Isolation of Albumin from Whole Human Plasma and Fractionation of Albumin-Depleted Plasma

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The dye Cibacron Blue F-3-GA was conjugated to Sepharose to provide an affinity column for serum albumin. Passage of whole human plasma through a column of Cibacron Blue–Sepharose results in the removal of approx. 98% of the albumin. The latter can be quantitatively recovered by desorption with NaSCN. Albumin-depleted plasma can be readily resolved into discrete fractions by a combination of conventional biochemical techniques. In particular, the resolution of plasma proteins with properties similar to those of native human plasma albumin can readily be accomplished by ion-exchange chromatography of the Sepharose–dye-treated plasma on DEAE-cellulose.

The isolation of proteins from blood plasma or serum is often complicated by the presence of albumin as a contaminant. Because this protein generally makes up more than half of the total plasma or serum proteins, conventional purification procedures are not reliable for the production of albumin-free fractions. For example, the popular Cohn fractionation scheme, commonly used for the isolation of plasma proteins (Cohn *et al.*, 1946), yields six heterogeneous fractions, each of which contains considerable quantities of albumin (Schultze & Heremans, 1966). Other techniques for plasma fractionation, such as precipitation with $(NH_4)_2SO_4$, organic compounds (Schultze & Heremans, 1966) or ion-exchange chromatography on DEAE-cellulose (Peterson & Sober, 1960), have also been disappointing. Although some products can be obtained free of albumin, most of the fractions isolated are severely contaminated with this protein.

Of particular importance in plasma fractionation is the fact that many proteins of current biological interest have physical properties similar to those of albumin, especially with regard to molecular weight and electrophoretic mobility. These proteins, which include α_1-proteinase inhibitor, α_1-acid glycoprotein, α_1-anti-chymotrypsin and Gc-globulins, as well as numerous others present in trace quantity, are poorly separated from albumin unless techniques such as preparative electrophoresis are used. Unfortunately, this also leads to poor yields and the possible isolation of denatured proteins.

Previously we described a procedure for the separation of albumin from other plasma proteins by adsorption on columns of Sepharose–Blue Dextran

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(Travis & Pannell, 1973; Pannell *et al.*, 1974). This technique had three major disadvantages: the efficiency of binding of albumin was low (7–8 mg of albumin bound/ml of Sepharose–Blue Dextran), the elution of the bound albumin required denaturing conditions, and Blue Dextran slowly leached from the columns. In the present paper we recommend a modification of the affinity gel, which gives a much greater capacity for albumin (40 mg of albumin bound/ml of gel) and results in a more stable Sepharose–ligand conjugate. We also present information on the fractionation of albumin-depleted plasma, including its comparison with whole plasma. Finally, we describe the rapid isolation of a homogeneous preparation of human albumin by desorption from Sepharose–ligand columns by using non-denaturing conditions.

Experimental

Materials

DEAE-cellulose (DE-52) was from Whatman Chemicals, Maidstone, Kent, U.K. Cibacron Blue F-3-GA was a gift from Ciba Geigy Corp., Ardsley, NY, U.S.A. Commercial human albumin preparations were from Sigma Chemical Co., St. Louis, MO, U.S.A. Antisera to specific plasma proteins were from Behring Diagnostics, Somerville, NJ, U.S.A. Outdated blood-bank plasma was given by St. Mary's Hospital, Athens, GA, U.S.A. All glassware used for the initial separation of albumin from the remainder of the plasma proteins was silicone-treated (Siliclad; Clay Adams, New York, NY, U.S.A.) to prevent activation of the clotting system by glass.

Methods

Preparation of Cibacron Blue–Sepharose. The coupling of Cibacron Blue F-3-GA to Sepharose followed the procedure of Böhme *et al.* (1972). Because the coupling of the dye to Sepharose requires heating to 80°C, a cross-linked gel, stable to high temperatures, was utilized (Porath *et al.*, 1971).

In a typical preparation 1 litre of washed Sepharose 4B (other porosities can be substituted) was mixed at room temperature (23°C) with 1 litre of 1 M-NaOH containing 5 g of $NaBH_4$. To this gently stirred mixture was added 20 ml of epichlorohydrin (1-chloro-2,3-epoxypropane) and the mixture heated to 60°C for 1 h. The cross-linked gel was then washed on a coarse-glass Buchner filter with hot water until the washings were neutral.

The Sepharose–dye conjugate referred to as Cibacron Blue–Sepharose was prepared by first suspending 500 ml of cross-linked gel in an equal volume of water and warming the mixture to 60°C. The dye (500 mg in 50 ml of water) was added dropwise with vigorous stirring and, after 15 min, 50 g of NaCl was added. The mixture was heated to 80°C and 10 g of Na_2CO_3 added. After 30 min the dyed gel was washed with hot water, followed by 0.05 M-Tris/HCl/0.05 M-NaCl, pH 8.0, until the washings were colour-free. Traces of blue dye were still eluted from freshly coupled gels during the first passage of plasma through columns of this material. Subsequent usage, however, indicated no further dye leakage.

Preparation of albumin-depleted plasma. In a typical experiment, a column (5 cm × 40 cm) of the Sepharose–dye conjugate prepared as described above was equilibrated with 0.05 M-Tris/HCl/0.05 M-NaCl, pH 8.0, and a sample of human plasma (50–200 ml) applied. As was reported elsewhere with Sepharose–Blue Dextran (Pannell *et al.*, 1974), some fractionation of plasma proteins did occur, not only because of molecular-weight differences, but also because of a tendency for weak ionic interactions of immunoglobulins with the dye conjugate. The latter may be decreased by using buffers containing 0.5 M-NaCl. However, in the experiments described here, all eluted protein was collected as a single fraction until the E_{280} was less than 0.1 and the eluate was then concentrated to the original plasma sample volume by ultrafiltration with an Amicon UM-2 membrane.

