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Binding of bromosulphthalein sodium by human serum albumin using a continuous diafiltration technique

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Summary

1. The recently introduced ultrafiltration-cum-dialysis technique termed continuous diafiltration has been used to obtain detailed binding isotherms of equilibrium solution concentration of bromosulphthalein (BSP) versus the number of molecules of BSP bound per molecule of human serum albumin for pure human serum albumin solutions (albumin concentrations: 1·59–3·39 g/100 ml) and for three dilutions of pooled human serum (albumin concentrations: 1·45–2·85 g/100 ml) in 310 ideal milliosmolar phosphate buffer at pH 7·4 and 22° C.

2. Qualitative analysis of the isotherms seems to indicate that there is a competition between the polymerization of the albumin to dimers and high oligomers and the binding of BSP by albumin.

3. The binding capacity of pure human serum albumin at total BSP (bound and free) concentrations below 2·3 mM is greater than that of the pooled serum at an equivalent albumin concentration, possibly indicating the blockage of binding sites by more strongly binding ligands.

4. The binding capacity of pure albumin at total BSP concentrations greater than 2·3 mM is less than that of pooled serum at an equivalent albumin concentration, suggesting the presence of other non-ultrafilterable materials in the serum capable of binding BSP.

Introduction

Crawford & Hooi (1968) and Crawford, Davies & Davies (1971) have studied the binding of bromosulphthalein sodium (BSP) by human serum from the following groups: men, pregnant women, women taking oral contraceptives and newborn infants. The techniques used were, respectively, equilibrium dialysis and a simplified form of the technique developed by Hummel & Dryer (1962) for gel filtration of macromolecules—small molecule complexes. All Crawford's work has been performed at 37° C with 125 μM equilibrium solution concentrations of BSP. Crawford *et al.* (1971) observed that under the latter conditions, in the albumin concentration range 2·7 g/100 ml–5·5 g/100 ml, the BSP binding capacity in mol/mol of albumin decreased as the albumin concentration was increased according to the following equation:

$$y=k+a_i+bx+cx^2$$

where y is the logarithm of the number of molecules of BSP found per molecule of albumin ; k, b and c are constants common to all groups of sera tested ; a_i is a constant characteristic of the group from which the serum was taken, and x is the logarithm of the albumin concentration. The differences in the constant a_i for the various groups were statistically significant. Crawford (1969) has called for a more fundamental study of the albumin concentration effect and of the observed differences between sera of different groups. This communication reports on the first stage of the more fundamental investigations.

Crawford & Hooi (1968) and Crawford *et al.* (1971) using dialysis and the simplified Hummel and Dryer gel chromatography technique were only obtaining the molecular binding ratio at one equilibrium solution of BSP. Thus for a series of different albumin concentrations, they were effectively studying a section through the family of binding isotherms for that range of albumin concentrations. Our aim has been to choose a technique which could give us a detailed binding isotherm for each serum sample or each pure albumin solution used and which would be relatively rapid and simple. These requirements seemed to us to be well fulfilled by the technique of continuous diafiltration (outlined in **Methods**) introduced by Blatt, Robinson & Bixler (1968). In an attempt to understand the binding mechanism, the binding isotherms of the amount of BSP bound versus the equilibrium solution concentration (unbound) of BSP at three different albumin concentrations and at three dilutions of pooled human serum have been analysed quantitatively using the procedure of Nichol, Jackson & Winzor (1967) which is based on the concepts of multiple equilibria.

Whilst there have been numerous studies of multiple binding equilibria in protein systems (see for example the monograph by Steinhardt & Reynolds (1969) which reviews the whole field), there have been comparatively very few studies of the effect of variation in protein concentration on the protein binding capacity. Blatt *et al.* (1968), Breyer & Bauer (1953) and Ray, Reynolds, Polet & Steinhardt (1966) confined their work to albumin concentrations below 2 g/100 ml. It seems remarkable that only Crawford & Hooi (1968), Crawford (1969) and Crawford *et al.* (1971) appear to have made a detailed study of albumin binding capacity in the physiological albumin concentration range. Despite the considerable practical and theoretical interest of binding studies within the physiological range, no one has previously attempted to study the molecular mechanisms of binding in the latter range of albumin concentrations.

