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Studies on the Mechanism of Binding of Serum Albumins to Immobilized Cibacron Blue F3G A

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The interaction of Cibacron Blue F3G A-Sepharose 4B with several serum albumins was studied. Although all albumins used were found to bind to this adsorbent, human serum albumin was bound to a far greater extent than were the others. From the results of competition experiments and n.m.r. studies of Cibacron Blue and/or bilirubin binding to human serum albumin it is proposed that the mechanism of the interaction between human serum albumin and Cibacron Blue is consistent with Cibacron Blue binding to bilirubin-binding sites. In contrast with these findings with human serum albumin, there is little or no interaction of Cibacron Blue and the bilirubin-binding sites of albumins from rabbit, horse, bovine or sheep sera, although some interaction occurs between Cibacron Blue and the fatty acid-binding sites of these proteins. Structural analogues of Cibacron Blue have been used to investigate the binding of albumins to these ligands.

The sulphonated polyaromatic dye Cibacron Blue F3G A (see Fig. 5 below) has been found to interact with many proteins, and numerous workers have utilized this property to purify proteins by affinity chromatography (Dean & Watson, 1979).

On the basis of the binding of Cibacron Blue to several proteins, it has been proposed that Cibacron Blue binds specifically to the dinucleotide-binding domain ('dinucleotide fold'). Binding of a protein to immobilized Cibacron Blue has been taken as evidence that the protein contains a dinucleotide-binding domain (Thompson *et al.*, 1975; Wilson, 1976). However, other proteins such as interferon (Maeyer-Guignard *et al.*, 1977; Jankowski *et al.*, 1976) and human serum albumin (Travis & Pannell, 1973; Travis *et al.*, 1976), which may not possess a dinucleotide-binding domain, have also been shown to bind to Cibacron Blue.

The interaction of immobilized Cibacron Blue with human serum albumin has been used not only to isolate the latter from plasma, but also to remove albumin before the isolation of other plasma-protein fractions, allowing albumin-free preparations (Travis *et al.*, 1976). Furthermore, it has been found that this is not a general property of serum albumins as a class, since only human serum albumin is reported to bind to Cibacron Blue–Sepharose, whereas albumins from rabbit, chicken and bovine sera do not bind to the same adsorbent (Iqbal & Johnson, 1977).

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Because of the widespread interest in the use of immobilized Cibacron Blue in plasma fractionation, the present study was undertaken to examine the different interactions of serum albumins with Cibacron Blue–Sepharose and to attempt to elucidate the mechanism of binding of human serum albumin to Cibacron Blue.

The known physiological functions of albumin include the binding and transport of long-chain-fatty acid anions, and the binding and detoxification of unconjugated bilirubin (Peters, 1975). The binding of these two ligands occurs primarily at separate sites, with fatty acids not interfering with bilirubin binding until five or more fatty acid molecules are bound per albumin molecule (Wooley & Hunter, 1970). Human serum albumin can bind 7 mol of fatty acid/mol with high affinity and then more with lower affinity (Goodman, 1958), or 3 mol of bilirubin/mol (Jacobson, 1969). Thus the effect of these molecules on the binding of human serum albumin to Cibacron Blue was studied.

Experimental

Materials

Sepharose was obtained from Pharmacia. Palmitic acid was obtained from BDH. Bilirubin, rabbit serum albumin (crystallized and freeze-dried; lot 27C-3963) and horse serum albumin (fraction V; lot 108C-8120) were obtained from Sigma; sheep serum albumin (crystallized) was obtained from Miles Laboratories; and bovine serum albumin (fraction V; pure; batch 76638) was obtained from Koch–Light. Human serum albumin was either purchased

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from Sigma (fraction V) or prepared from plasma on Cibacron Blue–Sepharose 6B columns by using the method of Travis *et al.* (1976). Cibacron Blue F3G A was obtained from Ciba–Geigy. Other triazine-based compounds were a gift from ICI. Outdated blood-bank plasma was obtained from The Royal Liverpool Hospital Blood Bank.

