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Chromatography of plasma proteins on immobilized Cibacron Blue F3-GA

Mechanism of the molecular interaction

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Fractionation of plasma proteins on immobilized Cibacron Blue F3-GA (Affi-gel Blue) under different conditions of pH, ionic strength and temperature was studied. At acidic pH the unbound proteins were eluted in order of increasing pI (the Affi-gel Blue behaving as ion-exchanger); at basic pH and at low ionic strength they were eluted in order of decreasing molecular weight (separation by diffusion–exclusion). For the proteins that were either retarded in comparison with substances of similar molecular characteristics, or that were bound to the resin, pseudo-ligand affinity or hydrophobic interactions were also implicated.

Pseudo-ligand affinity chromatography on immobilized Cibacron Blue F3-GA (Haff & Easterday, 1978) has been extensively applied to the purification of enzymes. In their review on the subject, Dean & Watson (1979) listed 80 enzymes whose interaction with the dye had been described in 118 papers. Little interest was shown in the application of this technique to the fractionation of plasma proteins: Dean & Watson (1979) reviewed only twelve such papers, which dealt with a total of 19 proteins.

For the purification of plasma components, Cibacron Blue was first recommended for simple group separation; conditions were used that ensured complete binding of albumin while the unbound protein was collected in a single fraction (Travis & Pannell, 1973; Travis *et al.*, 1976). Owing to the high concentration of albumin, this step resulted in a 2-fold purification; more important, subsequent separation procedures were simplified, since albumin has molecular characteristics similar to many other plasma components. Because of its practical and theoretical importance, albumin binding to Cibacron Blue has been studied in detail (Angal & Dean, 1977; Young & Webb, 1978; Leatherbarrow & Dean, 1980). As for other plasma components, Angal & Dean (1978) described the elution of 11 proteins from Cibacron Blue–Sepharose by a pH gradient, and Vician & Tishkoff (1976) and Gee *et al.* (1979) the interaction between components of the classical pathway of complement activation and immobilized Cibacron Blue. For preparative purposes, the technique has been used for the purification of α_2-macroglobulin (Virca *et al.*, 1978) and of α_1-antichymotrypsin (Travis *et al.*, 1978).

Our group has recently described the fractionation of plasma proteins on Affi-gel Blue under close-to-physiological conditions (Gianazza & Arnaud, 1982). The present paper describes the influence of the pH of the equilibration buffer, of the elution procedure, of the ionic strength and of the temperature.

Abbreviations used: α_1AT, α_1-antitrypsin; α_1S, α_1-acid glycoprotein; α_1X, α_1-antichymotrypsin; α_2M, α_2-macroglobulin; C3, third component of complement; C4, fourth component of complement.

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Experimental

Materials

Affi-gel Blue (cross-linked agarose gel with covalently coupled Cibacron Blue F3-GA; degree of substitution 4.5 μmol/ml) was kindly provided by Bio-Rad Laboratories (Richmond, CA, U.S.A.). In order to make possible a direct comparison of the results, plasma was obtained from a single healthy volunteer with his informed consent. The sample was collected on citrate/soya-bean trypsin inhibitor as described by Harpel (1973). Poly- and mono-specific antisera against plasma proteins were purchased from Dako (distributed by Accurate Chemical and Scientific Company, Hicksville, NY, U.S.A.).

Methods

A 100ml portion of Affi-gel Blue was packed in a column 2.5 cm in diameter and 20 cm in height. The

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gel was equilibrated with a 50m-equiv./litre buffer, pH5.0, 7.0 or 9.0; in one case, the 'low-ionic-strength' experiment, a 1:1 dilution of the pH7 buffer was used. A 15ml portion of plasma was dialysed overnight at 4°C against 800ml of the starting buffer; 12ml were applied to the column. The experiments were run at 25°C, except for the 'low-temperature' experiment, which was carried out at 4°C. The unbound fraction was washed from the column with the starting buffer; the bound proteins were eluted by increasing the ionic strength (salt gradient), the pH (pH gradient) or the hydrophobicity of the solvent (glycerol gradient) at constant ionic strength. Details of the elution protocols are listed in Table 1. The flow rate was 0.5ml/min; fractions (2.5ml) were collected. The eluate was analysed by fused rocket immunoelectrophoresis against poly- and mono-specific antisera as described by Svendsen (1973). At the end of each cycle the column was 'stripped' of albumin and tightly bound proteins with 0.5M-NaCNS.