Isolation of albumin. The bound albumin was desorbed from the Cibacron Blue–Sepharose column by several methods. These included washing with 0.05 M-Tris/HCl, pH 8.0, containing one of the following: 0.5 M-$CaCl_2$, 0.5 M-$MgCl_2$, 1 mM-sodium decanoylate or 0.5 M-KCl. Various mixtures of these were also used. However, in all cases the removal of albumin was judged to be incomplete since further washing with 5 M-guanidine hydrochloride also eluted variable amounts of this protein. However, when 0.05 M-Tris/HCl/0.2 M-NaSCN, pH 8.0, was used

complete desorption of the albumin occurred. The column could then be re-equilibrated with the starting buffer and a new sample of plasma applied. This procedure was followed for as many as ten applications of 200 ml samples of plasma with complete reproducibility. Occasionally, the columns were stripped with 5 M-guanidine hydrochloride followed by 0.05 M-Tris/HCl/0.05 M-NaCl, pH 8.0, to remove traces of lipoproteins, which were precipitated at the top of the column bed. Such treatment had no deleterious effect on subsequent plasma application.

Fractionation of plasma and albumin-depleted plasma. In order to determine the effect of albumin removal on plasma fractionation, both whole plasma and albumin-depleted plasma were subjected to a variety of standard treatments. These included: (*a*) electrophoresis on polyacrylamide gels (Brewer & Ashworth, 1969); (*b*) immunoelectrophoresis against antisera to whole plasma; (*c*) Cohn fractionation (Cohn *et al.*, 1946); (*d*) ion-exchange chromatography on DEAE-cellulose with an NaCl gradient at pH 8.0; (*e*) salt fractionation with $(NH_4)_2SO_4$. In addition, fractions isolated by the last three techniques were tested by gel electrophoresis and by immunodiffusion for the presence or absence of particular plasma proteins.

Results

(*a*) *Separation of albumin from plasma*

When human plasma was passed through a Cibacron Blue–Sepharose column, the dark-blue colour of the column turned to a lighter blue when albumin was bound. Further, the yellow–orange colour of the original plasma was replaced by a light brown after the pooled eluate from the Cibacron Blue–Sepharose column was concentrated to the original volume of the material applied.

When the column was subsequently stripped with NaSCN, the albumin eluted had a bright yellow colour, which remained during dialysis against water. The final product after freeze-drying, however, was a fluffy white powder. From 200 ml of plasma, between 7 and 8 g of albumin could be obtained as a routine, representing essentially all of the bound albumin.

Examination of the various fractions by gel electrophoresis (Plate 1) indicated that nearly all of the original proteins present in normal plasma were retained in plasma after passage through a column of Cibacron Blue–Sepharose. Virtually all of the albumin (98%) and some lipoprotein appear to be the only components bound to Cibacron Blue–Sepharose. After recovery the albumin was found to migrate essentially as a major (97.6%) and minor component (albumin dimer, 2.4%) in electrophoresis (Plates 1–3), with no evidence of the presence of *α*- or *β*-globulins (M. Bertolini, personal communica-

tion). A commercial sample of crystalline human albumin (Sigma) is shown for comparison (Plates 1–3, Fig. 1). This latter material contained more dimer as well as other minor contaminants. Fraction V human albumin or fatty acid-free human albumin gave similar patterns.

Immunoelectrophoresis of the albumin-depleted plasma (Plate 2) confirmed the fact that nearly all of the albumin had been adsorbed to the Cibacron Blue–Sepharose column. Only a weak fuzzy pre-

cipitin reaction could be detected in the albumin region. The unadsorbed albumin had a faster mobility than did human albumin isolated from the Cibacron Blue–Sepharose column by NaSCN treatment. This may represent either an electrophoretic artifact or an actual difference in the molecule, perhaps in terms of bound components; repassage through the affinity column did not result in binding of this residual albumin. However, as shown below, it presented little interference in the fraction-
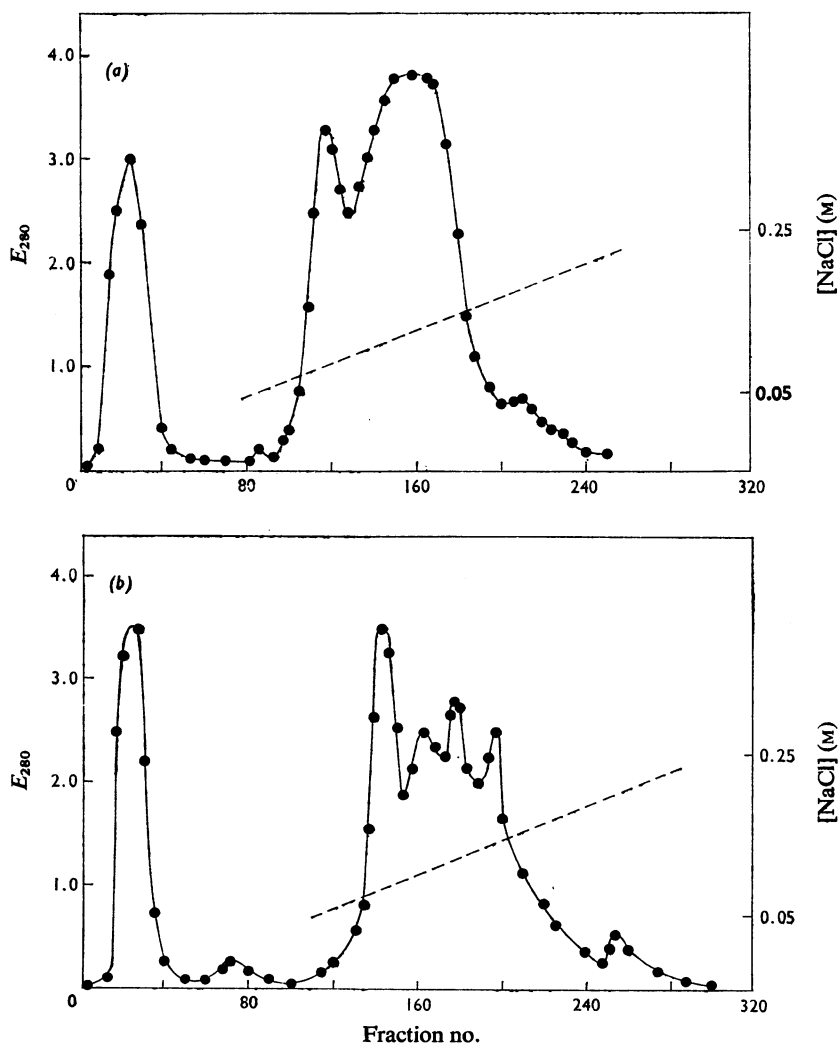