Methods

(*i*) *Preparation of matrices.* Dye–Sepharose derivatives were prepared by using the method of Heyns & De Moor (1974). Cibacron Blue was also coupled via its free amino group to epoxy-activated Sepharose 6B (Porath, 1974) after the reactive chlorine group had been removed to prevent coupling via the triazine ring. This was achieved by allowing Cibacron Blue to react with a solution of sodium methoxide in methanol for 2h at 60°C to produce 6-methoxytriazine Cibacron Blue. To show that none of the coupling achieved with this preparation was due to unchanged Cibacron Blue attaching via the triazine ring, a control was performed where the 6-methoxytriazine Cibacron Blue was incubated with unchanged Sepharose under the same conditions as with the activated Sepharose. No coupling was detectable.

The concentration of dyes bound to Sepharose was determined spectrophotometrically after hydrolysis of the agarose beads at 90°C in 50% (v/v) acetic acid.

(*ii*) *Preparation of palmitate/albumin solutions.* Palmitic acid was prepared as its potassium salt by dissolving it in a minimal amount of warm 0.05M-KOH and then making up the solution to the required volume with warm 0.025M-Tris/HCl, pH 8.6. Palmitate/albumin solutions of the desired molar ratio were made up and then left overnight at 5°C to allow equilibration to occur before applying them to the columns. Albumins were defatted by the method of Chen (1967).

(*iii*) *Preparation of bilirubin/albumin solutions.* The reported solubility of bilirubin is extremely low. Burnstein & Schmid (1962) report a value of 7mg/100ml and Martin (1949) as low as 0.1mg/100ml in phosphate buffer, pH 7.4. Since this is too low to prepare a sample of high bilirubin/albumin ratio in a small volume, the solubility of bilirubin was studied. It was found that by dissolving the bilirubin in a minimal amount of 0.02M-KOH and then making up to the required volume with 0.025M-Tris/HCl, pH 8.6, a solution of 100mg/100ml or greater could be prepared at a reasonable pH.

This solution was used immediately to give the required bilirubin/albumin ratios for the experiment with 10mg of albumin in a total volume of 0.45ml. The bilirubin/albumin solutions were allowed to stand for 4h at 5°C before applying them to columns, to allow equilibration to occur (Wooley & Hunter, 1970).

A_{280} measurements on bilirubin/albumin solutions were corrected for the contribution to the A_{280} by bilirubin. This correction factor was found to be 16.5% of the maximum absorbance at 450–470nm. All solutions containing bilirubin were protected from light.

(*iv*) *Column experiments.* All the micro columns used were obtained from Bio-Rad Laboratories (6mm diam.) and contained 1.0ml of settled gel. Albumin (10mg) in 0.45ml of solution containing bilirubin or palmitate as appropriate was applied to a column that had been pre-equilibrated with 0.025M-buffer. In the experiments where the pH was varied, the volume of the applied albumin solution was 0.20ml. Unbound protein was washed through with 10 column-volumes of the same buffer and the unbound albumin was measured. Albumin concentrations were determined on the basis of their A_{280}, with the appropriate albumin solutions as standards in each case. Bound albumin was calculated by difference and checked by determining the amount of albumin that was eluted by thiocyanate.

The use of 0.2M-NaSCN solution to elute albumin from the columns (Travis *et al.*, 1976) was found to cause significant 'tailing' and did not completely remove all bound protein. Therefore unless the albumin was required preparatively, the use of 0.5M-NaSCN/0.05M-Tris/HCl, pH 8.0, was found to be more satisfactory. All column experiments were carried out at 5°C.

(*v*) *N.m.r. studies.* N.m.r. spectra were recorded at 25°C in 0.01M-phosphate-buffered ^2H_2O [pH* (apparent pH) 8.0]. Measurements were made by using a Bruker WH 270 machine in Fourier-transform mode over a 4000Hz sweep width. Selective decoupling was applied to diminish the $^1H^2HO$ peak. Unless stated, the number of scans used was 10000. The protein solution used contained 1.0M-human serum albumin (0.5ml) and had been allowed to exchange in ^2H_2O overnight.

Human serum albumin was extracted from plasma for n.m.r. studies on Cibacron Blue–Sepharose 6B columns at pH 8.5 by using the method of Travis *et al.* (1976). It was then further purified using a second, smaller, Cibacron Blue–Sepharose column by applying a saturating amount of albumin solution in order to eliminate binding of any other protein contaminants by competition. Any remaining contaminants were removed with 0.2M-KCl/0.025M-Tris/HCl (pH 8.5) (losses of human serum albumin are minimal under these conditions). Human serum albumin desorption was effected by using 0.2M-NaSCN/0.05M-Tris/HCl, pH 8.0. The eluate was rapidly dialysed by using a hollow-fibre cartridge (Amicon H10P10). Prolonged con-

tact of human serum albumin with NaSCN was found to result in the formation of dimers and oligomers that do not bind to immobilized Cibacron Blue to the same extent as does the monomer (E. George & P. D. G. Dean, unpublished work). The purified human serum albumin was then defatted and freeze-dried before use. On polyacrylamide-gel electrophoresis (Ornstein, 1964) the purified human serum albumin was found to migrate as a single band.