Methods

Materials

The albumin used was dried, purified, human serum albumin (100% standard for electrophoretic analysis) supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. It was not subjected to any further purification procedures. Bromosulphthalein sodium salt was supplied by Lloyd's Research Ltd., London as 50 mg/ml solution. This concentration was diluted using 310 ideal mOM phosphate solution at pH 7·4 to a millimolar stock solution. The albumin was also dissolved in similar phosphate buffer solution. The nitrogen used for pressurizing the eluant reservoir and diafiltration cell was pure (' white spot ') nitrogen from British Oxygen Company Ltd. The phosphates used were of analytical reagent grade and were

supplied by B.D.H. Ltd., Poole, Dorset. Glass distilled water was used to prepare all the solutions. The ultrafiltration membranes used were 'Diaflo' PM-10 membranes manufactured by Amicon Ltd., High Wycombe, Bucks. They were used in an Amicon model 12 ultrafiltration cell which was connected via an Amicon CDS-10 Concentration/Dialysis Selector valve to an Amicon RS-4 Stainless Steel Reservoir of 4 l. eluant capacity.

The procedure for diafiltration was first described by Blatt *et al.* (1968). In the simple techniques of dialysis and ultrafiltration, the molecular binding ratio for the binding of a drug to a protein is normally determined at a single equilibrium solution concentration of the drug. If a binding isotherm is to be constructed, a whole series of experiments must be performed at different equilibrium solution concentrations. The disadvantage with dialysis is that it is very slow and wasteful of serum or binding protein, if a binding isotherm is desired. The principal disadvantage of ultrafiltration, which is particularly serious, is that the protein concentration is increased as the experiment proceeds and phenomena such as those described in this paper would be difficult if not impossible to observe. As with dialysis, it is wasteful of material since a large number of experiments must be performed to construct a reasonable binding isotherm.

Continuous diafiltration combines the advantages of ultrafiltration and dialysis without their disadvantages. In this technique, the protein solution or the serum is ultrafiltered using an anisotropic semipermeable membrane but the rate at which the solution is ultrafiltered is exactly balanced by the rate at which drug solution is added from a reservoir. Thus the protein solution is ultrafiltered at a constant volume by the balanced addition of drug solution. If the ultrafiltration rate is slow enough for equilibrium to be maintained between the free (unbound) drug and the drug bound to protein, then analysis of the eluate from the ultrafiltration cell enables us to determine the free drug concentration for a given addition of drug from the reservoir. We can thus calculate the amount retained by the protein in excess of that which would be retained in the cell in the absence of protein as described below.

The data obtained from the diafiltration experiments consisted of eluate concentration versus fraction number. Typical eluation curves are illustrated in Fig. 1; curve A is the curve obtained with a given volume of buffer (containing no albumin) in the ultrafiltration cell, curve B is a typical curve obtained with the same volume of a buffered albumin solution in the cell. The position of curve B for albumin in the cell is always below curve A, its absolute position depending upon the albumin concentration. The elution curve data were converted into binding isotherm data by summation of the differences between the elution curve in the absence (A) and in the presence (B) of the binding protein. The amount bound c_B at a given eluate concentration, c'_n (Fig. 1), was obtained by summing the differences between c_n and c'_n for all the fractions up to and including fraction number n, multiplying by the eluate fraction volume and the molecular weight of the binding protein (albumin) and dividing by the product of the ultrafiltration cell volume and the protein concentration in the cell. In effect, this represents the excess amount of BSP retained (as mol BSP/mol of albumin) in the ultrafiltration cell when albumin is present. The equation for this summation is:

$$c_B = \sum_n (c_n - c'_n) \frac{\Delta v \,.\, M}{V \,.\, c_p} \qquad (1)$$

where c_B=number of mol of BSP bound/monomer gramme molecular weight of albumin, n=fraction number, Δv=fraction volume in litres, c_n=eluate concentration of BSP in molar units at fraction number n for buffer alone in the utrafiltration cell, c'_n=eluate concentration of BSP in molar units at fraction number n for albumin or serum in the cell, M=molecular weight of albumin, V=volume of albumin solution, c_p=concentration of albumin in the cell in grammes per litre.