Fig. 1. *Ion-exchange chromatography of whole human plasma (a) and Cibacron Blue–Sepharose-treated plasma (b) on DEAE-cellulose (pH 8.0)*

The column (1.9cm×40cm) was equilibrated with 0.05M-Tris/HCl, pH8.0. After application of the sample (50ml) the column was washed with equilibration buffer and eluted with a linear gradient (----) of 0–0.25M-NaCl (1 litre total); 5ml fractions were taken. ●, E_{280}.

ation of other plasma proteins by virtue of its low concentration.

(b) Ethanol fractionation of normal and albumin-depleted plasma

Although the Cohn ethanol-fractionation procedure (Cohn et al., 1946) is probably deleterious for the isolation of many plasma proteins in a native state, it is still the most widely used process for their initial fractionation. As shown in Plate 3 the fractionation of normal and of albumin-depleted plasma gave striking differences. None of the precipitates obtained from albumin-depleted plasma contained albumin, whereas all of those from normal plasma had varying amounts of this protein. Only the supernatant from fraction V of albumin-depleted plasma contained albumin. Other differences occurred in the degree of precipitation of certain plasma protein components from the two starting materials. These differences are almost certainly due to the lower protein concentration in Cibacron Blue–Sepharose-treated plasma, because of the loss of most of the albumin.

Cohn fractionation of albumin-depleted plasma as a general purification is not recommended. However, it may be useful for the isolation of specific plasma proteins which are not denatured by ethanol treatment.

(c) DEAE-cellulose chromatography

The fractionation of both normal and albumin-depleted plasma by DEAE-cellulose was performed at pH 8.0 in order to give a general scheme of the type of separations obtainable. The samples were dialysed against 0.05 M-Tris/HCl, pH 8.0, and then applied to identical columns of DEAE-cellulose equilibrated against the same buffer. After adequate washing, a linear gradient of 0–0.25 M-NaCl, in the same buffer, was introduced.

The comparative results of the fractionation obtained by this technique are shown in Fig. 1 and Plate 4. In Fig. 1, the protein elution profiles (E_{280}) indicate the resolution obtainable when the albumin concentration is decreased (Fig. 1b). The electrophoretic separation of several individual fractions (Plate 4) clearly reinforces the suitability of removing albumin from plasma before fractionation. Not only are virtually all fractions free of albumin (Plate 5), but also the resolution of α_1-proteinase inhibitor, α_1-acid glycoprotein, and Gc-globulins are easily demonstrated. Further, the one fraction found to contain albumin in the chromatography of albumin-depleted plasma (Plate 5, fraction 210) is easily resolved from other plasma proteins by virtue of its low concentration in comparison with that in whole plasma.

(d) Fractionation of plasma proteins with properties similar to those of albumin

After resolution of Cibacron Blue–Sepharose-treated plasma into high- (>80000) and low-molecular-weight fractions on Sephadex G-75, the residual albumin is confined to the latter. (In contrast, a similar chromatographic separation of whole plasma results in albumin contamination of both fractions.) The low-molecular-weight fraction from Cibacron Blue–Sepharose-treated plasma, equivalent to 100 ml of whole plasma, was divided into two portions, which were dialysed separately against 0.05 M-Tris/HCl/0.05 M-NaCl, pH 8.8, or 0.03 M-sodium phosphate pH 6.5, and applied to columns of DEAE-cellulose equilibrated with the same buffers. In both cases the mixture was separated into an unadsorbed and an adsorbed fraction. The unadsorbed fraction contained only transferrin (Plate 6e). At pH 6.5 gradient chromatography resolved the remainder of the proteins into four fractions (Fig. 2a); the first fraction (I pH 6.5) contained Gc-globulins and a number of other unidentified α-globulins, as well as traces of albumin (Plate 6f). The second fraction (II pH 6.5) contained α_1-proteinase inhibitor, the remainder of the albumin and, again, other unidentified proteins (Plate 6g). The third fraction (III pH 6.5) was composed mainly of α_1-acid glycoprotein and α_1-anti-chymotrypsin (Plate 6h). The final peak (IV pH 6.5) was identified as pre-albumin (plate 6i). This fraction was resolved into two components; however, only electrophoretically homogeneous pre-albumin was detected.

