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GROUP FRACTIONATION OF PLASMA PROTEINS ON DIPOLAR ION EXCHANGERS

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SUMMARY

The preparation of arginine– and sulphanilic acid–agarose is described. These dipolar ion exchangers have been characterised and applied to the fractionation of human serum by employing a modified displacement technique called pulse elution.

INTRODUCTION

A series of investigations dealing with the adsorption and desorption of proteins on group specific adsorbents[1] and dipolar ion exchangers[2] was initiated at this Institute. The programme was directed towards the development of improved fractionation methods for mixtures of proteins in general and plasma proteins in particular. In dipolar ion exchangers the distribution of ions rapidly reaches equilibrium as judged from the chromatographic behaviour of salts. Changes in bed volume due to alteration in ion concentration are usually very small or scarcely detectable under the appropriate conditions. These facts are of great importance for large-scale applications. There are also reasons to believe that suitable conditions for linear chromatography may be found.

Dipolar ion exchangers can be synthesised in many ways, for instance by reacting amino acids with an hydroxyl-containing polymer activated by cyanogen bromide[2,3]. In the present study we have used another simple 2-step procedure. Agarose was converted to an oxirane derivative by reaction with epichlorohydrin:

$$\text{(Polymer)–OH} + \text{CH}_2\text{–CH–CH}_2\text{Cl} \rightarrow \text{(Polymer)–O–CH}_2\text{–CH–CH}_2 + \text{HCl}$$

(O bridge on the oxirane carbons)

After excess reagent had been removed, coupling was effected in alkaline solution:

$$\text{(Polymer)–O–CH}_2\text{–CH–CH}_2 + \text{RNH}_2 \rightarrow \text{(Polymer)–O–CH}_2\text{–CH(OH)–CH}_2\text{–NHR}$$

(where R contains at least one acidic group).

This method can be used not only to attach amino acids but also proteins and nucleotides containing an amino group, etc. to agarose, Sephadex, cellulose and other

hydroxyl-containing polymers. At this Institute we have produced biospecific adsorbents and matrix-linked enzymes. Oxirane groups can also be introduced in the polymer via bisepoxides or by other means.

EXPERIMENTAL

Material and methods

Chemicals. Analytical grade chemicals were used.

Arginine–agarose. 100 ml of sedimented Sepharose 6 B (obtained from Pharmacia Fine Chemicals, Uppsala, Sweden) was transferred to a glass filter funnel and thoroughly washed with distilled water and drained. After suspension in 80 ml 1 *M* NaOH, 10 ml epichlorohydrin was added and the mixture heated on a water bath at 60° for 2 h in a round-bottomed flask under vigorous stirring. Excess epichlorohydrin was removed by extensive washing with distilled water until no odour could be detected. The drained gel was suspended in 100 ml 0.01 *M* NaOH containing 10 g arginine. After being kept at 60° for 2 h, the mixture was allowed to cool overnight. The nitrogen content was determined and found to be 4.15 % (*ca.* 750 μmoles/g dry gel). The gel particles retained their spherical shape in aqueous solutions, but could not be freeze-dried without breakage.

Sulphanilic acid–agarose. 100 ml Sepharose sediment was treated as above and suspended in 80 ml 1 *M* NaOH. 10 ml epichlorohydrin was added and the suspension left for 24 h at room temperature under vigorous stirring. After washing with a large amount of water, the gel was suspended in 100 ml 2 *M* potassium carbonate buffer (pH 10) containing 3 g sulphanilic acid, and the pH was readjusted to 10. Coupling was allowed to take place without stirring for a period of 6 days. Nitrogen and sulphur contents were determined on dried samples of gel and found to be 0.93 and 1.78 %, respectively (*ca.* 600 μmoles/g dry gel). As in the case arginine–agarose, the gel particles retained their shape after the introduction of the dipolar substituents.

