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BINDING OF CORTISOL TO HUMAN ALBUMIN AND SERUM: THE EFFECT OF PROTEIN CONCENTRATION

U. W. MUELLER and JULIA M. POTTER

Department of Pharmacology, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, 6009, Western Australia

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Abstract—Using equilibrium dialysis at 37°, the binding of two cortisol concentrations to both human albumin and serum were examined as a function of protein concentration. The range of protein concentration included the normal physiological range for albumin and cortisol. The use of dextran outside the dialysis sacs enabled the inside protein concentration to be stabilized against dilution resulting from colloid osmotic fluid movement. Furthermore, this enabled the protein mass within a given series to remain constant whilst allowing predetermined concentration changes. Scatchard analyses show that as the protein concentration decreases in either serum or albumin the binding affinity and/or number of binding sites for cortisol increases. Colloid osmotic fluid movements indicate that albumin behaves anomalously at relatively high concentration. This anomaly indicated by the presence of an apparently higher molecular weight species appears to be related to the concentration of cortisol present. In serum, the influence of corticosteroid binding globulin on cortisol binding is concentration dependent. This was shown by an apparent increase in binding site availability with protein dilution.

Cortisol has been shown to be transported in plasma by two major proteins, namely albumin and corticosteroid binding globulin. The differentiation of these two proteins in terms of their cortisol binding ability results from a relatively large difference in both affinity and capacity [1].

The traditional method of measuring ligand–protein interaction usually involves maintaining a fixed protein concentration whilst varying the ligand concentration. This approach assumes that the apparent association constant K and the number of binding sites n are independent of protein concentration. However, there is evidence that cortisol [2] and other drugs [3–5] show albumin-concentration dependence of these parameters. In view of this evidence both albumin and serum were examined to ascertain the binding kinetics for cortisol using varying protein concentrations in the presence of two fixed cortisol concentrations. The range of protein included physiological concentrations of albumin, whilst the cortisol concentrations selected were a normal level found within the diurnal variation and a 10-fold dilution thereof.

MATERIALS AND METHODS

Human serum. Samples of whole blood were withdrawn by venepuncture from healthy male volunteers and the serum harvested after standing at room temperature (23°) for 30 min and centrifugation at 2000 *g* for 10 min. Endogenous cortisol of the pooled sera was determined by competitive protein binding assay [6].

Human albumin. Normal serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) was received as a concentrate (250 mg/ml) in sterile bottles containing no preservatives. Only one batch was used throughout all experiments. No endogenous cortisol was detected by the competitive protein binding assay. The purity of this albumin preparation with regard to the presence of other protein contaminants was determined by the microimmunoelectrophoretic technique of Scheidegger [7] against two rabbit antiserums, namely whole human plasma and human corticosteroid binding globulin. No such contaminants were detected. However, albumin polymers (namely dimer and tetramer) were found to constitute about 4 per cent of the total protein. This was measured by passage through Sephadex G-200, with gel permeation performed by downward elution in a 2.6 × 100 cm column with 20 mM Tris–phosphate buffer, pH 8.0 containing 150 mM sodium chloride and 0.5% (w/v) sodium azide at room temperature (23°). Symmetry of the optical adsorption profile for both monomer and dimer of albumin suggested they were not in dynamic equilibrium with one another. Repeated application of any albumin polymer gave the same elution volume as found in the original sample, suggesting there was no dissociation to monomeric albumin.

Equilibrium dialysis of cortisol

Dextran. In order to control the osmotic movement of buffer into the dialysis casing during equilibrium dialysis, which thereby effectively dilutes the initial protein concentration, dextran was placed in the external buffer medium. Dextran T-70 (Pharmacia, Australia) is a defined polysaccharide polymer with a number average molecular weight of 42,500. Using known Dextran T-70 concentrations in the external buffer medium, its generated colloid osmotic pressure allowed the protein concentration

within the dialysis casing to be varied. The concentration of Dextran T-70 used varied from 0.75 to 8% (w/v).

