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A general method for fractionation of plasma proteins

Dye–ligand affinity chromatography on immobilized Cibacron Blue F3-GA

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The chromatographic behaviour of 27 different plasma proteins on fractionation of human plasma on immobilized Cibacron Blue F3-GA was studied. The column was eluted by using a three-step procedure. First, a low-molarity buffer (30mM-H_3PO_4/Na_3PO_4, pH 7.0, *I* 0.053) was used, then a linear salt gradient (0–1.0M-NaCl in the buffer above) was applied, followed by a wash with two bed volumes of 1.0M-NaCl. Finally, bound proteins were 'stripped' with 0.5M-NaSCN. Up to 1 ml of whole plasma could be loaded per 5 ml bed volume. No denaturation of proteinase inhibitors or complement fractions was observed. The recovery of individual proteins ranged between 52 and >95%. Enrichment of four individual plasma components (α_1-antitrypsin, caeruloplasmin, antithrombin III and haemopexin) was between 10-fold and 75-fold These results indicate that chromatography on immobilized Cibacron Blue F3-GA can be a useful initial step in the purification of plasma proteins.

The specific interaction between phosphofructokinase and Cibacron Blue F3-GA (Blue A) was first reported by Kopperschlager *et al.* (1968). Further studies showed that this property was shared by a number of other enzymes, all of them with a specific secondary conformation, the 'dinucleotide fold' (Thompson *et al.*, 1975). As a result, chromatography on immobilized Blue A has been used extensively in the past 5 years for enzyme purification (Haff & Easterday, 1978; Lowe *et al.*, 1981).

Interaction of plasma proteins with immobilized Blue A was first studied by Travis & Pannell (1973), who showed that plasma albumin would bind to the dye, thus making albumin-depleted plasma easily available. Further studies by the same investigators indicated that chromatography on agarose-linked Blue A was a useful step in the isolation of albumin (Travis *et al.*, 1976) and in the purification of α_2M (Virca *et al.*, 1978) and α_1X (Travis *et al.*, 1978). Angal & Dean (1978) also reported the effect of pH on the adsorption of plasma by immobilized Blue A.

We have systematically studied the fractionation of plasma components on immobilized Blue A by monitoring 27 individual plasma proteins, in order to assess the general characteristics of this separation procedure and its use as a preparative method.

Abbreviations used: Ig, immunoglobulin; α_1S, α_1-acid glycoprotein (orosomucoid); α_1AT, α_1-antitrypsin; α_2HS, α_2-HS glycoprotein; α_2M, α_2-macroglobulin; α_1X, α_1-antichymotrypsin; $\alpha_1\beta$, $\alpha_1\beta$-glycoprotein; VLD lipoprotein, very-low-density lipoprotein; LD lipoprotein, low-density lipoprotein; HD lipoprotein, high-density lipoprotein; SDS, sodium dodecyl sulphate.

Material and methods

Plasma

Blood samples (150ml) from healthy volunteers were collected (with their informed consent) on citrate/soya-bean trypsin inhibitor as described by Harpel (1973), centrifuged at 500 *g* for 15 min at 4°C, and dialysed overnight at 4°C against 4 litres of H_3PO_4/Na_3PO_4 buffer (0.03M, pH 7.0, *I* 0.053).

Chromatography on immobilized Blue A

Cross-linked agarose gel (bead diameter 150–300 μm) with covalently coupled reactive Blue A dye (colour index 61211) was kindly provided by Bio-Rad Laboratories (Affi-gel Blue). The degree of substitution was 4.5 μmol of dye/ml of gel; its binding capacity for albumin was evaluated at 11.3 mg/ml of settled gel.

The gel (400ml) was packed in a column of dimensions 2.5 cm (diameter) × 100 cm (height). The column was first equilibrated with 5 bed volumes of 0.03M-H_3PO_4/Na_3PO_4 buffer, pH 7.0. The chromatographic experiments were run at room temperature, and the A_{280} of the column effluent was

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monitored. The flow rate was 1.2 ml/min; fractions (4.8 ml) were collected at 4°C. After loading the sample, the column was washed with 800 ml of the buffer. Then a linear salt gradient (from 0.0 to 1.0 M-NaCl in the buffer, total volume 1000 ml) was applied with a Pharmacia Gm 10 gradient mixer. After an additional wash with 900 ml of 1.0 M-NaCl in the buffer, the tightly bound proteins were removed with 0.5 M-NaSCN and the column was re-equilibrated with 3 bed volumes of the starting buffer.