Titration. Potentiometric titrations were performed at room temperature with a pH-meter 26, Radiometer (Copenhagen) employing an alkali-resistant glass–calomel electrode (GK 2302 B). The titration vessel is schematically drawn in Fig. 1. It was provided with a lid with inlet for nitrogen, an ascarite trap to exclude carbon dioxide and an opening for the burette tip. A magnetic stirrer was introduced into the vessel to allow rapid mixing.

Human serum. The human serum was supplied by the Academic Hospital of Uppsala and kept frozen at —20° until used. Most experiments were performed with serum kept for less than two weeks.

Electrophoresis. The HJERTÉN apparatus was used[4] with a revolving tube of 36.5 cm length and 0.3 cm I.D. All analyses were performed with 4 μl samples in 0.1 *M* Tris–HCl (pH 8.5). 1200 V were applied across the electrodes which resulted in a current of 5.0 mA. By scanning the tube every 8th min, the progress of electrophoresis was conveniently followed. For identification the diagrams were compared with that of the serum obtained under identical conditions. (40-min run.)

Other equipment. Columns similar to that described by PORATH AND BENNICH were used[5]. The light transmission (254 nm) of the effluent was recorded on LKB-Uvicord (LKB-produkter AB, Bromma, Stockholm) and samples were collected in a time-based fraction collector (AB Stålprodukter, Uppsala). Absorption in the ultra-

violet or the visual range was measured by Hitachi spectrophotometer Model 101 and the conductivity was determined with a conductometer of type Philips PR 9501. The samples were concentrated with the Aminco Diaflo membranes.

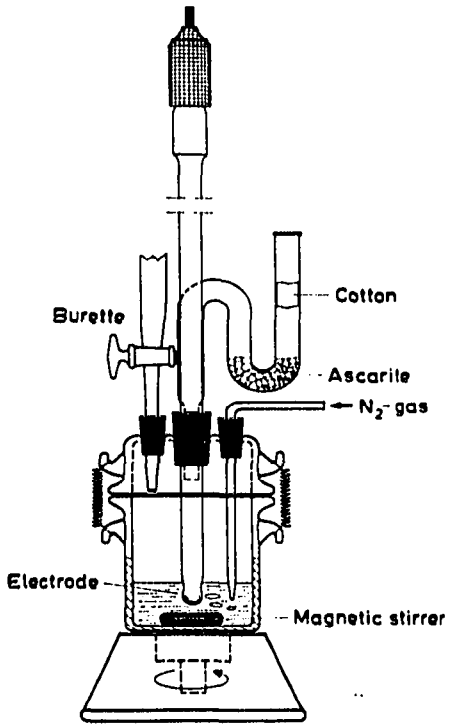

Fig. 1. Titration vessel (see text).

RESULTS

Characterisation of the ion exchangers

Titration. Gel was allowed to swell in excess water and then to settle in a cylinder. 20 ml of 0.5 *M* sodium chloride were added to 10 ml of the sediment, and the slurry was transferred to the titration vessel. The vessel was closed and nitrogen introduced in a slow current, and the stirring was started. Hydrochloric acid (1 *M*) was added to a pH below 2.0. Titration was effected by 0.05-ml portions of 0.1 *M* sodium hydroxide. The titration curves for arginine–agarose and sulphanilic acid–agarose are shown in Figs. 2 and 3, respectively.

From the curve in Fig. 2 for arginine–Sepharose, p*K* values of 2.95 and 8.10 were calculated for the carboxylic and amino groups, respectively. Due to the low concentration of guanidino groups in the gel, it was not possible to obtain a good estimate of the p*K* of this group. In a separate experiment in 0.5 *M* sodium chloride, the following p*K* values for the titratable groups of free arginine were obtained: 2.80, 9.07 and 12.65.

The p*K* values for sulphonic and amino groups were found to be 2.95 and 7.25, respectively, from the titration curve for sulphanilic acid–agarose (Fig. 3).