At pH 8.8, a linear salt gradient also eluted the bound proteins into four fractions (Fig. 2b). The first fractions (I pH 8.8) contained α_1-anti-chymotrypsin, Gc-globulins, and traces of α_1-acid glycoprotein (Plate 6j). The second fraction (II pH 8.8) was composed of the rest of the α_1-acid glycoprotein, all of the α_1-proteinase inhibitor, and smaller amounts of Gc-globulins and other α-globulins (Plate 6k). The third fraction (III pH 8.8) contained the albumin that could not be adsorbed to Cibacron Blue–Sepharose (Plate 6l) and which, as previously stated, had an unusual immunoelectrophoretic pattern (Plate 2). Traces of other unidentified proteins were also present in this fraction. The final fraction (IV pH 8.8) contained only pre-albumin.

(e) $(NH_4)_2SO_4$ fractionation

Fractionation of both normal and Cibacron Blue–Sepharose-treated plasma with $(NH_4)_2SO_4$ by addition in 10% increments up to 90% saturation gave essentially identical results, with the sole exception that albumin was present in all of the precipitates from normal plasma, whereas it was only found in the precipitate obtained between 60 and 70% saturation of Cibacron Blue–Sepharose-treated plasma.
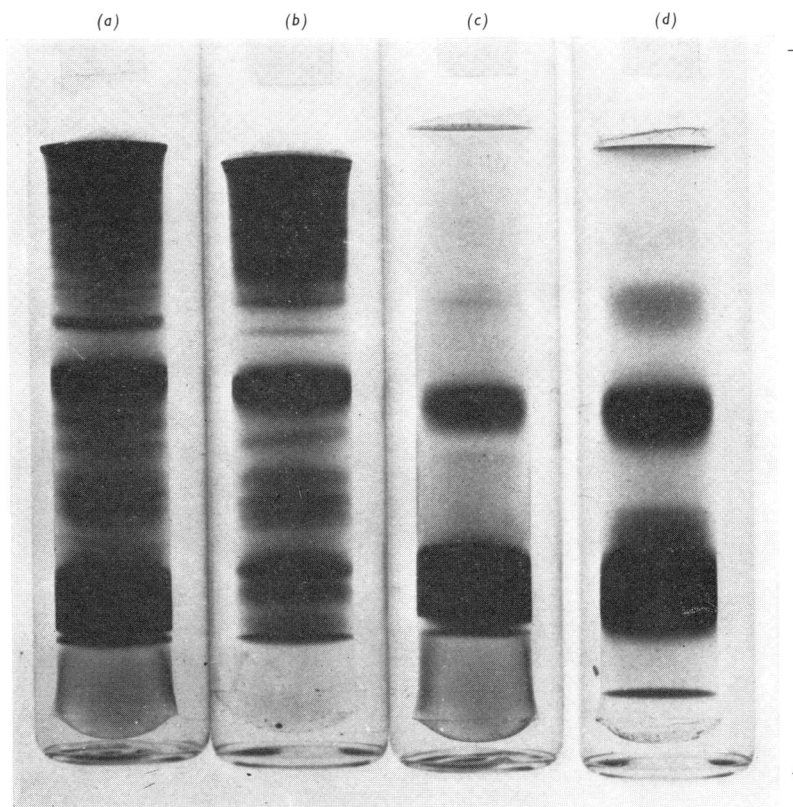
EXPLANATION OF PLATE I
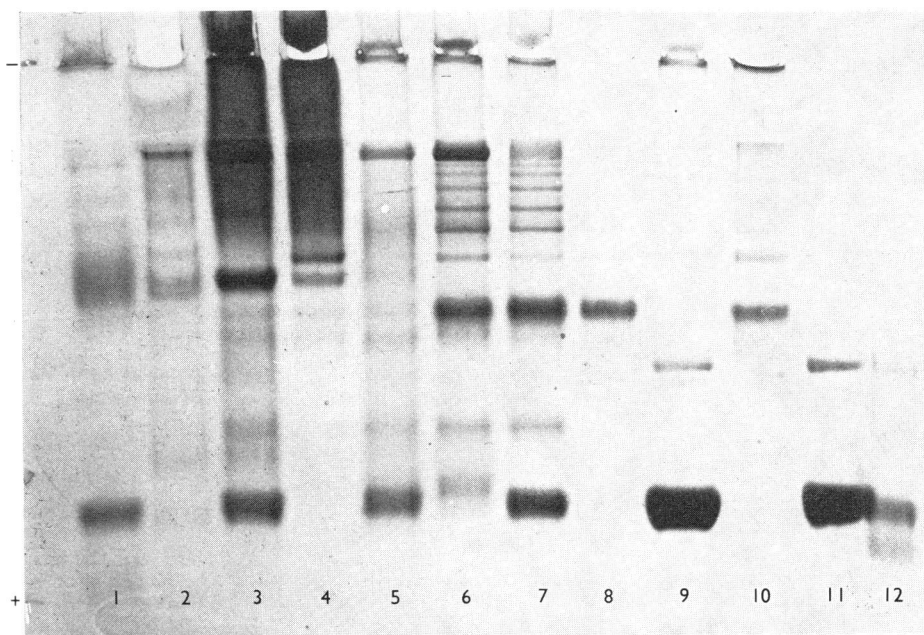
Disc electrophoresis of human plasma and fractions

The running pH was 8.9 in a 7.5% polyacrylamide gel. Gels were stained in Coomassie Brilliant Blue G. Direction of migration is from cathode (top) to anode (bottom). (*a*) Whole human plasma (10 μl); (*b*) Cibacron Blue–Sepharose-treated plasma (10 μl); (*c*) albumin eluted with NaSCN from Cibacron Blue–Sepharose (400 μg); (*d*) crystalline human albumin (Sigma) (200 μg).