Protein analysis

SDS/polyacrylamide-gel electrophoresis was performed with the discontinuous buffer system of Laemmli (1970) in a horizontal set-up using the LKB Multiphor apparatus (Görg *et al.*, 1980). The stacking gel was T4 C2.5 (Hjérten, 1962), and the separation gel was T7.5 C4 (T = % acrylamide; C = % bisacrylamide). The thickness of the slabs was 0.5 mm. Two-dimensional electrophoresis was performed as described previously (Görg *et al.*, 1980; Emerson *et al.*, 1980). Immunoelectrophoresis was performed as described by Grabar & Williams (1953) and fused rocket immunoelectrophoresis by the method of Svendsen (1973), with antisera from Dako (Accurate Chemical and Scientific Co., Hicksville, NY, U.S.A.) and Behring Diagnostics (Somerville, NJ, U.S.A.). Antisera against lipoproteins were kindly provided by Dr. Maria Lopes-Virella. Total protein concentration was measured by the method of Bradford (1976), with bovine serum albumin as a standard. The concentration of individual plasma proteins was measured by electroimmunoassay (Laurell, 1966) and compared with a standard obtained from Calbiochem–Behring (La Jolla, CA, U.S.A.).

Results

Fig. 1 shows the elution profile of plasma proteins fractionated on Affi-gel Blue. The locations of 27 different proteins were determined by analysis of the eluted fractions by fused rocket immunoelectrophoresis using poly- and mono-specific antisera (Figs. 2 and 3, Table 1).

Washing of the column with 0.03 M-phosphate buffer led to a complex elution profile of at least three overlapping peaks (I–III), followed by a progressive return toward the baseline. α_1AT and α_1S were the major components of peak I. Peak II contained mostly prealbumin, together with α_2-HS. Transferrin was the major component of peak III, overlapping with group-specific component and caeruloplasmin; these three proteins were almost completely separated from those in peak I (Fig. 2). IgG and IgA were present in all the fractions, but their concentrations were maximal in the first tubes. No albumin was eluted in the first and second peaks, but a small amount was detected in the trailing part of the elution profile. Samples from each tube were pooled (tubes 40–160 and 161–245) and analysed by two-dimensional electrophoresis (Figs. 4*a* and 4*b*).

When a salt gradient was subsequently applied to the column, a second series of peaks (IV–VIII) were obtained. Elution of the bound proteins began at a concentration of NaCl as low as 0.01 M. First to be removed were a group of high-molecular-weight proteins including haptoglobin (phenotype 2-2 in the donors used), which was the major component of peak IV, α_2M, IgM, IgG and IgA. Next, three proteins of mol.wt. ~65000, namely α_1X (peak V), antithrombin III (peak VI), and thyroxin-binding globulin, were detected. Almost completely separated from the high-molecular-weight proteins in peak VII were the C3 and C4 fractions of complement, thyroxin-binding globulin and $\alpha_1\beta$. The elution of fibrinogen began at fraction 350, continuing for about 100 tubes (peak VIII) and finally overlapping with the elution of properdin factor B, retinol-binding protein and haemopexin. The last protein identified in peak IX was the C5 fraction of complement. Albumin leaked out at a low constant concentration during the first part of the