Cortisol. Tritiated cortisol (1,2,6,7(n)-H^3), sp. act. 82 C/mmole (Radiochemical Centre, Amersham, Bucks, U.K.) was found to be 97% radiochemically pure using ascending thin layer chromatography on silica gel in a dichloromethane:acetone; 3:1 (v/v) solvent system. The specific activity of this material was reduced by the addition of unlabelled cortisol (Calbiochem, U.S.A.) The final working external dialysis buffer used contained about 5×10^4 cpm/ml.

Protein. One ml aliquots of either neat serum or albumin diluted with dialysis buffer to 40 mg/ml were placed in previously boiled Visking dialysis casing and tied with surgical linen thread. This means that within the dialysis series of either albumin or serum, the mass of protein within dialysis sacs was constant. The endogenous concentration of cortisol in serum was found to be 2.55×10^{-7} moles/l, whilst none was detectable in the albumin preparation. When the sacs containing one ml of neat serum were immersed in 10 ml of external dialysis buffer, the endogenous cortisol concentration was effectively diluted 11-fold becoming 2.32×10^{-8} moles/l. For the albumin series, unlabelled cortisol was added to the external dialysis buffer to yield an approximately equivalent final concentration, namely 2.77×10^{-8} moles/l. These two cortisol concentrations for both albumin and serum constituted the lowest examined in this study (Table 1). The higher cortisol concentration was achieved by addition of 2.76×10^{-7} moles/l to the external buffer for both albumin and serum. A trace amount of tritiated cortisol was added to the external buffer of all sacs giving a final content of 5×10^4 cpm/ml.

Dialysis. Dialysis was performed with 10 mM sodium phosphate buffer pH 7.1, NaCl 150 mM at $37 \pm 1°$ in a shaking water bath for 20 hr. Serial sampling of both inside and outside control sacs suggested that steroid equilibration was approached after 5 hr independent of the protein concentration examined. No demonstrable cortisol binding to dextran was found when dextran alone was dialyzed against buffer containing only cortisol.

Taking duplicate aliquots of both internal and external solutions for liquid scintillation counting at the conclusion of dialysis allowed calculation of protein bound cortisol. Liquid scintillation counting was in toluene based scintillant containing a non-ionic detergent, Teric X-10 (Robert Bryce, Australia) in the ratio of Toluene:Teric X-10; 2:1 (v/v) with six grams of 2,5 diphenyloxazole (Koch-Light Labs, U.K.) per litre. Counting in a Nuclear Chicago Isocap 300 had an efficiency of approximately 32 per cent. The counting error was kept to $\pm$ 1% for all samples.

Binding data was expressed either as a percentage or in the form of a Scatchard analysis [8], using the relationship

$$\frac{r}{[S]} = K(n - r) \qquad (1)$$

where r is the number of cortisol moles bound per mole of albumin, [S] is the molar concentration of free steroid, n is the number of cortisol binding sites per mole of albumin, and K is the apparent association constant. The molecular weight of albumin was assumed to be 66,250 [9]. For both serum and albumin the Scatchard plots are presented as a function of molar cortisol bound to moles of albumin in order to facilitate direct comparison between the two systems.

Protein concentrations. Human albumin and corticosteroid binding globulin (CBG) concentrations were determined by quantitative radial immunodiffusion [10], using antibodies raised separately to the two respective proteins. Radial immunodiffusion was performed in 1% (w/v) Type 1 agarose (Sigma Chem. Corp., U.S.A.), $M_r = -0.14$ in 50 mM sodium barbital buffer, pH 8.6. Using a nominal gel thickness of 1.1 mm, 4 μl of the appropriate antigen dilution was placed into 3 mm diameter holes with an intercentre distance of 1.6 cm. Radial diffusion occurred at 23° in a humid chamber for 48 hr. Calibration standards were obtained for albumin, using its extinction coefficient at 280 nm, $E^{1\%}_{1\,cm} = 5.31$, and its molecular weight 66,250 [9] and for CBG, $E^{1\%}_{1\,cm} = 7.10$ and molecular weight 49,500 [11]. Quantitation of steady state precipitin ring diameters was performed with vernier calipers on photographs obtained using dark-field illumination.