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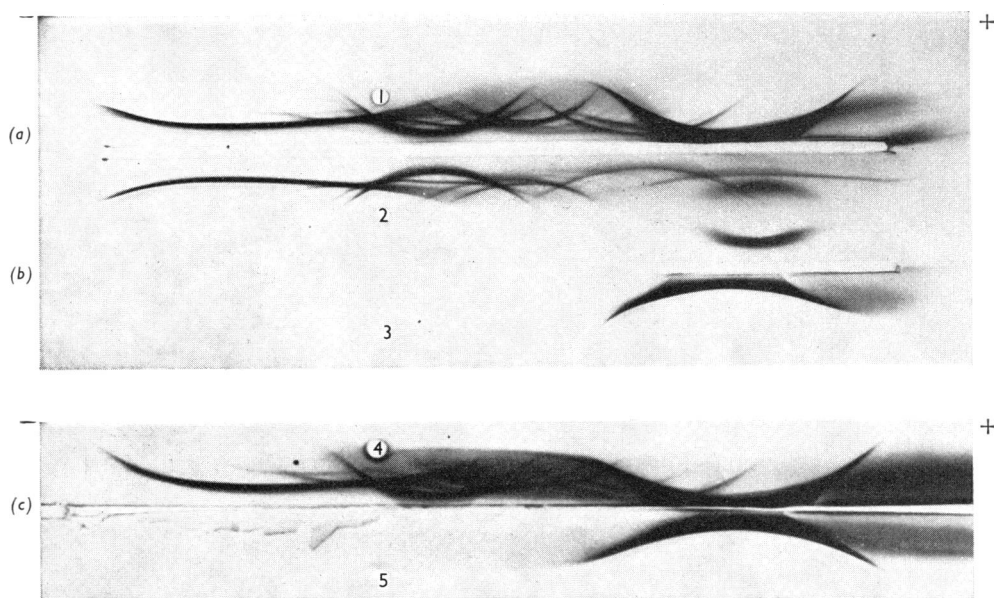
EXPLANATION OF PLATE 2

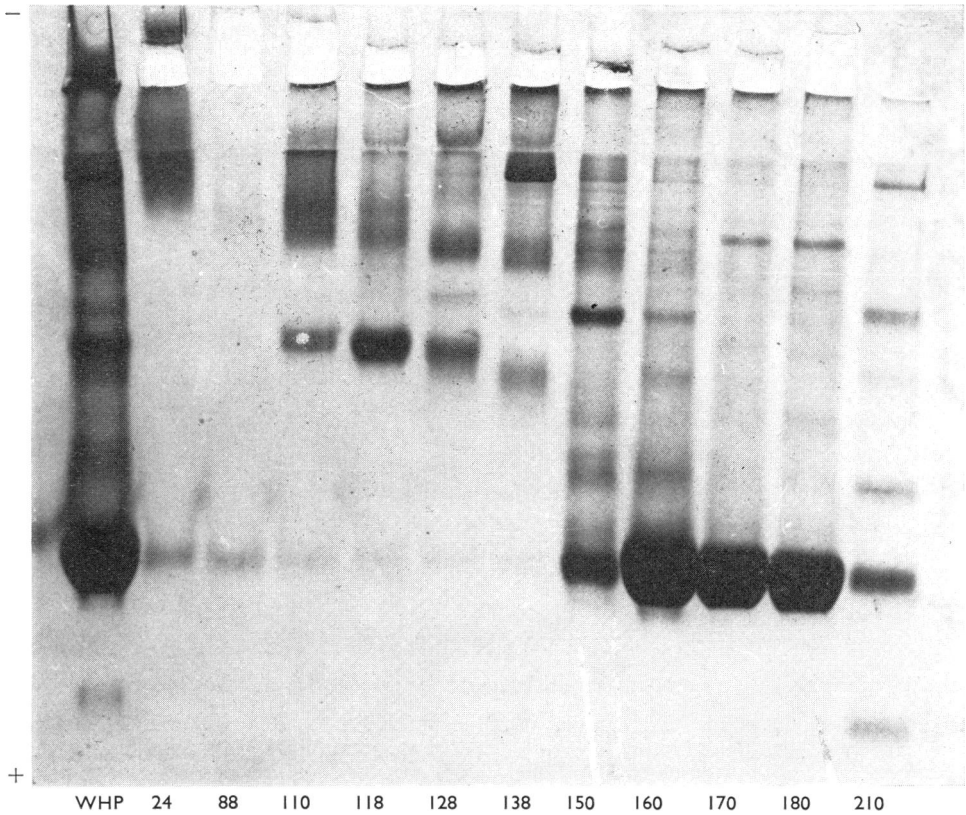*Immunoelectrophoresis of human plasma and fractions*

