we have investigated whether the contamination model can explain these earlier results. No satisfactory explanation has been offered so far for this observed dependence upon protein concentration with HABA.

MATERIALS AND METHODS

Binding of HABA to human albumin. The data obtained by Bowmer and Lindup (see Fig. 3 in Ref. 4) were used for this analysis and the binding of HABA to crystalline human albumin (fraction V) at

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pH 7.4 and 37° was measured by equilibrium dialysis as previously described [4]. The binding of HABA (5 to 450 μM) to four concentrations of human albumin (0.25, 0.5, 1 and 2% w/v) was measured.

Binding model. The competitive contaminant model of Pedersen and Pedersen [29] was used to analyse the binding data. This model is based on the proposition that a protein P which binds ligand X has some of its binding sites for X contaminated by a different ligand Y. The model assumes that the protein has two independent sets or groups of binding site: one (or more) high affinity site(s) for which the two ligands X and Y compete, and another group of sites with lower affinity to which only X binds. It is not essential that the contaminated site has higher affinity than the uncontaminated sites but the phenomenon would be more difficult to detect if this were not the case. The competition 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. It should be noted that a contaminant ligand Y is present in the experimental system in a fixed ratio to P since it is associated with the protein. Therefore with higher protein concentrations the ratio of contaminant Y to ligand X will increase and so inhibit the binding of X. The concentration ratio of Y to P is called the contaminated fraction (β) and so the uncontaminated fraction $\alpha = 1 - \beta$.

The binding to the contaminated high affinity site is described by the following reaction schemes where the association constants are given in parentheses:

$$P + X \rightleftharpoons PX\,(K_x) \tag{1}$$

$$P + Y \rightleftharpoons PY\,(K_y). \tag{2}$$

The fractions (r) of the site occupied by X and Y respectively are given by Eqns (3) and (4)

$$r_x = \frac{K_x x}{1 + K_x x + K_y y} \tag{3}$$

$$r_y = \frac{K_y y}{1 + K_x x + K_y y} \tag{4}$$

where the unbound ligand concentrations are denoted by x and y. Equations (3) and (4) are identical to those used for a standard competitor system although there is an important difference between a competitor, where various concentrations may be used experimentally, and a contaminant in that the latter has a constant total concentration which is governed by the protein concentration. This implies that the unbound concentration (y) of the contaminant can be expressed in terms of the unbound concentration of the ligand X as previously shown [29]:

$$\begin{aligned} K_y y = &-0.5\,(\alpha b + 1 + K_x x) \\ &+ 0.5\,[(\alpha b + 1 + K_x x)^2 \\ &+ 4b\beta\,(1 + K_x x)]^{1/2}. \end{aligned} \tag{5}$$

The dimensionless quantity b is defined as $b = K_y C_P$ and has a value much larger than one for a strongly bound contaminant; C_P is the initial total concentration of the protein. Equation (5) is inserted into Eqn (3) to obtain a binding curve of r vs unbound ligand concentration (x) and thus related plots such as that of Scatchard, r/x vs r, can also be obtained.

In equilibrium dialysis some of the contaminant will diffuse out of the protein containing compartment and this can be corrected for [29] by substituting C'_P for C_P in Eqn (5):

$$C'_P = C_P \frac{V_1}{(V_1 + V_2)} \tag{6}$$

where V_1 and V_2 are the volumes of the compartments with and without protein. In the case of the results for HABA analysed below the volumes of the two compartments were equal, i.e. $V_1 = V_2$.

RESULTS

Analysis of the experimental data

The data were fitted to several binding models by a non-linear regression method. In all cases the data to which the models were fitted were in the form of r vs the unbound ligand concentration x. However, the graphical representation used to decide upon the acceptability of a given fit was the Scatchard plot (r/x vs r). This plot shows the behaviour at low concentrations much more clearly than a plot of r vs log x. This plot also emphasises the experimental errors of the results obtained at low concentrations which in many cases lead to a large scatter of the data points near $r = 0$. The present data show very little scatter (Fig. 1) and a pronounced dependence upon ligand concentration which is an indication that the data are reasonably precise at low concentrations.

The Adair equation was the first model used, although this is not really a binding model but rather a thermodynamic description of the consecutive binding of the ligand to albumin from which the best binding site model can be derived as discussed previously [34]. This description is exact for the binding of a single ligand to a protein which does not polymerise. Any failure, however, of this description is indicative of the presence of either another competitive ligand or of protein–protein interactions. It should be noted that this description cannot give rise to any protein concentration dependence of ligand binding and therefore cannot explain the data for HABA. We have used it just to illustrate that this description cannot represent the form of the observed dependence (r/x vs r). Observation of a similar discrepancy may be helpful in deciding between models, in particular when only a single protein concentration has been used. It was found necessary for all protein concentrations to assume that albumin had three or more binding sites for HABA. The resulting fits are shown in Fig. 1 as broken lines. There was a clear discrepancy between the fits and the low concentration data ($r \leq 0.2$) for 0.25% and 1% human serum albumin (HSA). However, at first sight the fits apparently resemble the data quite well for 0.5% and 2% HSA, but a closer look reveals that these data also have a tendency to increase their slope as $r \rightarrow 0$, a pattern which the Adair description could not reproduce.