Table 1. Typical dialysis operation

	Albumin		Serum	
	Outside sac	Inside sac	Outside sac	Inside sac
At commencement				
Volume of solution (ml)	10	1	10	1
Dextran T-70 concentration (%)	2.5	—	4	—
Albumin concentration (moles/l)	—	6.04×10^{-4}	—	7.09×10^{-4}
Cortisol concentration (moles/l)	2.80×10^{-8}	—	2.76×10^{-10}	2.55×10^{-7}
At equilibrium				
Bound cortisol (moles/l)	—	5.25×10^{-8}	—	1.38×10^{-7}
Bound cortisol/ Albumin concentration	—	8.70×10^{-5}	—	1.95×10^{-4}

RESULTS

Albumin-cortisol binding. Figure 1 shows the percentage of cortisol bound to albumin and serum, expressed as a function of albumin concentration. Examining first the binding to albumin alone irrespective of which cortisol concentration is chosen, namely 2.54×10^{-8} moles/l or 2.51×10^{-7} moles/l, approximately 84 per cent is bound at 47 mg/ml albumin. This concentration of albumin is that which was found in the undiluted pooled serum used in these experiments. The normal albumin concentration range found in healthy adults is between 35 and 50 mg/ml [12].

Figure 2 is the same data for pure albumin presented as a Scatchard plot over the albumin range of 25 to 120 mg/ml for the two cortisol concentrations as above. Unlike classical protein–ligand interactions obeying the law of mass action, this plot has a positive slope for both cortisol concentrations examined. This observation may be interpreted as either increased affinity and/or binding sites for cortisol on albumin, the rate or degree of which appears to be intimately related to the cortisol concentration present. Although not as marked, a similar response namely a positive slope, is observed with points of equal protein concentration, as shown by the stippled lines at 25 (c), 50 (b) and 120 (a) mg/ml in Fig. 2. However, the magnitude of the positive slope decreases with protein dilution. These observations suggest that cortisol binding to albumin only approaches saturation at extremely dilute albumin concentrations

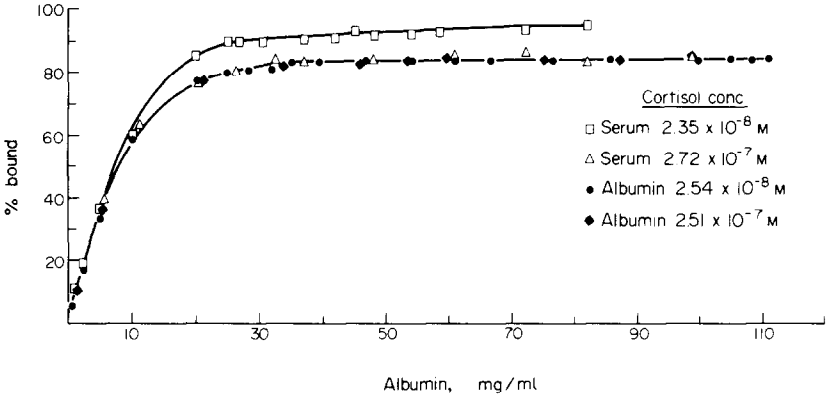

Fig. 1. Cortisol binding to human albumin and serum. Two concentrations of cortisol were examined in the presence of either varying serum or albumin concentrations. Serum is expressed as a function of its contained albumin concentration. The binding of cortisol is shown as the percentage bound to protein.