A computer programme was used to calculate (i) the c_B values corresponding to the various c'_n values, (ii) the $(c_B)^{-1}$ values corresponding to the various $(c'_n)^{-1}$ values and (iii) the values of total drug concentration (free and bound) at the various c'_n values. Subsequent plots of $(c_B)^{-1}$ versus $(c'_n)^{-1}$ (inverse isotherms) were used in the qualitative evaluation of the binding process by the method of Nichol *et al.* (1967).

A value of 69,000 for the monomer molecular weight of albumin (Tanford, 1968) has been used in the calculations described in this section.

The ultrafiltration cell was connected to a stainless steel reservoir via a concentration/dialysis selector valve attached to a high pressure supply of nitrogen (3 kg/cm²). The outlet from the ultrafiltration cell was a small bore plastic tube, the outlet of which was placed just above the fraction collector siphon (2·8 ml capacity).

In the 'continuous diafiltration' mode of operation of the Amicon system, the reservoir contained the buffered solution of BSP and the ultrafiltration cell contained the buffered albumin solution. Pressure was applied via the valve to both reservoir and cell simultaneously for a few seconds and then the valve was switched so that the pressure of gas was only on the liquid in the reservoir. Liquid then passed from the reservoir to the cell at the same rate as the eluate left the cell via the ultrafiltration membrane. To maintain the cell contents at an accurately constant volume, it was necessary to block the ultrafiltration cell pressure relief valve with paraffin wax. The cell contents were stirred continuously during the course of the experiment. The ultrafiltration rate was about 7 ml/hour.

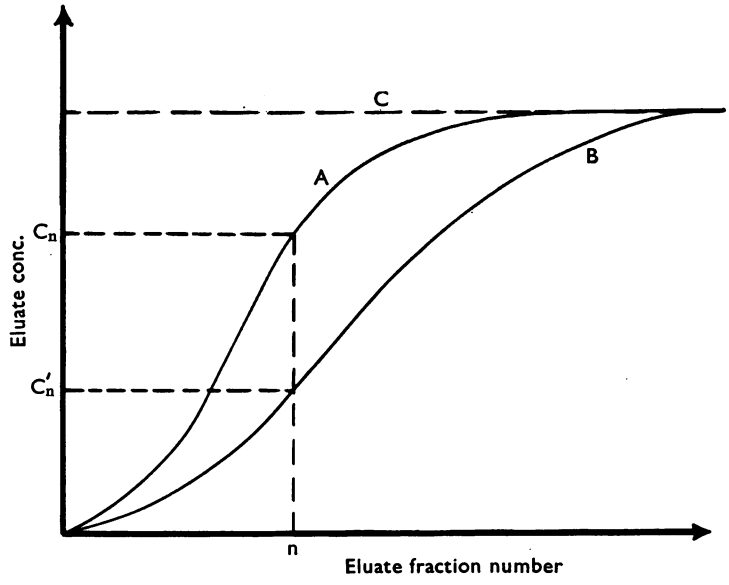

FIG. 1. Typical ultrafiltration cell elution curves for the 'continuous diafiltration' mode A with buffer in the cell but no albumin, B with buffer containing albumin in the cell. C is the reservoir concentration of the substance being bound.