Electrophoresis was carried out for 2 h at 8 mA, in 0.05 M-sodium veronal buffer, pH 8.6. Wells 1 and 4, whole human plasma; well 2, Cibacron Blue–Sepharose-treated plasma; wells 3 and 5, albumin eluted with NaSCN from Cibacron Blue–Sepharose; slots *a* and *c*, anti-(whole human serum); slot *b*, anti-(human albumin).
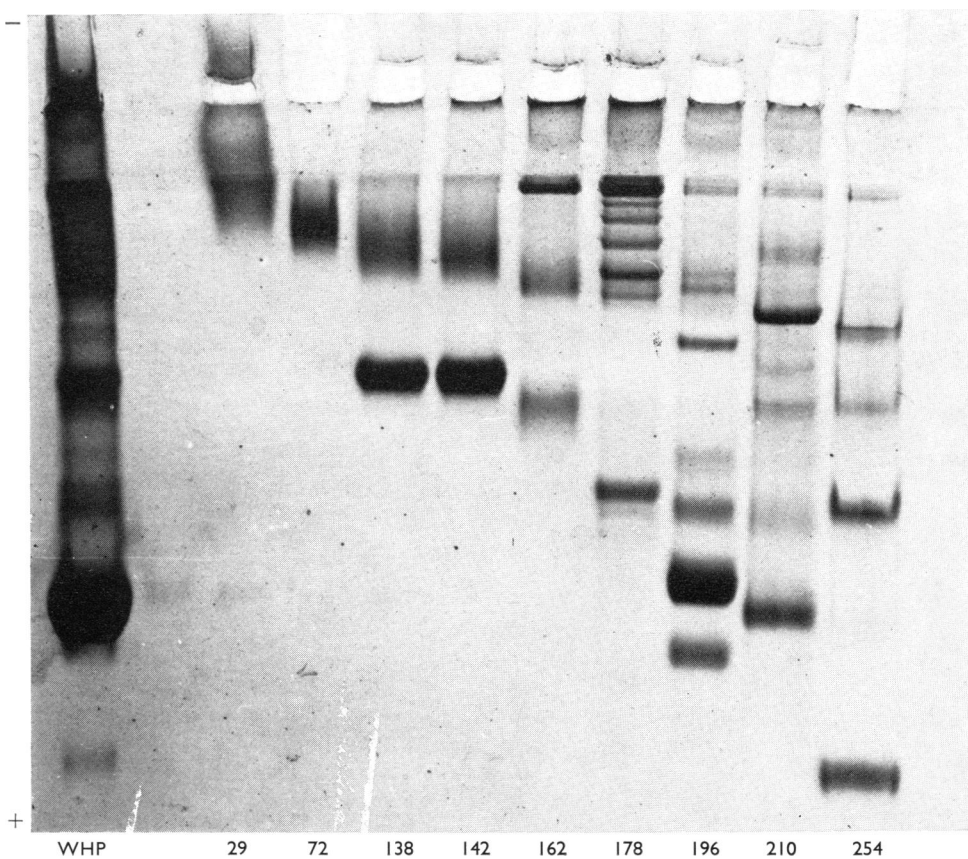
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EXPLANATION OF PLATE 3

Gel-slab electrophoresis of Cohn fractions from whole human plasma and Cibacron Blue–Sepharose-treated plasma

The polyacrylamide gel was made in a discontinuous system from 4% to 8%; the running pH was 8.8. The gel was stained as in Plate 1. Direction of migration is from cathode (top) to anode (bottom). Whole plasma was the starting material for the odd numbered gels. Cibacron Blue–Sepharose-treated plasma was used for the even-numbered gels. 1 and 2, Cohn fraction I; 3 and 4, Cohn fraction II and III; 5 and 6, Cohn fraction IV-1; 7 and 8, Cohn fraction IV-4; 9 and 10, Cohn fraction V; 11 and 12, Cohn fraction V supernatant.

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WHP   29   72   138   142   162   178   196   210   254

EXPLANATION OF PLATES 4 AND 5

Gel-slab electrophoresis of plasma fractions from DEAE-cellulose chromatography

Conditions of electrophoresis were as in Plate 3. (Plate 4), Samples from whole human plasma; (Plate 5), samples from Cibacron Blue–Sepharose-treated plasma. In both cases the numbers beside each slot are equivalent to the fraction numbers given in Fig. 1. WHP, whole human plasma.