Results and discussion

In Affi-gel Blue a sulphonated dye of the triazine class, Cibacron Blue F3-GA, is covalently coupled to cross-linked agarose gel. Owing to its properties, such a matrix is expected to interact with proteins according to one or another of the following mechanisms: (i) ion-exchange; (ii) hydrophobic interaction; (iii) exclusion–diffusion; and (iv) affinity binding. To probe the relative importance of these forces, we studied the effect of different experimental conditions (listed in Table 1) on the chromatography of human plasma. The elution pattern of eleven major proteins was monitored by fused rocket immunoelectrophoresis; the results are depicted in Figs. 1(*a*)–1(*g*).

If ionic interactions with the sulphonic groups of the dye were taking place, one would expect: (1) the amount of protein bound to the resin to decrease as the pH of the medium increases, and, in consequence, proteins are titrated to their anionic form; (2) bound molecules to be released when increasing the ionic strength of the medium; (3) the elution order to be dictated by the acid/basic behaviour of the proteins. All of these expectations come true. When comparing experiments run with buffers of the same ionic strength and different pH (Figs. 1*a*, 1*c* and 1*g*), the amount of protein washed with the starting buffer (unbound fraction) increases with the pH of the medium. The unbound protein is negligible at pH4 (result not shown); at pH5, it is 1/25 that at pH7 and 1/75 that at pH9 (in agreement with Angal & Dean, 1978). As for the molecules that do bind to the resin, they are eluted at higher ionic strength: e.g. caeruloplasmin and transferrin are bound with 25m-equiv./litre (Expt. *e*, Table 1 and Fig. 1.), and eluted with 50m-equiv./litre (Expt. *c*) at pH7. Moreover, the salt concentration at which a given protein is released lowers as the pH increases. For instance, C3 and C4 are not removed at pH5 by a NaCl concentration as high as 2M (Expt. *a*), whereas at pH7 they are eluted between 0.55 and 1M (Expt. *c*) and at pH9 between 0.45 and 0.95M (Expt. *g*). We have already shown that after extensive washing with 1M-NaCl at pH7, only albumin and some lipoproteins are still bound to the column and require a chaotropic agent (such as 0.5M-NaCNS) to be desorbed (Gianazza & Arnaud, 1982), which means that high ionic strength is effective on *all* proteins. The relationship between elution order and pI (Putnam, 1975; Righetti *et al.*, 1981) under the various experimental conditions tested is plotted in Figs. 2(*a*)–2(*g*). A good correlation is found between the two parameters when proteins are bound at pH5 and the elution is carried out either by increasing the ionic strength (Expt. *a*; $r = 0.73$) or raising the pH

Table 1. *Seven different protocols for the chromatography of plasma proteins on immobilized Cibacron Blue F3-GA*
pH was measured at 25°C. For all buffers (except for Expt. *e*) $I = 0.05$ (Long, 1961). Salt and glycerol gradients were in starting buffers.