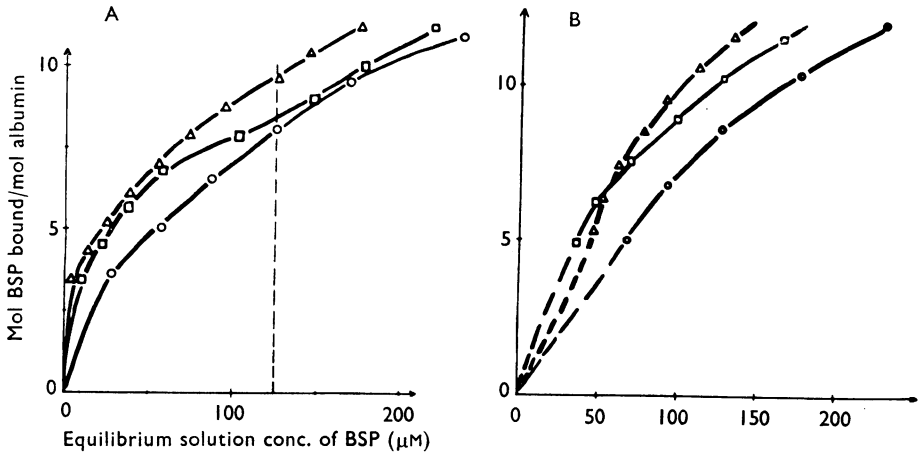

FIG. 2. The 22° C binding isotherms for BSP binding on to (A) human albumin at different albumin concentrations and (B) on to human serum at different dilutions.

(A) ○—○, 1·59 g% albumin
□—□, 2·31 g% albumin
△—△, 3·39 g% albumin
} in pure albumin solutions

(B) ●—●, 1·45 g% albumin
■—■, 2·29 g% albumin
▲—▲, 2·85 g% albumin
} in diluted pooled serum

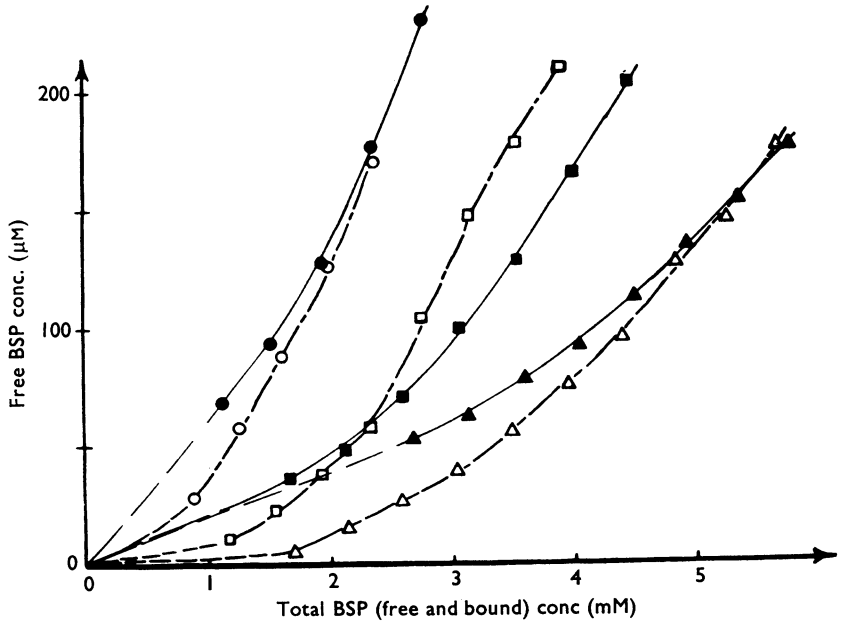

FIG. 3. The 22° C isotherms of free BSP versus total (free and bound) BSP for BSP binding on to pure human serum albumin at different albumin concentrations and on to various dilutions of pooled human serum.

○—○, 1·59 g% albumin
□—□, 2·31 g% albumin
△—△, 3·39 g% albumin
} in pure albumin solutions

●—●, 1·45 g% albumin
■—■, 2·29 g% albumin
▲—▲, 2·85 g% albumin
} in diluted pooled serum

Experiments were performed at room temperature (22° C) with an eluant reservoir concentration of BSP at 1 mM and with cell concentrations of pure albumin solutions at 0, 1·59, 2·31 and 3·39 g/100 ml, respectively. Experiments were also performed with three dilutions of pooled human serum. The serum was diluted with 310 ideal mOM phosphate at pH 7·4 to give final test dilutions of albumin in the serum in the ultrafiltration cell of 1·45, 2·29 and 2·85 g/100 ml respectively. The albumin content of the pooled human serum was determined by the single radial immunodiffusion technique of Mancini, Carbonara & Heremans (1965).