Adsorption capacity. The adsorption capacity was measured by frontal analysis. On a column of arginine–agarose (2.0 × 14.8 cm; V_0 = 21 ml; V_t = 47 ml) in 0.05 *M*

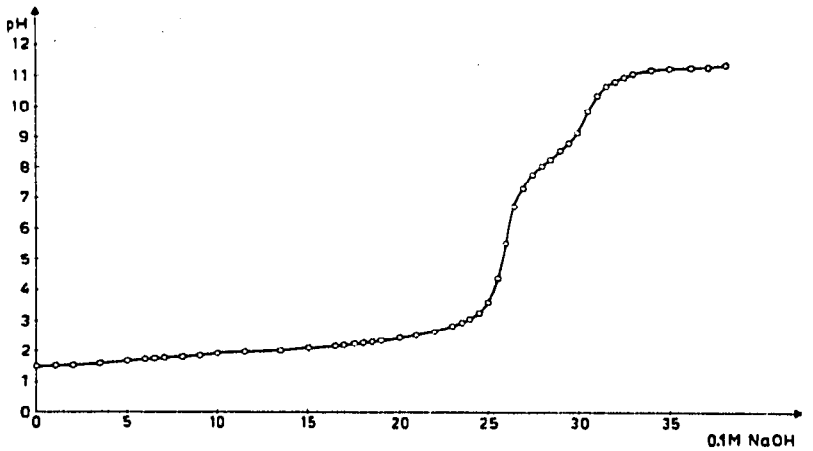

Fig. 2. Titration curve for arginine–Sepharose 6 B.

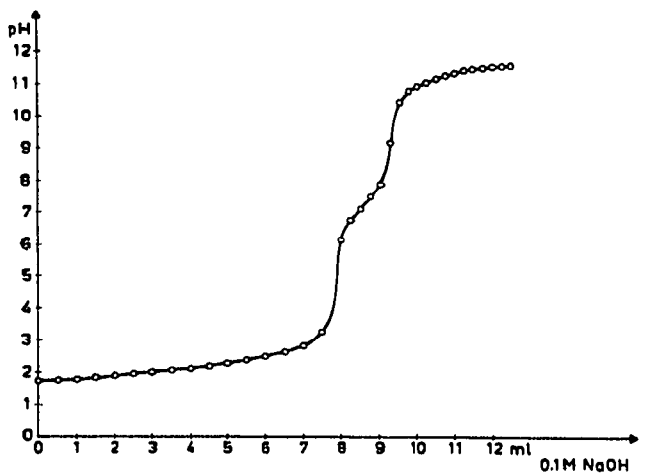

Fig. 3. Titration curve for sulphanilic acid–Sepharose 6 B.

Tris–HCl, pH 7.5, 0.5 % solution of serum albumin was continuously introduced at a speed of 24 ml per h. The absorption at 254 nm was recorded and the adsorption of albumin was calculated from the retention on the column. $V_e - V_0$ was found to be 218 ml which corresponds to 1.09 g of albumin. A similar experiment was made at pH 6.2 in 0.02 *M* imidazole–HCl, 1 *M* glycine. The column was then found to adsorb 2.31 g of albumin. After these two experiments the column was washed extensively with distilled water. The gel was transferred to a beaker and freeze-dried. The dry weight of the gel was found to be 1.717 g. The adsorption capacity of arginine–agarose for albumin was calculated to be 0.6 g and 1.3 g per g dry gel in the two buffers tested.

The adsorption capacity for γ-globulin on sulphanilic acid–agarose was measured in the same manner and found to be 0.02 g and 2.1 g in the Tris and imidazole buffers, respectively.

Flow properties. A column 2 × 15 cm of arginine–Sepharose 6 B was packed in 0.05 *M* Tris–HCl (pH 7.5) buffer by sedimentation[6].

The column was connected to a buffer reservoir. The pressure was adjusted by elevating or lowering the position of the reservoir. A series of flow-rate determinations were made at various pressure heads. Fig. 4 shows the flow rate as a function of the pressure.

Behaviour of salts and alkali. The chromatographic behaviour of buffer ions is of particular interest from a practical point of view. A "pulse" of salt, in high concentration, or of a different kind, migrates through the bed as a compact zone (Fig. 5).