	Expt.	Starting buffer	Volume (ml)	Eluent	Volume (ml)
(*a*)	pH5, salt gradient	Sodium acetate $pH_{25°C}$ 5.0	130	0–2M-NaCl	400
(*b*)	pH5, pH gradient	Sodium acetate, pH5.0	130	Sodium acetate, $pH_{25°C}$ 5.5	60
				+ Sodium phosphate, pH6.0	60
				+ Sodium phosphate, pH6.5	60
				+ Sodium phosphate, pH7.0	60
				+ 0–1M-NaCl	250
(*c*)	pH7	Sodium phosphate, pH7.0	250	0–1M-NaCl	250
(*d*)	pH7, low temperature	Sodium phosphate, pH7.0	260	0–1M-NaCl	250
(*e*)	pH7, low ionic strength	Dilution of (*c*) (1:1)	290	0–1M-NaCl	250
(*f*)	pH7, glycerol gradient	Sodium phosphate, pH7.0	290	0–50% (v/v) Glycerol	250
(*g*)	pH9	Tris/HCl, pH9.0	290	0–1M-NaCl	250

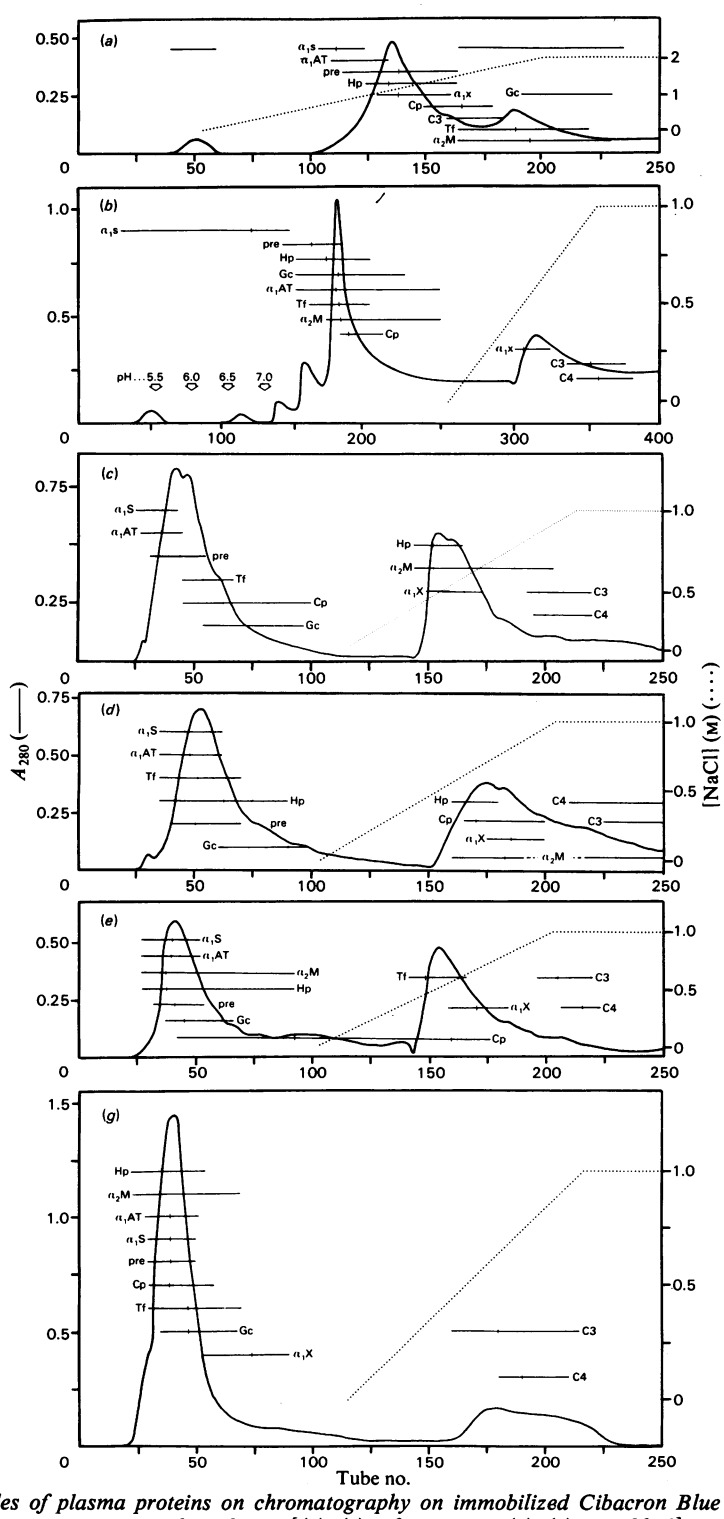

Fig. 1. *Elution profiles of plasma proteins on chromatography on immobilized Cibacron Blue F3-GA under different experimental conditions* [*(a)–(g) refer to expts. (a)–(g) in Table 1*]

Bars mark the range and peak of elution for eleven individual proteins. Additional abbreviations used: pre, prealbumin; Hp, haptoglobin; Cp, caeruloplasmin; Gc, group-specific component; Tf, transferrin. Data on Expt. (*f*) is not shown.

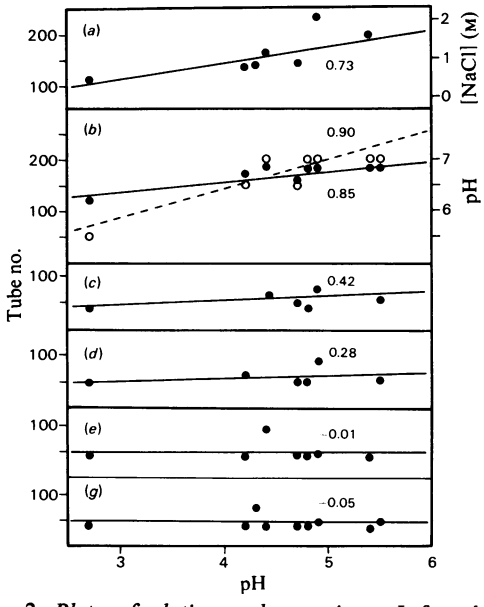

Fig. 2. *Plots of elution order against pI for eleven proteins after chromatography of plasma on immobilized Cibacron Blue F3-GA under different conditions* [(*a*)–(*g*) *refer to Expts.* (*a*)–(*g*) *in Table 1*]

In (*b*), open symbols and broken line refer to the relationship between the pH of elution and the pI of the proteins. The values tabulated on the right side of each section are the correlation coefficients, *r*, of the linear regression for every set of data.