The eluate fractions from the ultrafiltration cell were analysed for bromosulphthalein as follows: aliquots of 1 ml volume from each fraction were added to 3 ml aliquots of 0·005 N aqueous sodium hydroxide solution and the optical density at 540 nm was read using a Zeiss PMQ-II single-beam spectrophotometer at room temperature in 1 cm path-length cells. Concentrations of BSP were obtained using a previously prepared calibration curve of BSP concentration versus optical density. The mean values of duplicate diafiltration runs were taken for each albumin concentration used in the ultrafiltration cell.

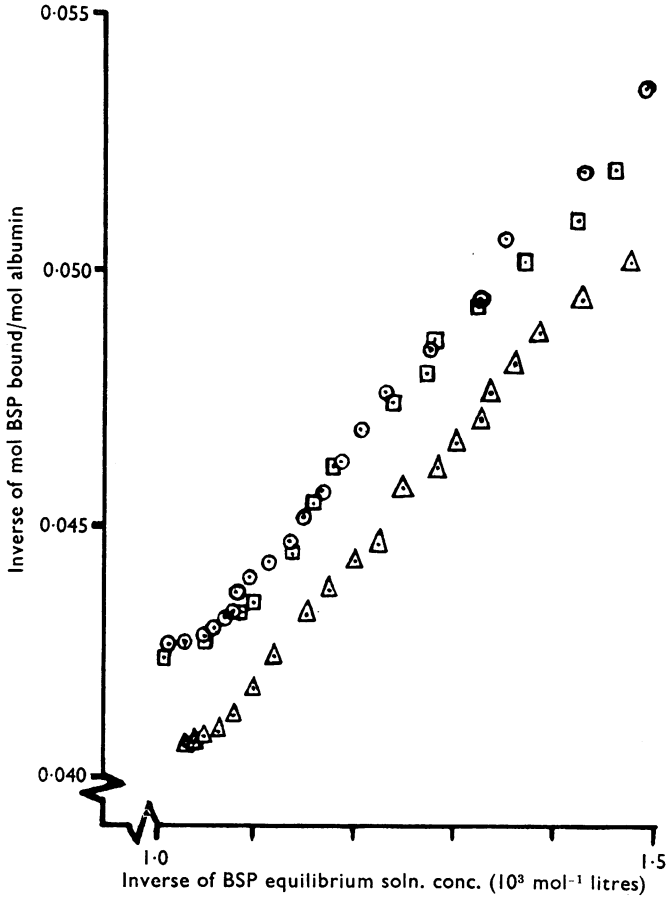

FIG. 4. The 22° C isotherms of the reciprocal of the mol of BSP bound per mol of albumin versus the reciprocal of the molarity of BSP equilibrium solution concentration (inverse isotherms) at different albumin concentrations. ○, 1·59 g% albumin; □, 2·31 g% albumin; △, 3·39 g% albumin.

After each run with albumin solution or with serum in the cell, the ultrafiltration membrane was deproteinized with an isotonic saline solution of pepsin overnight at room temperature. The membranes were stored in 10% aqueous ethanol at 4° C until required again. Before use, the membranes were placed in the cell as usual and the cell was flushed with 10 ml of phosphate buffer to remove any BSP remaining in the membrane. The cell was dismantled, thoroughly washed and then reassembled ready for use.

Results

The 22° C binding isotherms for BSP binding on to pure human serum albumin and on to pooled serum (expressed as amount bound in mol per mol of albumin) for the BSP concentrations of pharmacological interest are shown in Fig. 2. The corresponding curves showing the relationship between free drug concentration and total (free and bound) drug concentrations are illustrated in Fig. 3 for pure albumin solutions and for diluted pooled serum. The inverse isotherms for pure albumin solutions binding high concentrations of BSP (near mM equilibrium solution concentrations) are illustrated in Fig. 4.