Results

Preliminary experiments indicated that Cibacron Blue–Sepharose would remove essentially all the albumin from human plasma but not from sheep or bovine sera. The results of applying human and sheep sera to Cibacron Blue–Sepharose 4B columns (1.0ml) at pH 8.6 are shown in Fig. 1. In both cases albumin is the major protein bound, although the amount of binding is different for the two different serum albumins. Almost all the human serum albumin is removed from the serum and then desorbed with SCN^- (thiocyanate), whereas a lesser percentage of the total sheep albumin binds (and is desorbed).

Effect of pH on binding of different albumins to Cibacron Blue–Sepharose

Varying the pH of adsorption affects the relative binding of different proteins to Cibacron Blue–Sepharose in a way that does not reflect their pI (Angal & Dean, 1978). Therefore the binding of different albumins to Cibacron Blue–Sepharose 4B columns at various pH values was studied. The effect of pH on binding is shown in Fig. 2. It is seen that the behaviour of all the albumins is different with Cibacron Blue, showing the greatest affinity for human serum albumin.

Effect of competing ligands on binding on albumins to Cibacron Blue–Sepharose

In order to discover whether Cibacron Blue binds to any of the sites normally occupied by bilirubin or fatty acid, albumin was passed down Cibacron Blue–Sepharose columns after pre-equilibration with various amounts of bilirubin or fatty acid (palmitate) and the relative binding determined. Experiments were performed with 0.025M-Tris/HCl buffer (pH 8.6).

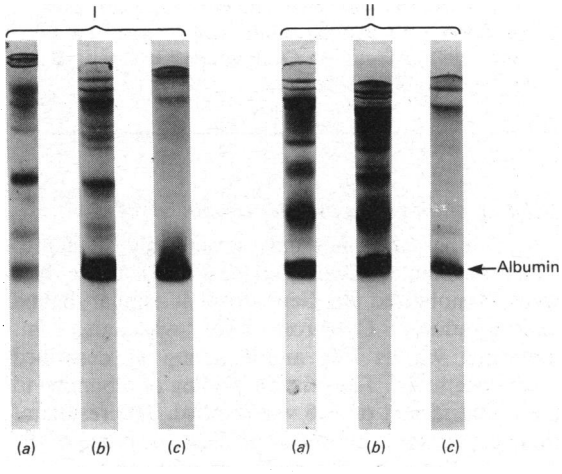

Fig. 1. *Polyacrylamide-gel disc electrophoresis of whole and Cibacron Blue–Sepharose-treated plasma*

I, Human plasma; II, sheep serum. Electrophoresis was carried out at pH 8.6 in 7.5% (w/v) polyacrylamide gels. The direction of migration was towards the bottom. Gels were stained with Naphthol Black. (*a*) Plasma proteins washed through a 1.0ml Cibacron Blue–Sepharose column at pH 8.6 after application of 0.03ml of plasma; (*b*) whole plasma; (*c*) proteins eluted from Cibacron Blue–Sepharose column with 0.5M-NaSCN, pH 8.0, after 0.30ml of plasma had been washed through with buffer (pH 8.6). All samples were diluted to the same extent, and the equivalent of 2μl of whole plasma was applied to each gel. The concentration of Cibacron Blue was 1.3μmol/ml of settled gel.