The data were also fitted to a model [35] describing HABA induced dimerization of albumin. This model

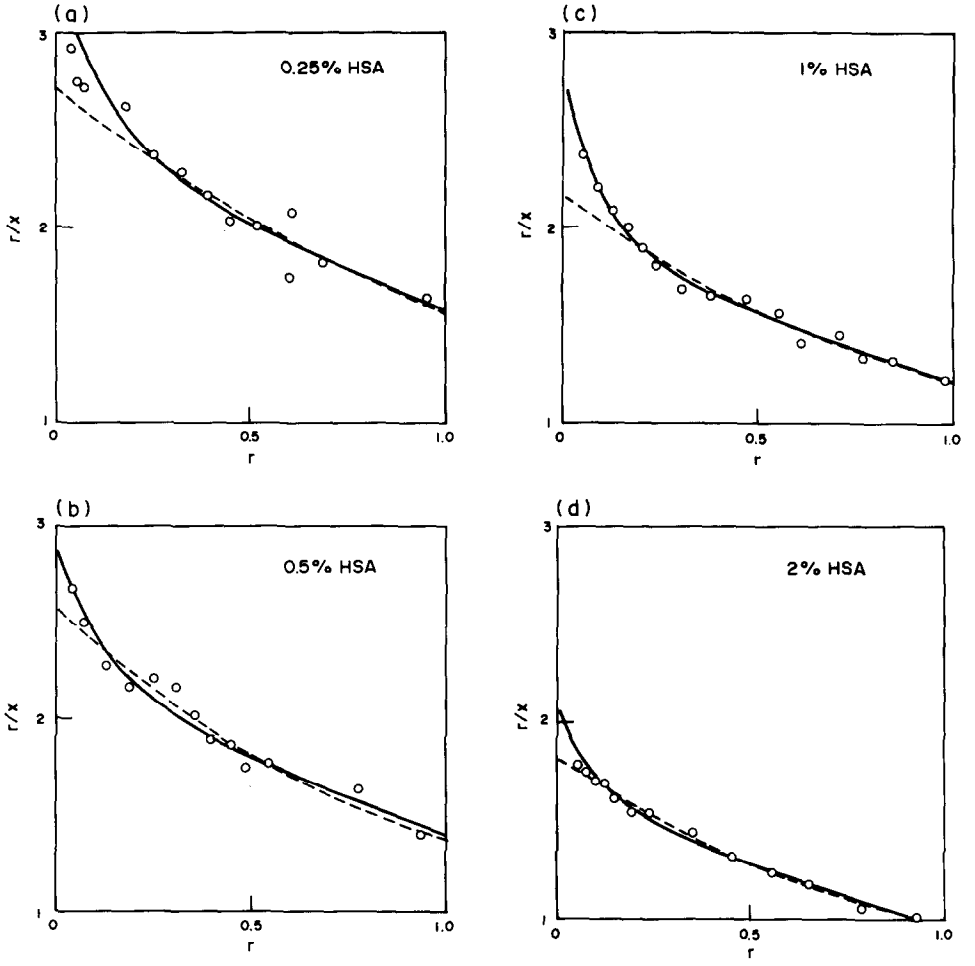

Fig. 1. Scatchard plots of the data for the binding of HABA (initial total concentration 10 to 450 μM), measured by equilibrium dialysis at pH 7.4 and 37°, to four concentrations of human albumin (a) 0.25%, (b) 0.5%, (c) 1.0% and (d) 2.0% (w/v). The unbound concentration of HABA denoted x, is given in units of 10^{-4} M. In each figure the fit produced by a non-linear least squares regression analysis of r vs x is indicated by a dashed line (---) for the Adair model and by a full line (——) for the contaminant model.

is known to give rise to a pronounced effect of albumin concentration, but it was unable to explain the present data. This conclusion can also be drawn by noting that this model predicts that the binding curves for different albumin concentrations all intersect at a point $r \simeq 0.5$, which is obviously not the case for the present data.