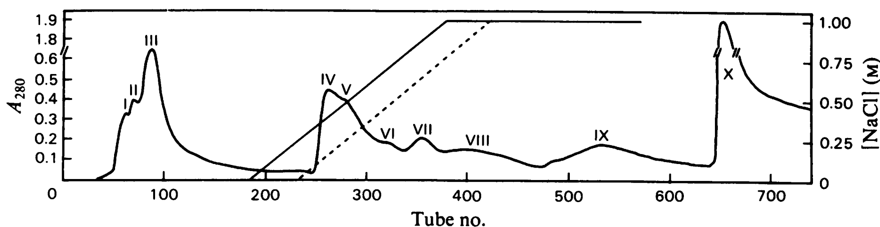

Fig. 1. *Elution profile of plasma proteins from immobilized Cibacron Blue F3-GA*
Plasma (80 ml) was run on a 400 ml column at room temperature. The equilibration buffer was 30 mM-H_3PO_4/Na_3PO_4, pH 7.0. Fractions (4.8 ml) were collected. At tube 185, a linear NaCl gradient (0–1.0 M, total volume of 1.0 litre) was applied. The broken line indicates the development of the gradient, taking into account the void volume of the column. At tube 380, the elution was continued with 1.0 M-NaCl. Starting at tube 575, the column was washed with 0.5 M-NaSCN.