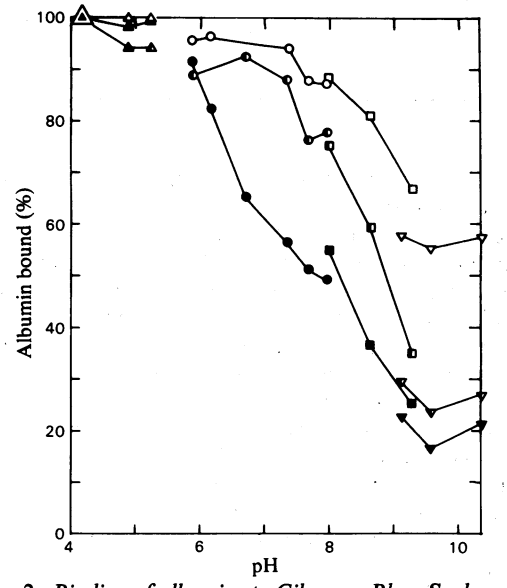

Fig. 2. *Binding of albumins to Cibacron Blue–Sepharose at various pH values*

The graph shows the binding of 10mg of albumin in a volume of 0.20ml when applied to 1.0ml Cibacron Blue–Sepharose columns (the concentration of Cibacron Blue was 1.5μmol/ml of settled gel). Open symbols, human albumin; half-closed symbols, sheep; closed symbols, bovine. Triangles, acetic acid/sodium acetate buffer; circles, H_2NaPO_4/HNa_2PO_4; squares, Tris/HCl; inverted triangles, glycine/NaOH.

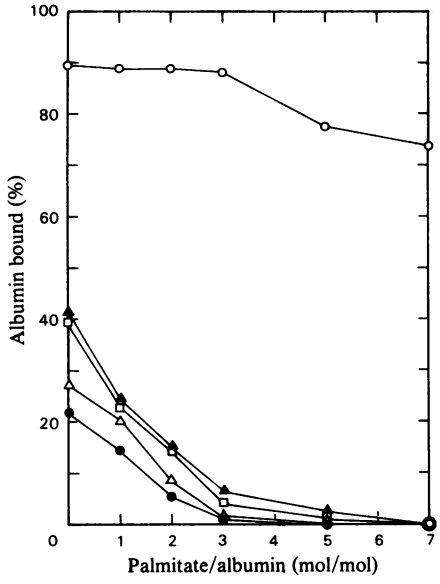

Fig. 3. *Effect of palmitate on the binding of albumins to Cibacron Blue–Sepharose at pH 8.6*
The graph shows the binding of 10mg of defatted albumin, in a volume of 0.45ml of buffered solution (pH 8.6) containing various amounts of palmitate, to 1ml Cibacron Blue–Sepharose columns (the concentration of Cibacron Blue was 1.3μmol/ml of settled gel). Albumins: O, human; □, sheep; ●, bovine; ▲, rabbit; △, horse.

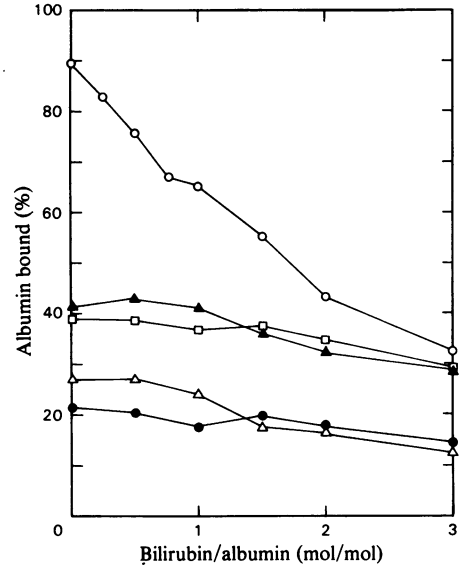

Fig. 4. *Effect of bilirubin on the binding of albumins to Cibacron Blue–Sepharose at pH 8.6*
The graph shows the binding of 10mg of defatted albumin, in a volume of 0.45ml of buffered solution (pH 8.6) containing various amounts of bilirubin, to 1.0ml Cibacron Blue–Sepharose columns (the concentration of Cibacron Blue was 1.3μmol/ml of settled gel). Albumins: O, human; □, sheep; ●, bovine; ▲, rabbit; △, horse.

(*a*) *Albumin/palmitate.* The effect of varying the concentration of added palmitate on the binding of previously defatted albumins to Cibacron Blue–Sepharose is shown in Fig. 3. There is a marked difference between human serum albumin, which binds strongly to the columns irrespective of palmitate concentration over the range used, and the other albumins, which exhibit marked reductions in binding with increasing concentration of added palmitate. There is a reproducible decrease in the binding of human serum albumin when the palmitate/human serum albumin ratio is 3:1 or greater. We have only observed these results at low ligand concentrations (such as 1.3μM-Cibacron Blue).