The competitive contaminant model, which is discussed briefly above was originally proposed by Brunckhorst and Hess [36] and later developed in terms of a binding equation and other useful relationships by Pedersen and Pedersen [29]. One of these relationships predicts that the free concentration of ligand corresponding to $r = 0.5$ depends linearly on the protein concentration if there is only a single site on the protein to which the ligand binds and this site is contaminated. However, the presence of more binding sites will change this behaviour. The present data did not show a linear dependence although the free ligand concentration at $r = 0.5$ did increase with increasing protein concentration. This could indicate that the model is applicable but that significant binding to other sites occurs. In the fitting procedure the binding (association) constants were varied until a best fit was obtained, but the fractional contamination was considered to be constant. In order to get an idea of the magnitude of the fractional contamination one should note that Pedersen and Pedersen [29] showed that if the low concentration data become linear, then the intersection of this line with the r-axis gives an approximate value of the uncontaminated fraction (α) of the sites. The present data continue to curve upwards, however, (and increase in slope) as r decreases so this procedure could not be used. It is nevertheless clear from Fig. 1c that $\alpha < 0.3$ and consequently the contaminated fraction $\beta > 0.7$. A plateau in a plot of r versus log x could also be used to estimate α [29] but in the present data no distinct plateau was observed. Instead the data were fitted to the approximate binding equation derived in Ref. 29. The advantage of this procedure is that it gives approximate values of all the parameters, including the contaminated fraction. The values so obtained were then used as

Table 1. Binding parameters for the interaction of HABA with human albumin obtained by the contaminant model on the basis of three binding sites (see Fig. 1a to d)

% HSA	β^*	$K_x/(10^4/M)$†	$K_y C_p'$‡	$K_1/(10^4/M)$§	$K_2/(10^4/M)$§	RMS‖
0.25	0.95	17	160	1.5	0.7	0.015
0.50	0.95	17	400	1.5	0.7	0.016
1.0	0.95	21	1000	1.4	0.7	0.009
2.0	0.97	17	1300	1.3	0.5	0.005

* The contaminated fraction of the high affinity site.
† The association constant of HABA for the high affinity site.
‡ Association constant of the contaminant for the high affinity site multiplied by the corrected protein concentration C_p', cf. Eqn (6).
§ Adair constants describing the ligand binding to the uncontaminated low affinity sites.
‖ The root mean square value which is the SD of r from the best least-squares fit to the data with Eqns (3) and (5).

initial values in the fitting program based on the exact binding equation, Eqns (3) and (5). The best fits are shown as the full curves in Fig. 1 and the corresponding values of the binding parameters are given in Table 1.

It was found that the data could only be fitted satisfactorily to the contaminant model by assuming three or more sites and of these a *single* high affinity site was contaminated rather than several. The contaminated fraction was found to be rather large 0.95, i.e. the high affinity site was almost completely contaminated by one or more inhibitory substances (acceptable fits were obtained for $0.90 < \beta < 0.98$ with the best fits given by the value above). This is the reason that the data were so difficult to fit and why the plateau was not observed, since this would have occurred at $r \simeq 0.05$ where too few experimental results were available. The most dramatic effect of a contaminant in a Scatchard plot is the increase in the slope for small r-values which appears around an r value equal to the uncontaminated fraction (Fig. 1). In the present case this occurred about r equal to 0.05, which was approaching the limit of experimental sensitivity.

An essential requirement of the applicability of the model is that the binding constants K_x, K_y and K_1, K_2 found by fitting are independent of the albumin concentration. This implies that the values of $K_y C_p'$ obtained by the fit should show a linear dependence upon C_p and as can be seen from Table 1 this requirement is well satisfied, allowing for experimental error and the associated errors of the estimated parameter values.

Another requirement for the proof of the model is that the model gives a statistical significantly better fit than the Adair description. That this is the case is evident from the figures, especially Figs 1a and 1c. This can also be shown by a statistical test for accepting an extra parameter since the difference in rms values between the two models is 8%.

The association constant for the binding of the competitive contaminant (K_y) was found from the estimated values of the product $K_y C_p'$ to be 5×10^6/M, an order of magnitude larger than the binding constant of 2×10^5/M for HABA to the high affinity site. The value of this high-affinity binding constant is an order of magnitude greater than that previously estimated from the Scatchard plot [4]. The Adair constants K_1 and K_2 for the binding of HABA to the low affinity sites were an order of magnitude smaller, i.e. 10^4/M.

DISCUSSION

The number of papers which include evidence, either direct or indirect, of an effect of protein concentration has continued to grow since the problem was reviewed briefly over ten years ago [2]. The number and diversity of compounds has also grown [3–25] and now includes an inorganic compound aurothiosulphate [25]. Although concern was expressed that the phenomenon was an artifact of the experimental system [4] it now seems unlikely that this is the case since inverse dependence has been observed with several different techniques.