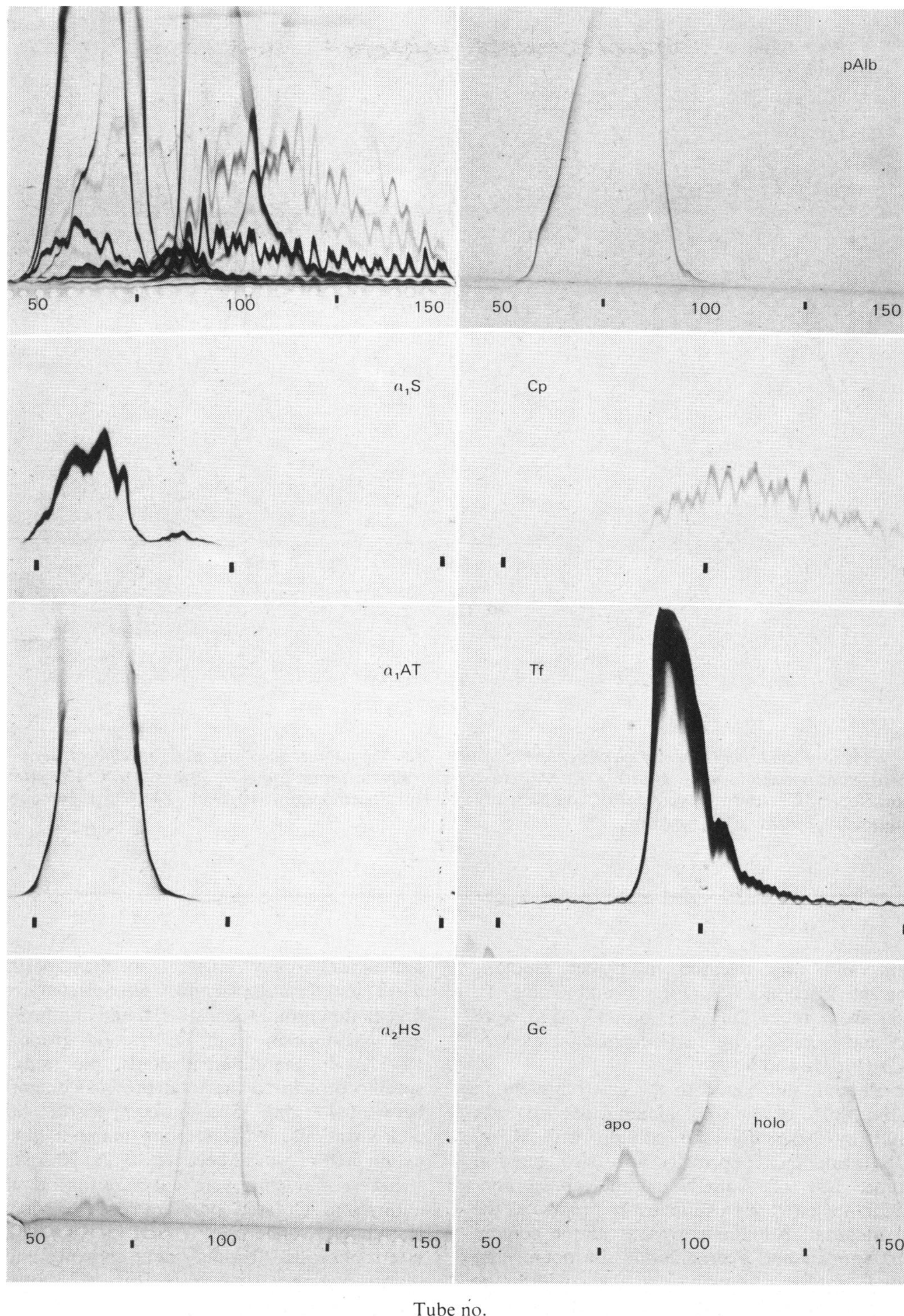

Fig. 2. *Fused rocket immunoelectrophoresis of tubes 43–153 developed against poly- and mono-specific antisera*
Gels were 1% agarose (1.5 mm thick) in 7.88 mM-sodium barbital/1.40 mM-barbital/93.67 mM-glycine/46.6 mM-Tris, pH 8.6. Antisera concentrations: serum proteins, 50 μl/ml; α_1AT, α_1S and Tf (transferrin), 20 μl/ml; Gc (group-specific component), 10 μl/ml; pAlb (prealbumin), 8 μl/ml; Cp (caeruloplasmin) and α_2HS, 6.67 μl/ml. Portions (2.5 μl) were applied to the wells. The plates were run overnight at 2 V/cm, pressed, washed twice, dried, and stained with 0.5% Coomassie Blue R250 in ethanol/water/acetic acid (9:9:2, by vol.). The anode is at the top.

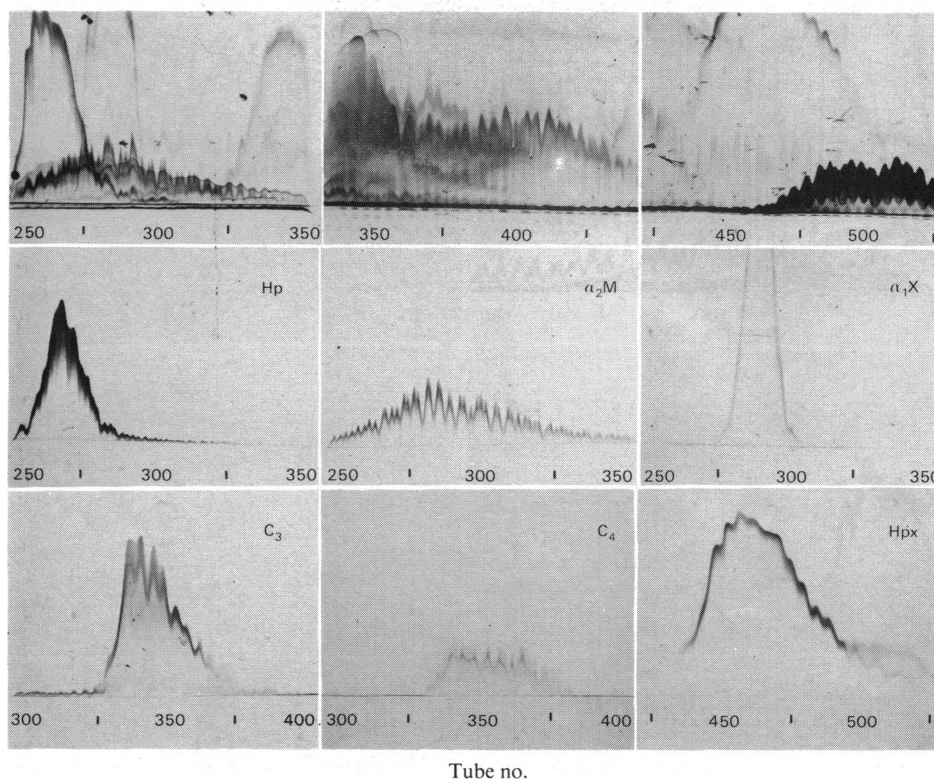

Fig. 3. *Fused rocket immunoelectrophoresis of tubes 248–556 against poly- and mono-specific antisera*
Experimental conditions were as in Fig. 2. Antisera concentrations: serum proteins, 50μl/ml; α_1X, 13.3μl/ml; Hp (haptoglobin), C3 (third component of complement) and Hpx (haemopexin), 10μl/ml; C4 (fourth component of complement), 8μl/ml; α_2M, 6.6μl/ml.

gradient and was released in higher amounts starting at fraction 375 (Fig. 3 and Table 1). Samples from tubes 246–375 and 376–575 were pooled and analysed by two-dimensional electrophoresis (Fig. 4*c* and 4*d*).

