The Effect of Matrix on the Binding of Albumin to Immobilized Cibacron Blue

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With Cibacron Blue as a ligand, the suitability of Sepharose, Sephacryl, cellulose and Ultrogel as column supports for the affinity chromatography of human serum albumin was examined. Sepharose and Sephacryl were the most effective matrices. Albumin was readily removed from such columns by using an electrophoretic desorption technique.

Numerous workers have utilized the ability of the sulphonated aromatic dye Cibacron Blue (Fig. 1) to purify proteins by 'affinity chromatography' (Bohme *et al.*, 1972; Ryan & Vestling, 1974; De Maeyer-Guinard & De Maeyer, 1976; Lamkin & King, 1976). The interaction of human serum albumin with Cibacron Blue has been used to isolate the former from plasma (Travis & Pannell, 1973). The process is selective, since albumin from rabbit, chicken or bovine sera did not bind to the same adsorbent (M. J. Iqbal & M. W. Johnson, personal communication; S. Angal, unpublished work).

In the present paper we have examined the effect of different support materials on the interaction of human serum albumin with immobilized Cibacron Blue. We have also investigated the possibility of avoiding thiocyanate desorption with a view to improving the clinical acceptability of the purified albumin.

Materials

Sepharose (a galactose–anhydrogalactose copolymer, normally referred to as agarose) was obtained from Pharmacia (G.B.) Ltd., London W.5,

U.K. Sephacryl (an acrylamide-cross-linked agarose) was a gift from Dr. R. Bywater, Pharmacia, Uppsala, Sweden. Ultrogels AcA54 and AcA44 (co-polymerized polyacrylamide–agarose mixed gels) were obtained from LKB Instruments, Croydon, Surrey, U.K., and cellulose was from Whatman Biochemicals, Maidstone, Kent, U.K. Cibacron Blue 3G-A [as the free triazine; see Bohme *et al.* (1972)] was given by CIBA-GEIGY, Manchester, U.K. Plasma was generously given by Professor A. Kloppe, University of Aberdeen Medical School. All other chemicals were of the highest purity available from BDH Chemicals, Poole, Dorset, U.K., and were used without further purification.

Methods

Cibacron Blue derivatives of cellulose, Sephacryl, Ultrogels AcA54 and AcA44, Sepharose 6B, Sepharose 4B and Sepharose 2B were prepared by the method of Heyns & DeMoor (1974). Cibacron Blue derivatives of the epichlorohydrin-cross-linked Sepharose CL 6B and Sepharose CL 2B were prepared by the method of Travis *et al.* (1976). The same method was also used in the preparation of a

Fig. 1. Structure of Cibacron Blue 3G-A

Cibacron Blue derivative of Sepharose 4B with prior cross-linking (Travis *et al.*, 1976).

Frontal analysis chromatography was performed at 4°C by using 1 ml of settled gel in columns with a diameter of 6 mm. Adsorption was effected with 0.5 g of human serum albumin/ml of buffer (0.05 M-Tris/HCl/0.05 M-NaCl, pH 8.0) and followed by measurement of the A_{280} . When the absorbance of the eluted fractions reached that of the applied solution, the columns were washed with the same buffer until the A_{280} declined to zero.

Desorption was effected with 0.2 M-sodium thiocyanate/0.05 M-Tris/HCl buffer, pH 8.0. Protein content of the eluates was determined by the method of Lowry *et al.* (1951), with human serum albumin as standard.

Samples of all blue gels except cellulose and Sephacryl were hydrolysed by boiling for 10 min in 50% (v/v) acetic acid. The A_{610} of the resulting solutions was measured with appropriate controls, to give an estimate of ligand concentration. Cellulose and Sephacryl samples were suspended in 10% (v/v) glycerol and the ligand concentration was determined directly at 610 nm against the appropriate blank in a Pye-Unicam SP.1800 spectrophotometer.

Human serum albumin was isolated from plasma on a 60 ml column of Cibacron Blue-Sepharose CL 6B (Travis *et al.*, 1976). Its purity was checked by electrophoresis on polyacrylamide gels (Ornstein, 1964).

Results and Discussion

Affinity column supports were compared by frontal analysis (Lowe *et al.*, 1973; Nicol *et al.*, 1974). This method supplies information about the

apparent association constants and maximum capacities and differs from pulsatile methods in that, during the process, equilibrium is established between the immobilized ligand and the protein. When human serum albumin was applied to the various blue-gel columns by frontal application, widely separated saturation curves were obtained (Fig. 2). From the half-saturation point for each curve and knowing the ligand concentration, one can calculate the apparent association constant (Nicol *et al.*, 1974). This constant is presented in Table 1, together with ligand concentrations, molar and maximum capacities.