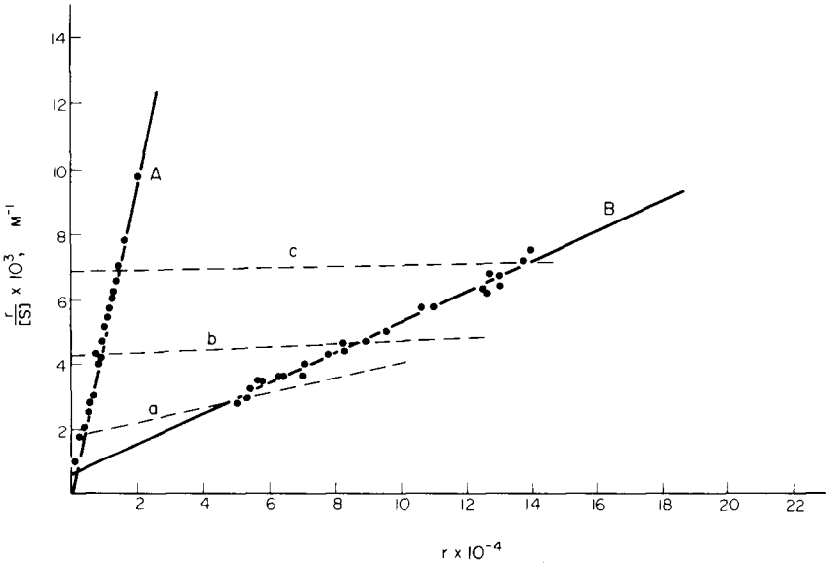

Fig. 2. A Scatchard plot for the binding of cortisol to human albumin. The albumin concentration shown is 25–120 mg/ml, with cortisol concentrations of 2.54×10^{-8} and 2.51×10^{-7} moles/l. Representative data points from 80 individual determinations for each cortisol concentration are shown. A describes the binding with the lower cortisol concentration, and B the higher cortisol concentration. The stippled lines join points of equal protein concentration; a is 120 mg/ml, b is 50 mg/ml and c is 25 mg/ml.

and the activity coefficient of albumin is therefore not independent of concentration. Even at an albumin concentration of 20 μg/ml (3.02×10^{-6} moles/l), saturation of albumin with either cortisol concentration examined here could not be demonstrated.

Colloid osmotic pressure. Dextran was used to oppose the colloid osmotic pressure generated by albumin within the dialysis sac. As there is an inverse relationship between colloid osmotic pressure and molecular weight (Van't Hoff Law), it is possible to derive a number average molecular weight for albumin at any given dextran concentration. This is achieved knowing the number average molecular weight for dextran and the equilibrium concentration of both dextran and albumin. The slope of this line will represent the reciprocal of the theoretical number average molecular weight for albumin. This is shown in Figure 3, as a plot of equilibrium dextran concentration against equilibrium albumin concentration. Figure 3 also shows the experimentally obtained values for albumin at both cortisol concentrations examined. At the higher cortisol concentration (2.51×10^{-7} moles/l), the albumin molecular weight derived from the slope of the line approximates 72,000 and at the lower cortisol (2.54×10^{-8} moles/l) is 81,000. These deviations from ideal osmotic behaviour are more apparent at relatively high albumin concentrations i.e. above the normal physiological levels. The phenomenon appears to be dependent upon the cortisol concentration present, with less apparent protein interaction occurring in the presence of the higher cortisol concentration studied.

Serum cortisol binding. When fresh serum is dialyzed under the same conditions as those described for isolated albumin in the presence of 2.35×10^{-8} moles/l cortisol, considerably more steroid is bound than to albumin alone (Fig. 1). This observation supports the view that serum contains at least one high affinity cortisol binding protein. However, by virtue of there being only a small difference in binding between albumin and serum at a cortisol concentration of 2.75×10^{-7} moles/l the second binding protein is of small capacity. The existence of this high affinity, low capacity cortisol binding protein in serum was originally described and named corticosteroid binding globulin (CBG) by Daughaday in 1958 [1].