The albumin still bound to the gel (representing more than 80% of the total albumin applied) was removed by 'stripping' the column with 0.5M-NaSCN (peak X). Lipoproteins were also eluted at this time. Use of NaSCN at the above concentration appeared to be sufficient to remove all the bound material. Additional washes of the column with 6.0M-guanidine hydrochloride did not release additional proteins, although some blue colour in the eluate indicated that this treatment could be detrimental to the bound dye.

The recoveries measured for several proteins are shown in Table 2; they ranged between 52 and >95%. The purification factor (Table 3) was calculated for four proteins chosen because of the difficulties usually inherent in their purification. α_1AT and caeruloplasmin were selected from the first elution group (peaks I–III) and antithrombin III and haemopexin from the second group (peaks IV–IX). In the different pools, the ratio of the specific protein to the total protein content varied between 17 and 35%. In comparison with their concentrations in the starting material, the purification factors ranged between 10 and 72-fold.

Several proteins were checked for possible denaturation. Crossed pore-gradient-electrophoresis–immunoelectrophoresis, SDS/polyacrylamide-gel electrophoresis (results not shown) and two-dimensional electrophoresis (Fig. 4) showed that fragmentation of caeruloplasmin, a common problem with other purification techniques, did not occur during this chromatographic process. α_1AT was studied for its trypsin-inhibitory capacity (Dietz *et al.*, 1974), α_2M for its binding capacity (Ganrot, 1966), and complement components C3, C4 and C5

Table 1. *Identification of 27 plasma proteins fractionated on immobilized Cibacron Blue F3-GA*
This is a summary of the results from fused rocket immunoelectrophoresis of the fractions obtained (see the legend to Fig. 2). When a protein was eluted in two peaks, the percentage of each (shown in parentheses) was calculated by integration of the areas.

Protein	Tube no. Range	Tube no. Peak
α_1AT	50–83	66
α_1S	48–78 (95%)	67
	78–96 (5%)	89
Prealbumin	60–99	80
Transferrin	83–115	95
α_2HS	62–78 (4%)	69
	88–110 (96%)	99
Transferrin	83–115	95
Caeruloplasmin	83–160	110
Group-specific component	65–95 (4%)	85
	95–155 (96%)	125
Haptoglobin	248–285	262
α_2M	248–360	280
α_1X	272–300	285
Antithrombin III	285–305 (15%)	296
	305–338 (85%)	325
Thyroxin-binding globulin	315–353	334
Third component of complement	330–385	342
Fourth component of complement	335–385	348
$\alpha_1\beta$	330–390	365
Fibrinogen	345–365	395
Properdin	390–460	430
Haemopexin	435–510	456
Retinol-binding protein	425–490	460
Fifth component of complement	495–540	515
Albumin		655
IgA		62
IgG		
Cathodal		60, 305
Anodal		70, 280
IgM		290
VLD, LD and HD lipoproteins	650–700	

Table 2. *Recoveries of seven individual plasma proteins after chromatography on immobilized Cibacron Blue F3-GA*
Samples (100 μl) from the tubes in which individual proteins were detected by fused rocket immunoelectrophoresis were pooled. The amount of each protein was measured by electroimmunoassay (Laurell, 1966) and compared with the amount of protein in the starting material, after correction for dilution factors.