(Expt. *b*; $r = 0.85$; in this case the peak concentration of a given protein is found about 2 pH units above its pI). As expected, the opposite is the finding for Expt. (*g*), at pH 9, where proteins bear a high density of negative charges and most of them are eluted by the starting buffer; in this case *r* equals −0.05. At pH 7 (Expt. *c*), *r* is 0.42 for the unbound fraction.

All these data suggest that ionic forces drive the mechanism of binding. Hydrophobic interactions, however, could also be expected, owing to the presence of polycyclic aromatic rings in the dye (Jankowski *et al.*, 1976; Begley *et al.*, 1981). One of the features of hydrophobic forces is that they are enhanced by high ionic strength (Hjertén, 1973), and in the example of Expts. (*e*) and (*c*), haptoglobin and α_2M become bound to the resin as the concentration of the buffer is doubled. Moreover, hydrophobic interactions diminish with a decrease in temperature: haptoglobin, again, is bound at 25°C (Expt. *c*), whereas at 4°C it is not (Expt. *d*). Even the elution by a pH gradient, discussed above, is not incompatible with such a mechanism: increasing the surface charge, in fact, would interfere with the removal of water molecules demanded for hydrophobic adhesion. To test this hypothesis further, elution was performed at pH 7 with a gradient of glycerol, a hydrophobic solvent (Expt. *f*). Only a small amount of a mixture of proteins, however, was released by washing with the highest concentration (50%) (results not shown). In comparison with 1 M-NaCl, the effectiveness of glycerol as eluant is about 5%. It thus seems that hydrophobic interactions play an accessory role to the dominant one played by ionic forces. It may be mentioned in this context that haptoglobin, for which the data above seem to prove hydrophobic binding, is known to bind its natural ligand, haemoglobin, with both hydrophobic (mostly directed towards α-chains) and ionic forces (towards β-chains) (Putnam, 1975).