Comparison of the 22° C isotherms for albumin and for diluted pooled serum (Fig. 2) shows that there are some differences between the two systems. In albumin solutions molar BSP binding capacity increases with increasing albumin concentration in the range studied, but it is clearly not a simple variation. In serum, we appear to be in a transition between a situation in which the molar BSP binding capacity increases with increasing albumin concentration and one in which it decreases with increasing albumin concentration (especially at equilibrium concentrations of BSP around 5×10^{-5}M). The molar BSP binding capacities for pure albumin and for pooled serum are very similar in the 50 μM free BSP concentration range but below this serum has a lower binding capacity per albumin molecule. At high free BSP concentrations (in the mM range), serum has a higher binding capacity per albumin molecule than pure albumin solutions have (not illustrated).

Discussion

It is clear from the form of the isotherms (Figs. 2 & 3) relating molecules of BSP bound/albumin molecule to the equilibrium solution concentration of freely available BSP for pure albumin and for diluted pooled serum that the situation is considerably more complex than Crawford and co-workers could have anticipated (1968, 1969, 1971) using one equilibrium solution concentration. The binding behaviour in serum resembles that of albumin in that the molar binding capacity is some function of dilution, but differences are also apparent. Other factors besides albumin concentration would seem to be playing a role, for example, competition in serum from strongly bound metabolites (at low BSP concentration) and/or the presence of other non-ultrafilterable materials which could bind BSP. Baker (1966) has suggested that the α_1-lipoproteins may be an important reservoir of BSP, she demonstrated this clearly for canine serum but not unambiguously for human serum. She even suggested that α_1-lipoprotein has a higher affinity for BSP than albumin does. However, the latter hypothesis is not supported by the isotherm data because serum has a lower BSP binding capacity than pure albumin solutions at low BSP concentrations (compare the curve in Fig. 3 for 2·29 g% albumin in serum with that for 2·31

g% pure albumin). Comparison of the same curves above a BSP total concentration of about 2·3 mM indicates that serum has a higher binding capacity than albumin in this concentration range. This may well be due to binding by α_1-lipoprotein. Crawford *et al.* (1971) have shown in electrophoresis experiments that BSP migrates principally with albumin but to a small extent also with α_1-globulins. Thus, evidence seems to be accumulating in favour of the hypothesis that other proteins besides albumin can bind BSP in serum.

Figure 4 illustrates the inverse isotherms for BSP binding on to pure albumin. The inverse isotherms are plots of the reciprocal of the molar BSP binding capacity versus the reciprocal of the BSP equilibrium solution concentration. The isotherms shown in Fig. 4 are for BSP equilibrium solution concentrations approaching 1 mM. The value of plotting detailed inverse isotherms in elucidation of binding mechanisms has been demonstrated by Nichol *et al.* (1967) in their theoretical study of binding equilibria. These workers showed that the shape of the inverse isotherms, as they approach the axis of the reciprocal binding capacity (at high equilibrium solution concentrations of the substance being bound) is indicative of the presence or absence of competitive or non-competitive isomerization or polymerization of the protein along with binding of the ligand. Examination of the isotherms of Fig. 4 shows that they curve towards the reciprocal binding capacity axis and that the isotherms for different albumin concentrations do not lie upon one another (all three curves diverge at lower eluate concentrations but this is not illustrated here). According to the classification of Nichol *et al.* (1967) this is indicative of a competition between the protein polymerizing and binding ligand (BSP).

Pedersen (1962) showed the presence of significant amounts of dimer, trimer, and higher oligomers (up to hexamers) in human serum albumin samples using Sephadex gel chromatography. The polymerization equilibrium should be dependent upon the albumin concentration and any disturbance of this equilibrium by competition for polymerization sites by BSP binding would also be expected to be dependent upon the albumin concentration.

It is concluded that the molar binding capacity of human serum albumin for bromosulphthalein varies with the albumin concentration in a manner which is indicative of a competition between albumin polymerization and binding of BSP by albumin. The binding capacity of albumin for BSP at low total BSP concentrations (less than 2·3 mM) is higher than the serum binding capacity. At high BSP total concentrations the serum binding capacity is higher than that of albumin, indicating the presence of other substances capable of binding BSP.

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