Protein	Recovery (%)
α_1AT	>95
α_1S	>95
Transferrin	>95
α_2M	52
Haptoglobin	62
α_1X	89
Third component of complement	58

Table 3. *Purification factors for four individual plasma proteins fractionated by chromatography on immobilized Cibacron Blue F3-GA*
Samples (100 μl) of the fractions in which a given protein was detected by fused rocket immunoelectrophoresis were pooled. The concentration of the protein was measured by electroimmunoassay (Laurell, 1966), and the total protein concentration was determined by the technique of Bradford (1976), with bovine serum albumin as standard. The ratio between individual protein and total protein content was compared with the same ratio in the starting material.

Protein	Concn. in plasma (%)	Concn. in the pool (%)	Purification factor
α_1AT	3.72	37.38	10.0
Caeruloplasmin	0.41	29.52	72.0
Antithrombin III	0.54	17.00	31.5
Haemopexin	1.41	36.9	26.2

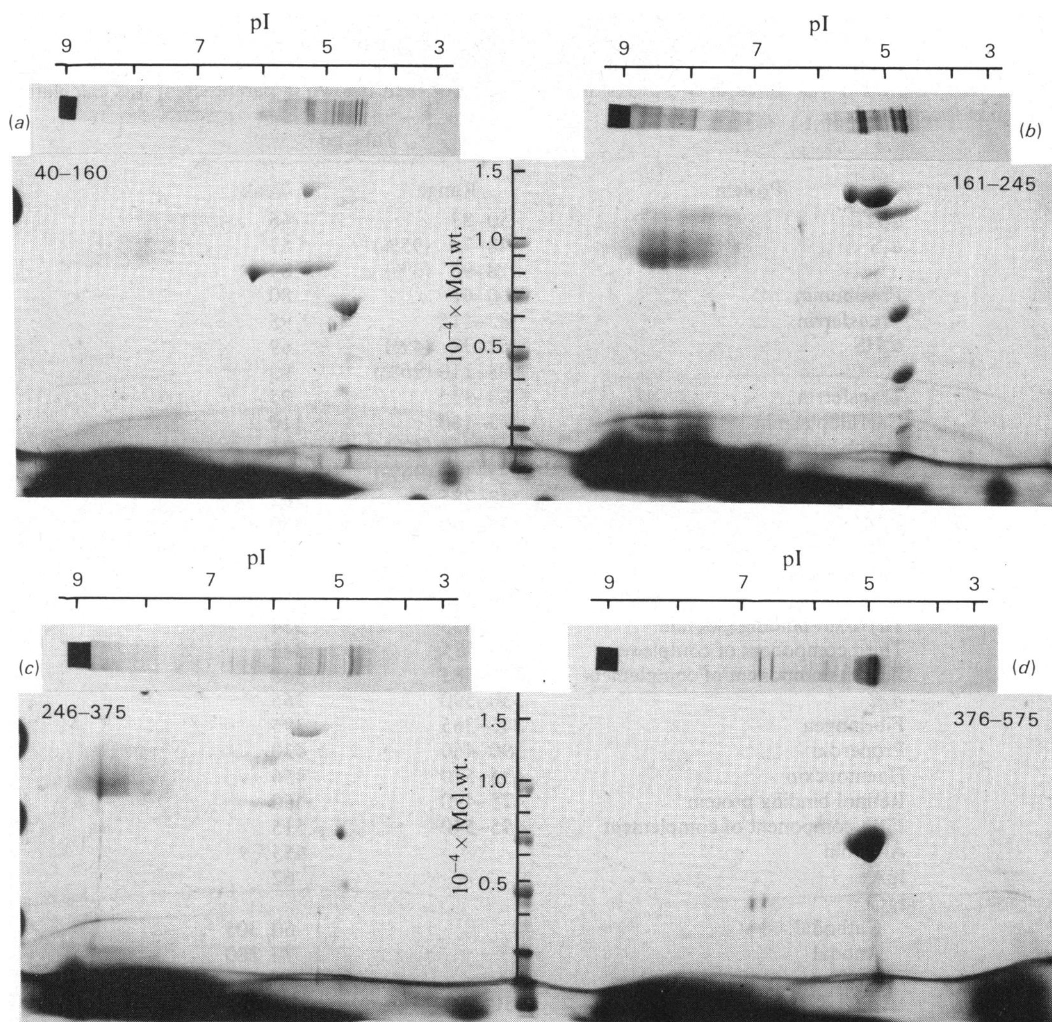

Fig. 4. *Two-dimensional mapping of fractions eluted from the column*

Portions (100 μl) were pooled from tubes 40–160, 246–375 and 376–575, and concentrated to 1–2 ml. Tubes 161–245 were pooled and concentrated to 5 ml. Isoelectric focusing was performed in 0.5 mm thick polyacrylamide gels (T4 C2.5) containing 2% LKB Ampholines (pH range 2.5–10) and 2 mM-Glu-Asp-Lys-Arg. A constant power of 5 W was applied for 2 h, with maximum voltage at 1600 V. The gel was then equilibrated for 5 min at room temperature with 0.0675 M-Tris/HCl, pH 6.8, containing 2% SDS and 5% 2-mercaptoethanol, and the strips from the first dimension were overlaid on the stacking gel of the SDS/polyacrylamide-gel plates. The separating gel was T7.5 C4 in 0.375 M-Tris/HCl (pH 8.8)/0.1% SDS; the stacking gel was T4 C2.5 in 0.125 M-Tris/HCl (pH 6.8)/0.1% SDS. The electrode buffer was 0.025 M-Tris/0.192 M-glycine (pH 8.3)/0.1% SDS. The gel thickness was 0.75 mm and the tracking dye was Bromophenol Blue. The gels were run at 200 V until the dye entered the separating gel, then at 600 V until the dye reached the electrode wicks. The gels were fixed in water/methanol/propanol (5:2:3, by vol.) solution containing 7% (v/v) sulphosalicylic acid and 2% (w/v) trichloroacetic acid. Staining was with 0.4% Coomassie Blue. Molecular-weight standards were: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase and soya-bean trypsin inhibitor.

by comparison of their concentrations and haemolytic activities (Williams & Chase, 1977). In each case the loss of biological activity was less than 5%.

Discussion