(*b*) *Albumin/bilirubin.* The effect of varying the concentration of added bilirubin on the binding of previously defatted albumins to Cibacron Blue–Sepharose is shown in Fig. 4. As for the albumin/palmitate experiments, there is a marked difference between the behaviour of human serum albumin and the other albumins. However, in this case the binding of human serum albumin diminishes with increasing concentration of bilirubin, whereas the other albumins show only minor decreases in binding.

Binding of albumins to other triazine dyes

A number of compounds structurally similar to Cibacron Blue are available (see Fig. 5). These dyes were immobilized on Sepharose at similar ligand concentrations. Cibacron Blue was also immobilized via its free amino group as described under 'Methods'. The relative binding of albumins to these matrices at pH 8.6 was studied. The results of these experiments are shown in Table 1. None of the matrices bound human serum albumin to the same extent as did Cibacron Blue, even though the structure of the ligands is similar. The amino-coupled Cibacron Blue showed very low binding to all albumins. Differences in ligand concentration account for changes in column capacity between experiments; however, these changes do not account for changes in the order of binding between different albumins, since the ligand concentration for any one experiment was the same.

^1H n.m.r. ligand-binding studies on human serum albumin/Cibacron Blue and human serum albumin/bilirubin

^1H n.m.r. spectra of human serum albumin (1.0mM), human serum albumin (1.0mM) in the

presence of 0.5, 1.0, 2.0 and 3.0mM-Cibacron Blue, and human serum albumin (1.0mM) with 2mM-bilirubin, were recorded and difference spectra of human serum albumin/Cibacron Blue minus human serum albumin and human serum albumin/bilirubin minus human serum albumin were plotted (see Figs. 6 and 7). These were compared with each other and with spectra of Cibacron Blue and bilirubin. Spectra of Cibacron Blue showed quite large changes with concentration, presumably due to intermolecular stacking.

Over the Cibacron Blue/human serum albumin range 0.5–2.0 the peak heights in the difference spectra are proportional to the concentration of Cibacron Blue, with the peaks occurring in identical positions.

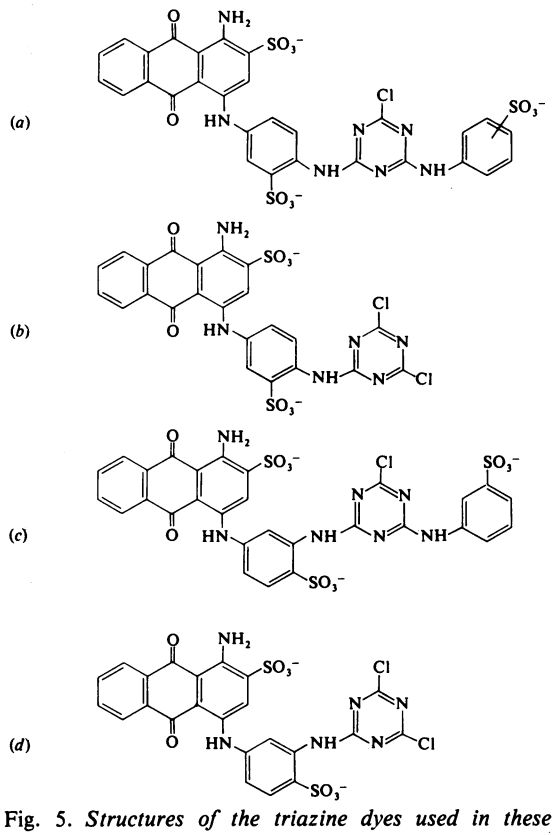

Fig. 5. *Structures of the triazine dyes used in these experiments*
(*a*) Cibacron Blue F3G A; (*b*) Procion Blue MX3G; (*c*) Cibacron Brilliant Blue; (*d*) Procion Blue MXR.

Discussion

Our results showed that removal of albumin by use of a Cibacron Blue–Sepharose column is more effective with human plasma than with any of the other columns used. This partly confirms the findings of Iqbal & Johnson (1977) that human serum albumin would bind to Cibacron Blue–Sepharose, whereas serum albumins from several other sources would not. The fact that we found some binding of the other albumins is probably due to the Cibacron Blue–Sepharose in our experiments being of higher ligand concentration. We estimate from their results of capacities for human serum albumin that our Cibacron Blue–Sepharose had a 2–3-fold greater ligand concentration. Ligand concentration was found to be very important in the relative binding of the different albumins, with higher ligand concentrations greatly facilitating binding of the weaker-binding albumins. The overall trends found in our experiments were, however, the same irrespective of ligand concentration.