The binding of cortisol to serum is transformed into a Scatchard plot in Fig. 4. The data is expressed in terms of serum albumin concentration, over a range of 25–84 mg/ml, enabling a direct comparison of cortisol binding between isolated albumin and serum. Any contribution by CBG to the total protein concentration will be negligible with respect to the albumin molar concentration. The CBG concentration found in the normal undiluted serum used in these experiments was 7.37×10^{-7} moles/l. Qualitatively the serum plot is similar to that for isolated albumin. Saturation of the higher affinity binding component (CBG) is demonstrated by joining points of equal albumin concentration i.e. 84 (d) and 50 (e) mg/ml (Fig. 4).

Comparing the Scatchard plots using the lower cortisol concentration for albumin and serum, the slopes of the two lines are divergent. This is more easily seen by the transformation of the data as in Fig. 5(A) in which nK, derived from the fraction of cortisol bound and albumin concentration, is plotted against the albumin concentration. The nK values for serum are expressed as if all binding is attributable to albumin. Similar data for the higher cortisol

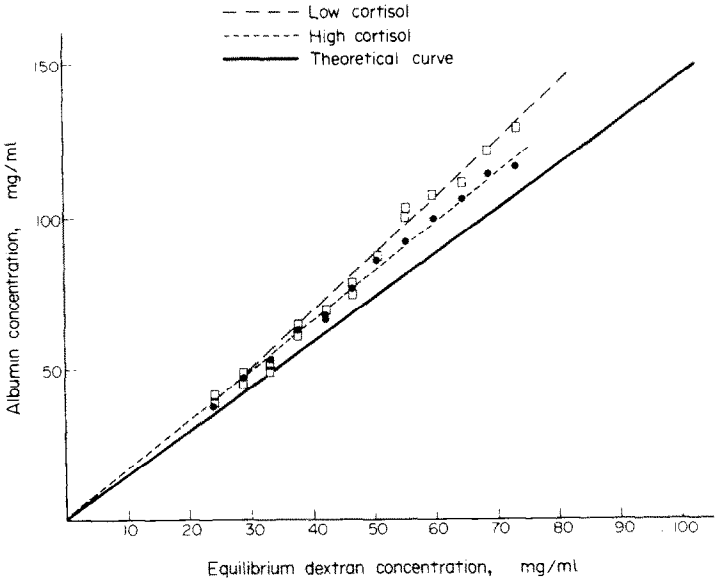

Fig. 3. The effect on protein concentration of dialyzing human albumin against various Dextran T-70 concentrations. Equal amounts of albumin were contained in each sac and dialyzed against known Dextran concentrations in the presence of cortisol either 2.54×10^{-8} (— — —) or 2.51×10^{-7} (– – – –) moles/l. The theoretical line relates the predicted albumin concentration with the known colloid osmotic pressure generated by Dextran. The reciprocal of the slope of the lines (both theoretical and experimental) give a measure of the number average molecular weight of albumin.

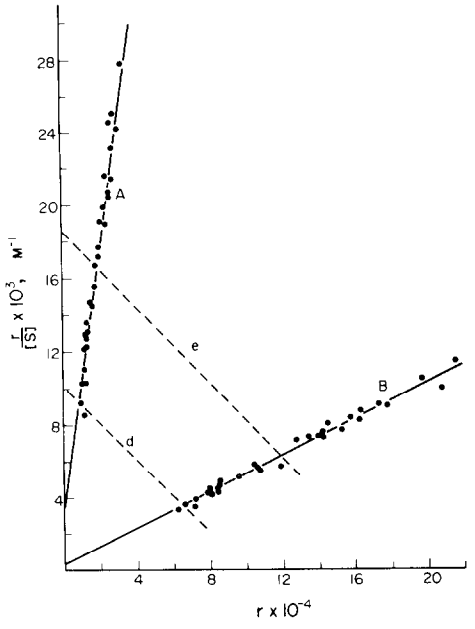

Fig. 4. A Scatchard plot for the binding of cortisol to human serum. The relative dilution and concentration of serum has been expressed as the albumin concentration, the range of which was 25 to 84 mg/ml. The cortisol concentrations used in dialysis were 2.35×10^{-8} and 2.75×10^{-7} moles/l. Representative data points from 80 individual determinations for each cortisol concentration are shown. A, described binding of the lower steroid concentrations and B, the higher. The stippled lines join points of equal protein concentration; d is 84 mg/ml and e is 50 mg/ml.