Chromatography of plasma proteins on immobilized Blue A results in substantial purification of several components. For example, in our studies, a pool of the α_1AT-positive fractions (tubes 50–78) contained 160 mg of α_1AT in a total of 425 mg of protein (10-fold enrichment from plasma with >95% yield). With other methods, similar enrichment can be obtained only after two or three steps, such as

precipitation with $(NH_4)_2SO_4$ and ion-exchange chromatography (Crawford, 1973; Morii *et al.*, 1978). Also, a pool of caeruloplasmin fractions (tubes 85–130) contained 28mg of this protein in a total protein content of 93mg. Moyer *et al.* (1980) more recently reported a similar purification only after a three-step procedure including precipitation with poly(ethylene glycol) followed by batch treatment with DEAE- and CM-Sephadex.

Other advantages of this technique include the volume of the sample (1ml/5ml of packed gel) and its protein concentration (60mg/ml), which are much higher than the usual loads in gel-filtration systems and of the same order of magnitude as those used for ion-exchange chromatography on agarose matrices. The high yield and the absence of denaturation (at least for the proteins studied) are noteworthy. In addition, the mechanical properties of the gel allow for high flow rates, and regeneration is easily accomplished. No apparent modification in the binding capacity of the gel appears to occur with time, and the same column has been used routinely in our laboratory for 1 year.

The exact mechanism by which proteins are fractionated by chromatography on immobilized Blue A is still unclear. This dye binds most, if not all, the enzymes with the so-called 'dinucleotide fold', and Thompson *et al.* (1975) have shown that the blue chromophore can assume a conformation that mimics the orientation and the anionic groups characteristic of NAD. More recent studies based on binding of dihydrofolate reductases led Subramanian & Kaufman (1980) to propose that Blue A binds to any protein possessing either a cluster of apolar residues that interact with the aromatic rings of the dye molecule, or of positively charged groups, which bind the sulphonate residues. As reported by others (Angal & Dean, 1977), and in our experience (C. Chapuis-Cellier & P. Arnaud, unpublished work), albumin is the plasma protein with the highest affinity for the dye. Studies by Leatherbarrow & Dean (1980) have shown that the binding of ligands such as fatty acids and bilirubin interferes with the ability of this protein to bind to the dye. A gross similarity between bilirubin and Blue A has been shown. However, it is difficult to extend these findings to the elution profile we obtained.