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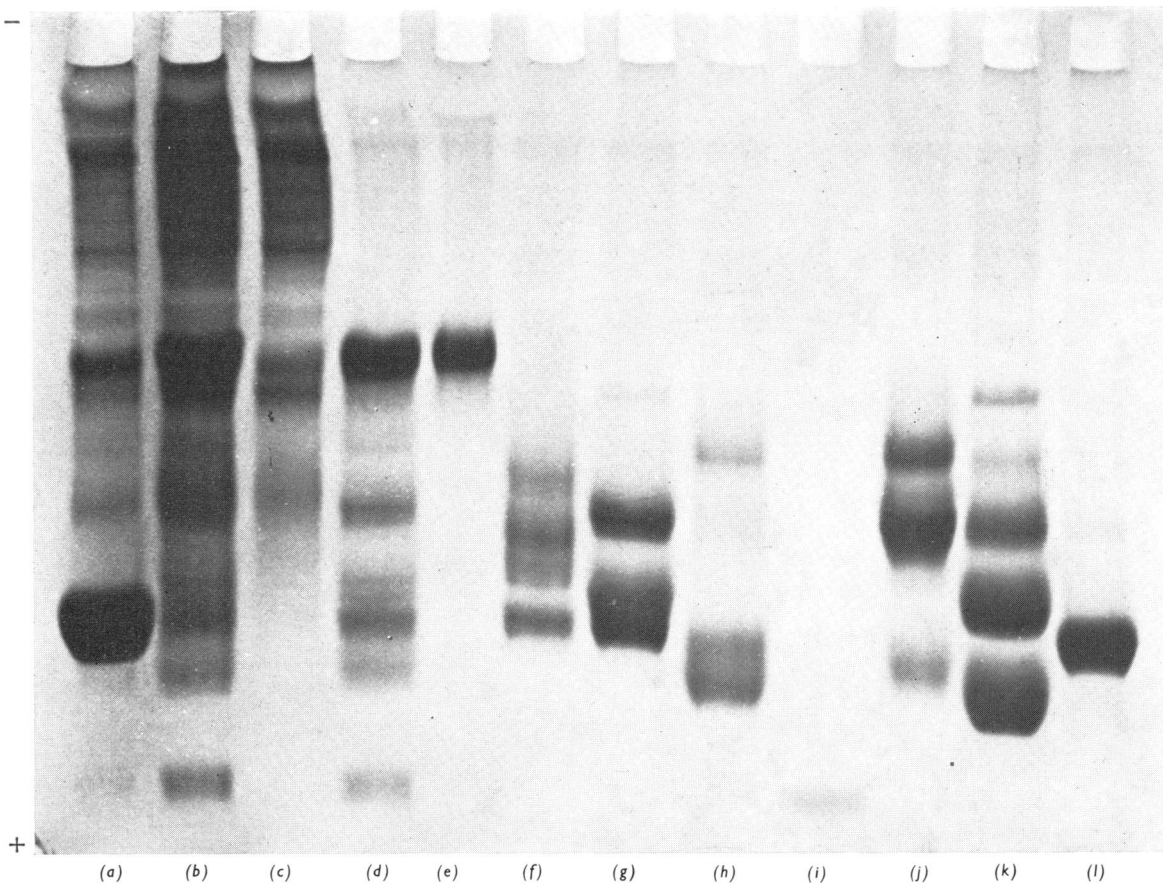
EXPLANATION OF PLATE 6

Gel-slab electrophoresis of human plasma and plasma fractions

Conditions of electrophoresis are as in Plate 3. (*a*) Whole human plasma; (*b*) Cibacron Blue–Sepharose-treated whole human plasma; (*c*) high-molecular-weight fraction from Sephadex G-75; (*d*) low-molecular-weight fraction from Sephadex G-75; (*e*) unadsorbed transferrin, from DEAE-cellulose, pH6.5; (the following Roman numerals refer to the pooled fractions of Figs. 2*a* and 2*b*) (*f*) I, pH6.5; (*g*) II, pH6.5; (*h*) III, pH6.5; (*i*) IV, pH6.5; (*j*) I, pH8.8; (*k*) II, pH8.8; (*l*) III, pH8.8.

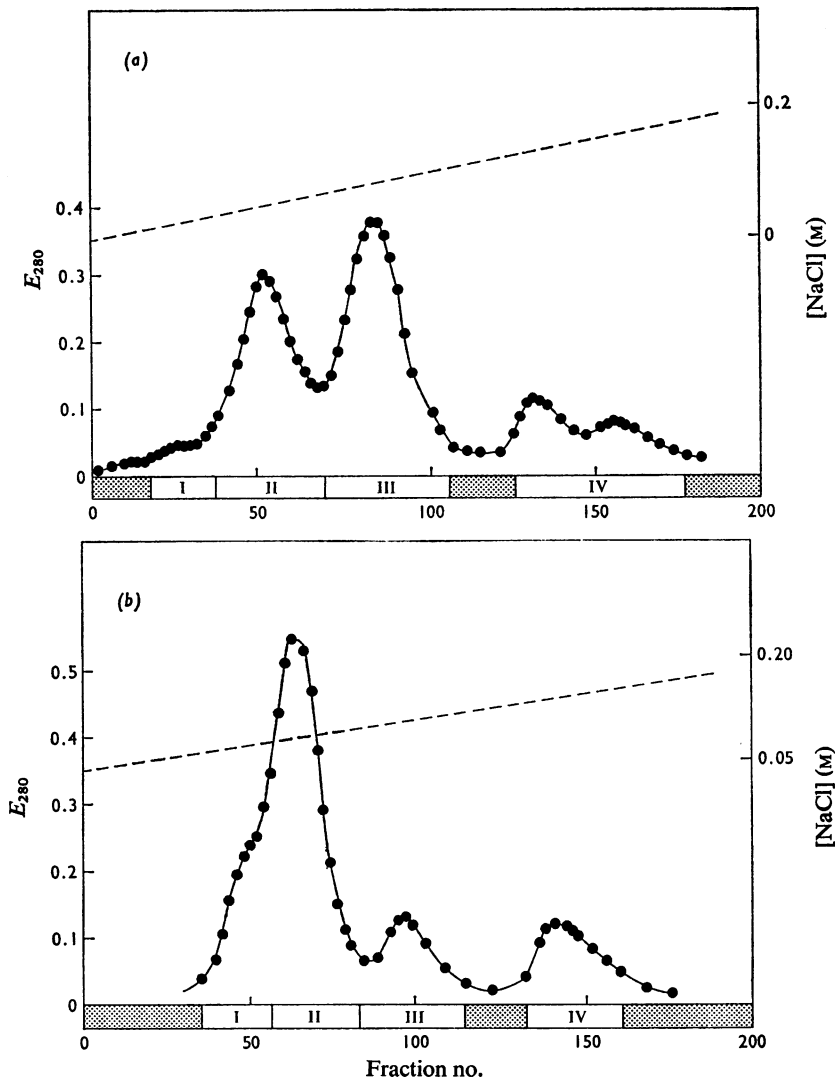J. TRAVIS, J. BOWEN, D. TEWKSBURY, D. JOHNSON AND R. PANNELL