A 16-fold range in ligand concentration was observed on immobilizing the dye to the various matrices, despite the similarity of the reaction conditions used. The values obtained for the cross-linked Sepharoses are appreciably lower than those obtained for the untreated agaroses, but the same general trend is observed, i.e. the higher percentage of agarose leads to greater ligand concentrations. The high value obtained for cellulose is notable. However, human serum albumin was very poorly adsorbed by the cellulose adsorbent and also the Ultrogel-based columns.

The molar effectiveness of the agarose adsorbents ranged between 0.064 and 0.157 mol of human serum albumin/mol of dye and was significantly higher than the result for cellulose and Ultrogels. The latter result is noteworthy, especially for cellulose, where the mode of dye immobilization should be similar to that observed for agarose. This could be related to the observations by Travis *et al.* (1976), who reported that the capacity of Blue Dextran agarose (where the triazine group of Cibacron Blue is modified with soluble dextran) was lower than that

Fig. 2. Frontal analysis chromatography of the Cibacron Blue derivatives of various support materials. Columns (diam. 6 mm, volume 1.0 ml) were equilibrated with 0.05 M-Tris/HCl/0.05 M-NaCl buffer, pH 8. Adsorption was effected with 0.5 mg of human serum albumin/ml of the same buffer until the absorbance of the fractions reached that of the applied solution. After removal of excess protein by washing, elution was effected with 0.2 M-NaSCN/0.05 M-Tris/HCl, pH 8. All fractions were 1.2 ml. (a) Δ , AcA54; $\blacksquare$, AcA44; $\bullet$, cellulose; $\triangle$, Sepharose CL 2B; $\square$, Sepharose CL 4B; $\circ$, Sephacryl. (b) $\circ$, Sepharose 2B; $\triangle$, Sepharose 4B; $\square$, Sepharose 6B.

Table 1. *Comparison of the properties of different support media*

See the ligand to Fig. 2 for experimental details. The value for human serum albumin adsorbed is calculated by difference and after elution with thiocyanate.

Support material	Ligand concn. ($\mu\text{g/ml}$ of gel)	Human serum albumin adsorbed (mg/ml of gel)	Human serum albumin eluted by desorption (mg/ml of gel)	Working capacity (mol of human serum albumin eluted/mol of ligand)	$10^{-3} \times$ Apparent association constant (M^{-1})
Cellulose	3.2	0.8	0.2	<0.01	1.1
Sephacryl	1.9	10.7	8.4	0.06	25.0
AcA54	0.3	0.2	0.2	0.01	3.1
AcA44	0.4	0.3	0.2	0.01	4.8
Sepharose 6B	2.3	32.4	20.7	0.14	45.3
Sepharose 4B	1.5	15.7	14.1	0.14	42.0
Sepharose 2B	0.9	9.1	8.4	0.14	35.0
Sepharose CL 6B	0.7	5.4	4.2	0.09	30.0
Sepharose CL 4B	0.4	5.0	4.6	0.16	46.8
Sepharose CL 2B	0.2	1.7	1.7	0.10	24.6

of blue agarose. Stellwagen *et al.* (1975) and Wilson (1976) have suggested that, although the dinucleotide fold in proteins specifically recognizes Blue Dextran, other structures besides this binding site also recognize blue agarose. Because of the similarity of dextran and cellulose our data could be explained by a similar increase in specificity for the blue cellulose.

It is harder to see why the Ultrogels do not behave in the same way as agarose. We have unsuccessfully tried to attach Cibacron Blue to polyacrylamide gels by the same method, indicating that the dye binds solely to the agarose moiety of Ultrogel. Cross-linking by epichlorohydrin, as in CL Sepharose, or by polyacrylamide, as in Sephacryl, does not have the dramatic effect on binding of human serum albumin observed with the Ultrogel matrices, where the polyacrylamide is thought to be interdigitated with the agarose.

These binding data are reinforced by the apparent association constants, which were similar for all substituted Sepharoses and Sephacryl (24×10^3 – $47 \times 10^3 \text{ M}^{-1}$) but at least a factor of 10 lower for cellulose and the Ultrogels. This probably explains the observed lower working capacities of these adsorbents.

Elution of human serum albumin bound to the columns was normally carried out with 0.2M-thiocyanate. However, considerable tailing occurred even at concentrations of 0.5M. The alternative, displacement by free Cibacron Blue, leaves undiffusible dye bound to the protein. We therefore investigated the possibility of electrophoretic desorption of the protein. The technique of electrophoretic desorption has been applied to a wide variety of affinity-chromatographic systems (Dean *et al.*, 1977). Separation of albumin from the gel was achieved by agarose slab electrophoresis within 2 h at 25 V/cm.

In conclusion, the choice of matrix for affinity chromatography remains an empirical one. The effects of changing the matrix can be unexpected and dramatic. Frontal analysis provides a versatile and rapid method for the comparison of different affinity supports.

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