The presence of two bands moving more slowly than albumin on the gels from the SCN^- eluates (Fig. 1) deserves some comment. It has been shown (Travis & Pannell, 1973; Travis *et al.*, 1976) that, as well as human serum albumin, Cibacron Blue columns also bind lipoprotein, which is not removed by 0.2M-NaSCN, pH 8.0. In our experiment, 0.5M-NaSCN, pH 8.0, was used, which seems also to remove this lipoprotein. This result shows that other plasma proteins bind to Cibacron Blue–Sepharose and that elution with 0.2M-NaSCN, pH 8.0, makes the procedure selective for albumin.

The pH of adsorption is shown to affect the

Table 1. *Binding of albumins to Cibacron Blue and other related dyes*
The Table shows the relative binding of 10mg of albumin in a total volume of 0.45–1.0ml of Sepharose–ligand at pH 8.6 (0.025M-Tris/HCl).

Ligand	[Ligand] (μmol/ml of settled gel)	Source of albumin ...	Albumin bound (%) Human	Bovine	Sheep	Rabbit	Horse
Cibacron Blue	1.3		90	22	39	42	27
Procion Blue MX3G	1.2		33	8	13	18	7
Procion Blue MXR	1.6		37	18	10	1	2
Cibacron Brilliant Blue	1.5		48	20	24	16	10
Amino-coupled Cibacron Blue	1.4		18	5	11	0	0

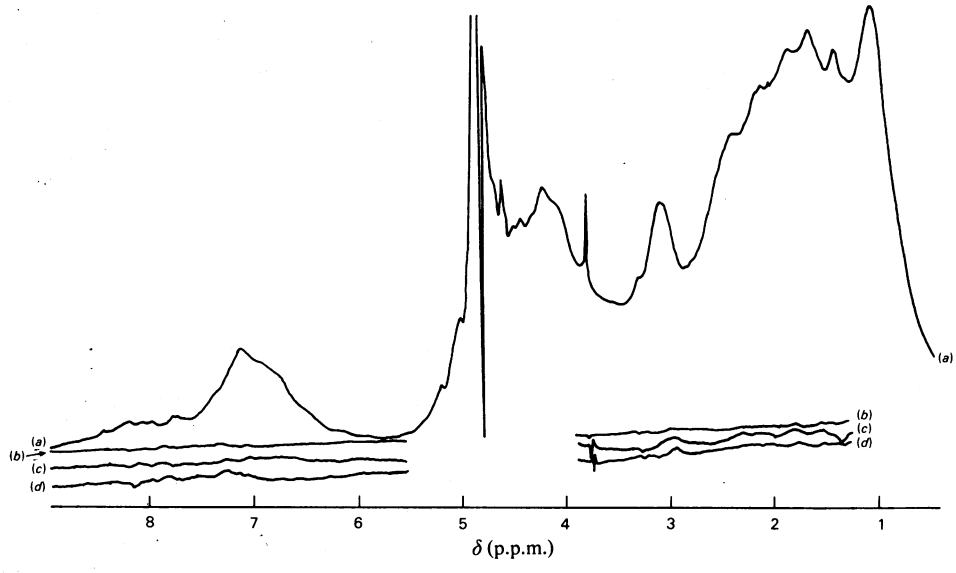

Fig. 6. *^1H n.m.r. spectrum of human serum albumin and difference spectra of human serum albumin/Cibacron Blue and human serum albumin/bilirubin minus human albumin*

Spectra were run in ^2H_2O at 25°C in the presence of 10mM-phosphate buffer, pH 8.0. Number of sweeps accumulated, 10000; concn. of human serum albumin, 1.0mM; volume, 0.5ml. (*a*) Human serum albumin, no additions; difference spectra of: (*b*) human serum albumin/Cibacron Blue (1.0mM) minus human serum albumin; (*c*) human serum albumin/bilirubin (2mM) minus human serum albumin; (*d*) human serum albumin/Cibacron Blue (2.0mM) minus human serum albumin. [In (*b*)–(*d*) the gain was in (*a*).]