We checked for a possible relationship between elution order and molecular parameters of seven major plasma proteins (α_1AT, α_1S, prealbumin, transferrin, α_2HS, group-specific component and caeruloplasmin) eluted by the low-molarity buffer. A weak correlation was found between elution order (i.e., the numbers of the fractions corresponding to the peak concentrations) and isoelectric point ($r = +0.40$) and electrophoretic mobility ($r = -0.56$). These data suggest some importance of the ionic interaction between charged dye and proteins. In contrast, the finding of a positive correlation between the logarithm of the molecular weight and elution order ($r = +0.40$) rules out a mechanism of exclusion–diffusion for the fractionation of unbound proteins. Although protein hydrophobicity is difficult to assess on the basis of raw analytical data, we tried to correlate elution order of the above proteins with percentage contents of hydrophobic amino acids (Phe + Ile + Leu + Met + Val + Tyr + Trp); no relationship was detected ($r = -0.05$). Only a loose correlation was found with a related parameter, percentage sugar content ($r = -0.53$) on the basis of standard analytical data (Putnam, 1975; Dayhoff, 1976; Bouillon *et al.*, 1976; Manolis & Cox, 1980).

Several proteins that were released at higher salt concentrations did possess some binding sites. These included α_2M, α_1X and antithrombin III, all of which bind proteinases; in contrast, α_1AT was eluted with the low-molarity buffer. Thyroxin-binding globulin and retinol-binding protein, which bind low-molecular-weight hydrophobic ligands, also bound Blue A, whereas vitamin D-binding protein (group-specific component) and prealbumin were eluted in the first part of the experiment. However, the apoprotein form of group-specific component was eluted after the holoprotein (C. Chapuis-Cellier, E. Gianazza & P. Arnaud, unpublished work). In contrast, metalloproteins such as transferrin and caeruloplasmin did not appear to interact strongly with the gel. Finally, haem-binding proteins such as haptoglobin and haemopexin were retained on this column. In addition, although some immunoglobulins (G and A) were eluted in the first peak, some remained bound and were eluted with the salt gradient.

It is our opinion that differential affinity of plasma proteins for Blue A dye involves complex interactions, probably of ionic and hydrophobic origin. Comparison of the structure of the binding site(s) of plasma proteins with that of Blue A will probably aid in the elucidation of these interactions. Since the dye is an artificial compound with no apparent biological relationship to the bound proteins, the designation 'pseudoligand' proposed by Haff & Easterday (1978) appears justified for Blue A.

It is noteworthy that the extent of substitution of the agarose matrix by the dye appears to have a major effect on protein binding. For example, with concentrations of dye in the range of 2μmol/ml of gel (Travis *et al.*, 1976), α_2M and haptoglobin were the first proteins to be eluted with the low-ionic-strength buffer. In contrast, with a highly substituted gel (4.5μmol/ml) as used in the present study, α_2M and haptoglobin were released only by the salt gradient. All the other proteins were eluted in the same order by both media, but their separation was improved in Affi-gel Blue. This was especially evident for α_1AT and α_1S, which were separated

from transferrin and caeruloplasmin on the high-substitution gel but co-eluted with them from the low-substitution gel. Thus it seems possible that the use of different conditions of ionic strength and/or pH may lead to further resolution of the protein peaks. In addition, as pseudoligand affinity chromatography appears to fractionate proteins by a mechanism different from that of exclusion–diffusion, ion-exchange, or isoelectric focusing, the latter techniques can be used for further purification of the proteins separated on immobilized Blue A. Indeed, preparative isoelectric focusing in a sucrose gradient (pH4–6) for the α_1AT fractions obtained here produced >95% pure protein with a final yield of 70%, and similar purification of the caeruloplasmin fraction was obtained with a second step of ion-exchange chromatography (E. Gianazza & P. Arnaud, unpublished work).

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