As for a mechanism of exclusion–diffusion, it would be expected to discriminate only between very large molecules. The fractionation range of a gel-filtration medium of the same agarose content as Affi-gel Blue (5%) is evaluated to span between 25 and 10000 kDa. In fact, only at pH 9 (Expt. *g*) is it observed that the unbound proteins are eluted in order of decreasing molecular weight (α_2M and haptoglobin polymers, then caeruloplasmin, then α_1AT, α_1S and prealbumin): even in this case the resolution is minimal (large sample volume, poor geometry of the column).

Cibacron Blue shows high affinity for a number of enzymes that require NAD, NADP or ATP (Dean & Watson, 1979; Haff & Easterday, 1978). Since the dye is an artificial compound with no biological relationship to the proteins it binds, it has been proposed to term its affinity as that of a 'pseudo-ligand' (Haff & Easterday, 1978). If such an interaction was to operate for any of the plasma proteins under investigation, this should be spotted as a deviation from the behaviour of molecules of similar molecular characteristics. This is the case for group-specific component (vitamin D-binding protein) that is consistently eluted after proteins of similar pI and/or molecular weight [see, for instance, the scatter of its data in Figs. 2(*a*) and 2(*c*); in Expt. *g*, its elution volume is 110% of the column V_t]. The hypothesis of specific affinity has been demonstrated: Cibacron Blue is in fact a competitive ligand at the binding site for vitamin D on group-specific component (C. Chapuis-Cellier, E. Gianazza & P. Arnaud, unpublished work).

A few proteins have been found to be eluted from the column in more than one peak. This is especially evident for α_1S at pH 5 (Figs. 1*a* and 1*b*) and for haptoglobin at low temperature (Fig. 1*d*). As for α_1S, differences in the sugar chains are likely to be implicated. For haptoglobin the hypothesis to be tested is a differential binding of polymers as against monomers, as demonstrated for albumin (Leatherbarrow & Dean, 1980). We have mentioned above that Affi-gel Blue is able to discriminate between the

holo- and the apo-form of group-specific component (C. Chapuis-Cellier, E. Gianazza & P. Arnaud, unpublished work) and the same phenomenon is suspected for other carrier proteins. Albumin itself is bound to a lower extent when saturated by its ligand, bilirubin (Leatherbarrow & Dean, 1980).

Fractionation on immobilized Cibacron Blue may be used for preparative purposes. The results reported in Figs. 1(*a*)–1(*g*) can help in the choice of optimal conditions for the purification of a given protein. As an example, α_2-macroglobulin could be easily separated by gel filtration from all plasma components, provided the few other high-molecular-weight proteins are removed. This can be achieved by chromatography on Affi-gel Blue at pH 5, followed by elution with salt gradient (Expt. *a*). Haptoglobin (and immunoglobulin M, results not shown) is eluted between 0.7 and 1.2 M-NaCl, where α_2M is released between 1.2 and 2 M-NaCl [details on the purification of α_2M from this material can be found in Arnaud & Gianazza (1982)].

The pattern of fractionation on Affi-gel Blue varies according to the experimental conditions, and it is tempting to suggest the sequential use of the same column with different protocols for the separation of individual proteins. As just one example, caeruloplasmin is the only major protein to shift from the bound to the unbound fraction when the temperature is raised from 4 to 25°C at pH 7. The advantages of the sequential procedure instead of a combination of different chromatographic procedures are the high yield and the absence of denaturation (Arnaud & Gianazza, 1982) offered by pseudo-affinity media.

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