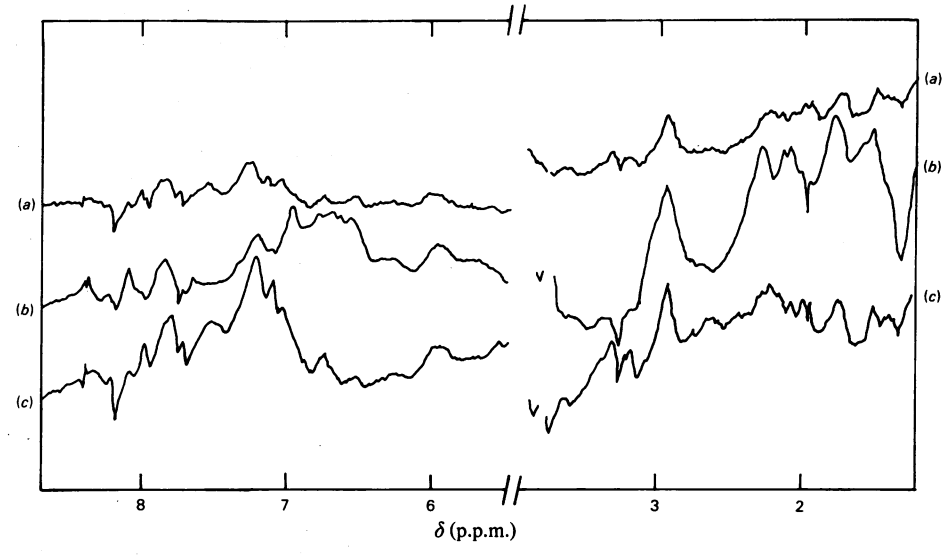

Fig. 7. *^1H n.m.r. difference spectra of human serum albumin/Cibacron Blue and human serum albumin/bilirubin minus human serum albumin*

Conditions were as in Fig. 6. Difference spectra of : (*a*) human serum albumin/Cibacron Blue (1.0mM) minus human serum albumin; (*b*) human serum albumin/Cibacron Blue (2mM) minus human serum albumin; (*c*) human serum albumin/Cibacron Blue (2.0mM) minus human serum albumin.

relative binding of the different albumins. It has been shown previously that in order to achieve selective binding of albumin from plasma, the pH should be above 8.0 (Angal & Dean, 1978). Above this pH the difference in affinity of immobilized Cibacron Blue for the different albumins is greatest. This difference in the binding of albumins at pH values of greater than 8.0 could be important in explaining the difficulties in separating albumins other than human serum albumin from plasma by this method.

The experiments involving binding of albumin/palmitate or albumin/bilirubin to Cibacron Blue–Sepharose give an insight into the mechanism of interaction of these albumins with Cibacron Blue–Sepharose. Furthermore, they provide a basis for explaining the different behaviour of human serum albumin from the other serum albumins.

The results indicate that the interaction of human serum albumin with Cibacron Blue–Sepharose is qualitatively and quantitatively different from the interactions of the other albumins with Cibacron Blue–Sepharose. The binding of the latter albumins falls drastically as the amount of palmitate bound is increased. This suggests that palmitate is perhaps occupying hydrophobic anion-binding sites that are able to bind Cibacron Blue. In this context, hydrophobic interactions between Cibacron Blue and proteins has been noted previously (Travis & Pannell, 1973; Seelig & Colman, 1977; Maeyer-Guignard *et al.*, 1977; Jankowski *et al.*, 1976). The binding of human serum albumin is largely unaffected by palmitate; however, it does decrease to some extent when the palmitate/albumin ratio is greater than 3:1. This behaviour is similar to bilirubin binding in the presence of fatty acid (Wooley & Hunter, 1970).

The binding of human serum albumin to Cibacron Blue–Sepharose falls with increasing concentration of added bilirubin, whereas binding of the other albumins is unaffected. This suggests that human serum albumin binds Cibacron Blue at, or close to, bilirubin-binding sites.

It is noteworthy that there is also an overall structural similarity between Cibacron Blue and bilirubin. Both molecules consist of planar aromatic ring systems, and the positions of negatively charged groups can be superposed in analogous positions (see Fig. 8). Although we hesitate to draw any conclusions from this, we would point out the similarity, as it offers a possible explanation for the binding of Cibacron Blue by human serum albumin.