The major possibilities suggested as an explanation for this phenomenon are protein–protein interaction, ligand-induced dimerization and contamination by one or more ligands which inhibit the binding of the ligand under investigation. Protein–protein interaction has been the subject of increasing interest and Zini *et al.* [37] have investigated this. The existence of two conformers of human albumin, dependent upon protein concentration has been suggested [19] but unfortunately the full details have not yet appeared. The thermodynamic activity of proteins in solution is altered by the presence of unreactive or inert macromolecules [38] and this hypothesis, also referred to in terms of either volume occupancy or excluded volume, has also been considered in relation to the binding of L-tryptophan and methyl orange to bovine albumin [39]. A better fit, however, to the data in Fig. 2 of Ford *et al.* [39] would have provided more convincing support for this hypothesis. Ligand-induced dimerization of the protein, with consequent inhibition of binding, has also been considered [35] but does not appear to have provided a satisfactory explanation for the inverse dependence of binding constants upon protein concentration.

The presence of an inhibitory ligand associated with the protein has been considered as a possible explanation but not until this model was applied to the interaction of aurothiosulphate with human albumin [25] had any convincing quantitative evidence appeared to support it. In some ways it is the

least attractive hypothesis since it is an uncomfortable reminder that proteins and receptor systems generally do contain interfering ligands.

The data for the binding of HABA to four concentrations of human albumin which, in common with other ligands, showed an inverse dependence of K upon the protein concentration has been fitted to several binding models by a non-linear regression method: Adair, ligand-induced dimerization and the competitive contaminant model. Only the contaminant model was able to explain the protein dependence and to reproduce the increasing slope of the binding curve at small values of r found in the Scatchard plot (Fig. 1) and so was the only model which could explain in a systematic way the binding curves and their inverse dependence upon protein concentration.

The importance of obtaining experimental results at low total ligand concentrations (and therefore low values of r) is apparent from the analysis presented here (Fig. 1). It would have been desirable to obtain results at r values even lower than that of 0.034 which was obtained for 0.5% human albumin.

The model produced its best fit on the basis of three or more sites with a single high affinity site being contaminated by one or more inhibitory substances to the extent of about 95%. The estimate of 5×10^6/M for the association constant of the contaminant suggests that residual substances such as fatty acids could be responsible. The fatty acids are commonly found in commercial albumin preparations at a ratio at least of 1:1 with the protein and often higher [30–32].

Previous work with cortisol [11] and HABA [20] has shown that Scatchard plots obtained by variation of the protein concentration at a fixed ligand concentration had lower slopes when higher ligand concentrations were used and that with HABA such a plot can be transformed to a negative one if the ligand concentration is raised sufficiently [20]. Although we have not yet been able to apply this contaminant model to these results it is consistent with them: the higher ligand concentrations would start to overcome the effects of an inhibitory contaminant present in a fixed ratio with the protein.

The phenomenon of inverse dependence of K and/or n upon protein concentration has been reported most frequently for anionic and neutral ligands with either bovine or human albumin, but they are not the only ligand–acceptor combinations which have been implicated. Basic drugs and albumin show the same binding behaviour [5, 16, 17] and this effect has also been observed with α_1-acid glycoprotein [21] and haemoglobin [12]. The primary function of albumin is probably to transport ligands [33] and so the better such an acceptor is at binding small molecules more likely it is to either remain or become contaminated with either endogenous or exogenous ligands, respectively. This property of albumin together with its frequent experimental use are likely to be the main reasons why the phenomenon is best documented for this protein.

We have not examined the receptor binding literature in detail but there are examples of this effect recorded with striatal homogenates [27, 28] and with the binding of steroid hormones to histones [40] and uterine cytosol [41]. The relatively crude nature of the membrane preparations used to study drug–receptor binding *in vitro* is likely to provide ample opportunity for endogenous ligands to affect binding. In such circumstances experiments with more dilute tissue preparations give a higher binding affinity. The usual design of such experiments would not, however, normally reveal an effect of protein concentration, but unless this effect is taken into account the apparent association constant could be in error by at least an order of magnitude. Assay sensitivity was, however, a limiting factor.

Non-integer values of n, obtained by a variety of fitting procedures, are usually attributed to experimental error and the difficulty of obtaining the best fit to the curve. These difficulties should also inevitably diminish and so, other things being equal, more attention should be paid to the possibility that values of n that are highly unlikely to be a whole number are in fact evidence for the presence of inhibitory contaminants.

The structural diversity of ligands showing inverse dependence has been an obstacle to finding a satisfactory explanation. The competitive contaminant model overcomes this problem, however, because it can account for such binding behaviour in terms of a property of the protein, rather than of the ligand, and so could be applied to any ligand–acceptor system where competitive inhibitors are present. It remains to be seen how widespread is the inverse dependence phenomenon but the contaminant model should find wider application to results which apparently show this effect.

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