Fig. 2. *Ion-exchange chromatography on Sephadex G-75 of low-molecular-weight fraction of Cibacron Blue–Sepharose-treated plasma*

(a) Separation at pH 6.5 by using a linear gradient (----) of 0–0.2 M-NaCl in 0.03 M-sodium phosphate, pH 6.5, (1 litre total); (b) separation at pH 8.8 by using a linear gradient of 0.05–0.2 M-NaCl in 0.05 M-Tris/HCl, pH 8.8, (1 litre total). Column size was 1.9 cm × 40 cm. Fractions pooled for electrophoresis (Plate 6) are indicated by Roman numerals. ●, E_{280}.

Discussion

Albumin represents at least 50% by weight of the proteins in plasma. The immunoglobulins and fibrinogen account for another 12% and 4% respectively. The remaining 32% is composed of more than 100 different proteins. Although in most classical fractionation schemes the immunoglobulins are cleanly separated from the bulk of the plasma proteins, albumin is found in a large number of fractions. For example, in method VI of Cohn *et al.* (1946), albumin is present in all fractions.

Ion-exchange chromatography of human plasma on DEAE-cellulose does not readily resolve the proteins into discrete fractions, because albumin is eluted as a broad peak and masks any resolution of other proteins. Further, since at least one-half of the plasma proteins have molecular weights near that of albumin, gel-filtration chromatography is also relatively ineffective for plasma fractionation.

The work in the present paper demonstrates that a number of advantages can be realized in any fractionation scheme for plasma proteins by first removing

albumin by affinity chromatography. The immediate advantage is that over one-half of the protein is removed in the affinity step, with only very minor mechanical losses of other proteins. Thus a smaller quantity of protein has to be processed in subsequent steps. Moreover, as clearly shown by the fractionation patterns presented in the present paper, the specific difficulties associated with the purification of plasma proteins whose molecular size and/or charge are similar to those of albumin are obviated in subsequent purification steps. Analytical advantages accrue from the lesser protein load and the detectability of components obscured by albumin. This is especially true in the resolution of α_1-proteinase inhibitor and α_1-acid glycoprotein.

Although the procedure presented here describes the bulk fractionation of plasma on Cibacron Blue–Sepharose, some resolution occurs analogous, primarily, to that obtained by gel filtration on Sepharose 4B. A major difference, however, is the trailing of immunoglobulins and lipoproteins due to retarding interaction with the ligand. The type of elution pattern obtained is identical with that demonstrated with Sepharose–Blue Dextran and described previously (Pannell et al., 1974).

The purification of higher-molecular-weight plasma proteins (>80000) does not generally involve interference by albumin, and the combination of gel-filtration chromatography with $(NH_4)_2SO_4$ fractionation usually separates the larger proteins from this potential contaminant. Unfortunately, a large number of plasma proteins have properties similar to albumin in both hydrodynamic size and ionic behaviour. The results demonstrated here clearly show that, if the albumin concentration is decreased to a value where resolution by most protein-fractionation techniques can be obtained (about 200mg/100ml), there is no longer any interference from this protein. It is only in whole plasma that separation problems occur, where the albumin concentration is at least tenfold higher than that of the majority of low-molecular-weight proteins.

The mechanism by which human plasma albumin and Cibacron Blue–Sepharose interact is of significance, but has not yet been determined. It has been shown (Böhme et al., 1972) that the dye has an affinity for enzymes which utilize AMP for reactions. Further, it has been suggested that the dye may resemble a nucleotide in shape and thus bind to any protein which has a 'nucleotide fold' (Thompson et al., 1975). This tenacious binding of albumin to dye would, on this basis, indicate that it is also capable of binding nucleotides, perhaps in the role of a transport agent. However, few data are currently available on this possible function of albumin.

Recent experiments indicate that other dyes linked to Sepharose will also bind albumin. These include Cibacron Brilliant Blue, Cibacron Scarlet and Cibacron Orange (D. Johnson, unpublished work), members of a series of dyes known as reactive dyes. These compounds are all derived from cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) and have one reactive chloride group. When they react with Sepharose the product is formed by synthesis of a stable azo-ester bond between the dye and hydroxyl groups of the Sepharose. Thus, in comparison with CNBr-derived conjugates, leakage of the ligand presents little, if any, problem. In our earlier report (Pannell et al., 1974) we stated that Cibacron Blue F-3-GA–Sepharose had a poor affinity for albumin. At that time, however, coupling was performed by CNBr activation of the Sepharose followed by reaction with the single amino group on the dye. Presumably this substituent is involved in albumin binding.

The technique described here for albumin removal is simple, inexpensive and easily repeated. Moreover, it is a mild procedure, so that denaturation is minimized. The products obtained, albumin and albumin-depleted plasma, are important as immediate sources for therapy (albumin) and for the isolation of other plasma proteins (albumin-depleted plasma). It is hoped that this technique will prove useful in plasma fractionation for research and for therapy, and as an aid in the investigation of other fluids, including cerebrospinal fluid, urine and intestinal fluids, all of which contain a high proportion of albumin.

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