The above results would indicate that the bilirubin-binding sites of human serum albumin are different from any such sites on the other albumins. Differences between the bilirubin-binding sites of human serum albumin and bovine serum albumin have been demonstrated previously (Wooley & Hunter, 1970).

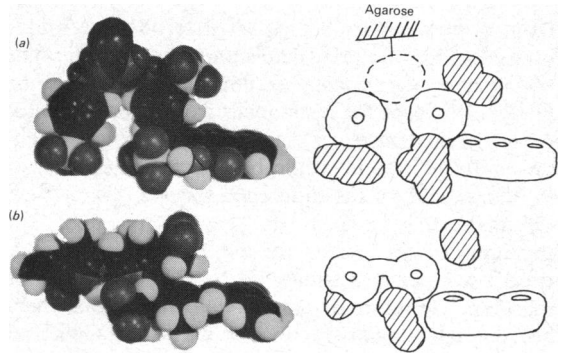

Fig. 8. *Comparison of Cori–Pauling–Koltun models of Cibacron Blue (a) and bilirubin (b)*

The models are arranged to be in analogous positions. The line diagrams (right) show the corresponding flat aromatic (⬭) and negatively charged (▨) regions. Note that the arrangement of the triazine ring (⌬) allows it to act as a spacer arm.

None of the other triazine dyes bound human serum albumin to the same extent as did Cibacron Blue. This indicates that the structural requirements for the affinity ligand are reasonably stringent, as the whole of the Cibacron Blue structure seems to be required. Comparison of these results with those obtained from the binding of such triazine dyes to nucleotide-dependent proteins (Beissner & Rudolph, 1978) suggests that these structural requirements are more rigid for human serum albumin than for those proteins that bind nucleotides, at least in so far as the differences demonstrated by the ligands used in the present study. Procion Blue MX3G and MXR both showed lower capacities for albumins than the other dyes. This could be due to the MX3G and MXR dyes being less negatively charged than Cibacron Brilliant Blue or Cibacron Blue and so bind less well to albumins, which are known to bind anions.

The amino-coupled Cibacron Blue showed very low binding to all albumins. The decreased binding of human serum albumin by this ligand suggests that, when Cibacron Blue binds, the anthraquinone ring system is buried in the protein and so attachment of a spacer arm to this portion restricts binding. Alternatively the amino group itself could be important in binding. This decreased binding of human serum albumin to amino-coupled Cibacron Blue has also been noted with CNBr-activated Sepharose (Travis *et al.*, 1976; Pannell *et al.*, 1974).

In order to assess whether Cibacron Blue and bilirubin bind to the same site(s) of human serum albumin, ligand-binding studies were performed by using n.m.r. If these ligands bind to the same site(s), then it would be expected that the difference spectra

from human serum albumin/Cibacron Blue minus human serum albumin, and human serum albumin/bilirubin minus human serum albumin would be identical (except for resonances from Cibacron Blue or bilirubin themselves), as the same proton resonances in the protein would be affected in each case. A comparison of the difference spectra reveals that, although they appear to be complicated, the positions of the peaks are the same in each case, except for peaks attributable to Cibacron Blue or bilirubin. Therefore in general we consider that there are enough similarities in the way that Cibacron Blue and bilirubin affect the human serum albumin proton resonances to conclude that they bind to the same or similar sites.

The large peak in the difference spectra at $\delta = 2.9$ p.p.m. is likely to be associated with alterations in the chemical shift of lysine ε-CH_2 proton resonances. Thus it is reasonable to suppose that these positively charged residues bind to the negatively charged Cibacron Blue or bilirubin molecules. This could offer an explanation for the decrease in binding of human serum albumin to Cibacron Blue–Sepharose at around pH 9 (Fig. 2), as at this pH the lysine residues could be becoming deprotonated.

In conclusion, there appear to be two types of interaction of Cibacron Blue with albumins. The first is demonstrated by all albumins and is with the hydrophobic fatty acid-anion-binding sites. The second, stronger interaction, which is demonstrated only by human serum albumin of those proteins presently studied, is to the bilirubin-binding site(s). Since this latter interaction is the stronger, and since it is not dependent on the fatty acid content of the albumin (which would vary depending on the diet of the animal in question), human serum albumin was found to be easier to separate from plasma than any of the other albumins when Cibacron Blue columns were used.

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