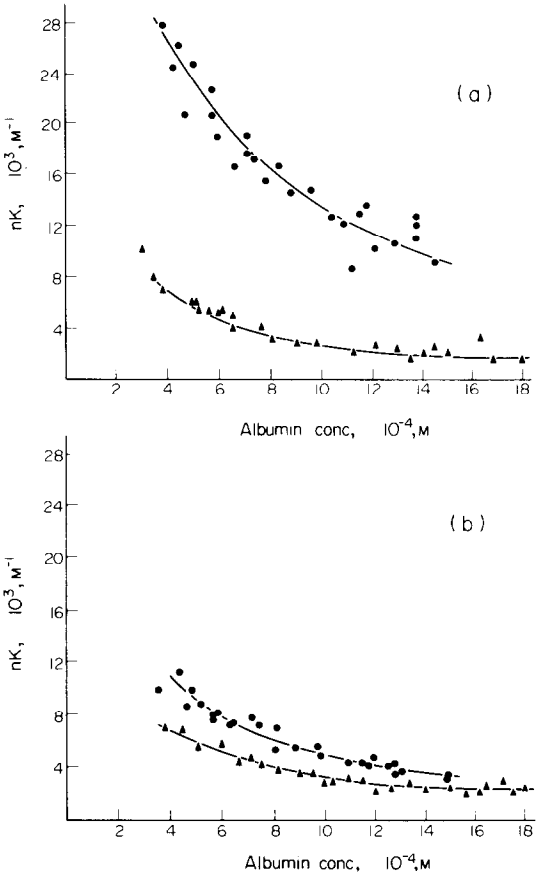

Fig. 5. Relationship between nK and human serum albumin concentration for the binding of cortisol. (A) The relationship of increasing albumin concentrations in the presence of the lower cortisol concentration examined, namely 2.35×10^{-8} moles/l for serum (●) and 2.54×10^{-8} moles/l for albumin (▲). (B) The same relationship with the two higher cortisol concentrations, namely 2.74×10^{-7} moles/l for serum (●) and 2.51×10^{-7} moles/l for albumin (▲). (A) and (B) are transformations of data contained in Figs. 2 and 4.

concentration is shown in Fig. 5(B). For both cortisol concentrations, nK decreases as the albumin concentration increases. In Fig. 5(A), comparing serum and albumin exposed to the lower cortisol concentration the decrease is more dramatic in the serum, presumably because of the presence of the high affinity binder CBG. It would seem that the behaviour of CBG itself in serum may be affected by concentration, as previously shown in the Scatchard plot in Fig. 4, with n decreasing as the protein concentration increases.

DISCUSSION

The experimental protocol used for this study on cortisol binding to albumin and serum represents a significant departure from conventional methods. The use of an inert material, namely dextran to oppose fluid movement during equilibrium dialysis experiments enables constant protein concentration to be maintained throughout. Coupled with this advantage is maintenance of a constant protein mass in a series, whilst allowing the opportunity to produce required variation in concentration within this mass. This overcomes the protein mass dependent Gibbs–Donnan effect when albumin concentration is altered in the presence of a fixed cortisol concentration. In the current study, the Gibbs–Donnan effect is itself minimised by the presence of a substantial quantity of saline in the dialysis buffer.

Albumin. The observation that the value of nK is a function of albumin concentration for the binding interaction with cortisol is in substantial agreement with the findings of Brunkhorst and Hess [2] and Kerkay and Westphal [13]. However, in this current study two concentrations of cortisol have been used to demonstrate that isolated albumin binding is not only dependent upon albumin concentrations, and therefore its variable activity coefficient, but also that cortisol itself plays a role in the determination of albumin activity. The effects are readily observable within the range of albumin found in clinical medicine, and may be associated with changes in steroid handling in patients who have very low albumin concentrations. In children suffering from severe nephrotic syndrome, the half-life of prednisolone, a synthetic steroid analogue of cortisol, is markedly increased in the presence of very low serum albumin [14].

Brunkhorst and Hess [2] have suggested that variable quantities of competitive inhibitors existed in the isolated albumin fraction. However, pre-dialysis of their albumin preparation against buffer prior to cortisol experiments suggests that any inhibitor would have to be either in a relatively large concentration or non-dialyzable. In this current study, if such a dialyzable inhibitor is present, due to experimental design it will remain constant throughout. Similarly a large molecular weight inhibitor (>10,000) would have revealed itself with gel permeation. However, the effects of a small tightly bound molecule such as fatty acid cannot be discounted.

Ray *et al.* [15] have reported that binding of dodecyl sulphate and dodecanol also exhibit bovine serum albumin concentration dependence. These results were subsequently shown to be due to a very slow approach to equilibrium by these ligands in the presence of high protein concentrations [16]. No such phenomenon with cortisol was observed in this study.

Positive co-operativity, in which the binding of one ligand to a macromolecule will create an energetically favourable site for another ligand molecule by some allosteric modulation has been demonstrated in other systems [17]. The operation of such a mechanism implies that if either protein or cortisol concentrations are varied with respect of each other then a similar response should be observed. The observations reported here support this form of interaction. Positive co-operativity is characterised by a positive slope on a Scatchard plot [17] as seen when either albumin or cortisol concentrations are held constant whilst the other is varied (Fig. 3). With protein dilution, nK increases. Furthermore, the apparent self association of albumin is demonstrated with an increase in the apparent number average molecular weight as shown by the colloid osmotic balance induced by dextran. This association also appears to be linked with the cortisol concentration present. The exact nature of this protein–protein interaction is not clear; however, they do not appear to be the covalently linked species found in small quantities in the starting preparation. The possibility that dimers of albumin not involving sulphydryl groups as described by Therriault and Taylor [18] and Hartley *et al.* [19] may be involved cannot be excluded. Other investigators have shown that if albumin concentration is varied with respect to the ligand concentration then a similar response, namely increase in binding with decrease in protein, is observed as described [3–5]. However, unlike this study no evidence is available to show that an increase in n and/or K exists if either cortisol or albumin concentration is varied with respect to the other.

Serum. The observation that at the higher cortisol concentration examined only a small increase in bound cortisol due to the presence of CBG in serum could be demonstrated was unexpected. However, the possibility that the isolated albumin binds more cortisol in the absence of other potential competing ligands cannot be excluded. Indeed the positive co-operativity of albumin for cortisol may well be considerably altered in the presence of tightly bound ligands such as fatty acids due to its flexibility or conformational adaptability [20]. In view of these observations it seems likely that cortisol binding in either serum or albumin cannot be adequately defined in terms of a single affinity constant or binding site unless due consideration is given to this apparent co-operativity.

Inspection of the Scatchard plots for both serum and albumin suggests that the other cortisol binding protein in serum, namely CBG, also demonstrates marked concentration dependent binding. It would seem most likely from the data that n changes in the presence of an unchanging affinity constant. The decreased binding of cortisol by CBG as a function of concentration is possible in as much as Werthamer *et al.* [21], have shown that dilution reversible polymers of CBG exist in whole human plasma. From our data, such polymers appear to be functionally reversible. The reason for their existence in plasma however remains obscure.

It appears the traditional approach of determining the binding kinetics of cortisol at only one protein concentration is inadequate and indeed may contribute to some erroneous conclusions regarding the concentration of unbound biologically active cortisol. Likewise, the use of dilute plasma and radioactively labelled cortisol to measure CBG concentrations in plasma (indirectly) may be misleading.

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