Conversion to a new buffer medium can therefore be accomplished within a fraction of the total volume of the column. Since the buffer ions move at a somewhat different rate through the bed, a "pH wave" will be created (Fig. 5). The amplitude of this wave is small enough to be neglected in practice, at least in preparative chromatography. For routine fractionations of plasma and serum, it is of great value that a short concentration pulse effectively displaces the adsorbed proteins so that the bed can be rapidly regenerated. Dipolar ion exchangers are well-suited for cyclic operations. We will call this form of elution *pulse elution.*

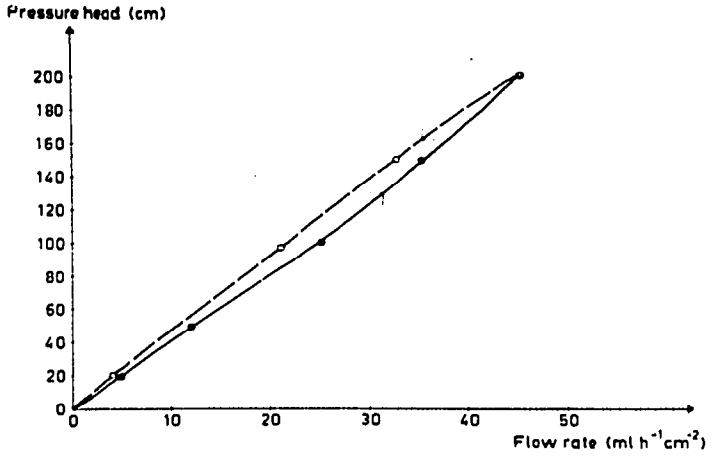

Fig. 4. Flow rate as a function of hydrostatic pressure. ———, increasing pressure; – – – –, decreasing pressure.

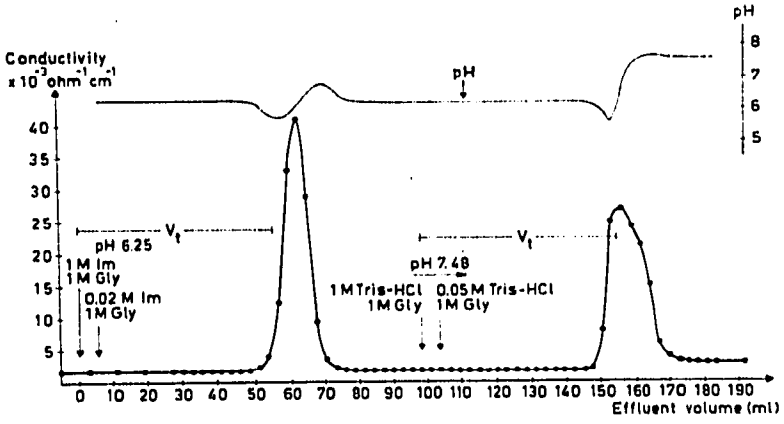

Fig. 5. Illustration of the rapid regeneration and buffer change on dipolar ion exchangers. Column: 2 × 36 cm, arginine–Sepharose 6 B. Flow rate: 27.6 ml/h.

Since buffer change can so easily be effected we considered the behaviour of alkali in the bed to be of great interest. The column from the previous experiment was loaded with 10 ml of 1 *M* sodium hydroxide followed by the original buffer, *i.e.* 0.05 *M* Tris–HCl in 1 *M* glycine, pH 7.6. The pH and conductivity of the effluent were measured and the results are shown in Fig. 6.

Fractionation of plasma proteins

In the experiments presented here, a simple strategy was chosen to achieve a group fractionation of the plasma proteins.

Step 1. 50 ml of serum, dialysed against 0.15 *M* Tris–HCl, pH 7.5, was introduced at a rate of 25 ml per h into a column, 2 × 39 cm, filled with arginine–agarose equilibrated with the same buffer. 8-ml fractions were collected. After 600 ml of buffer had passed, a pulse of 25 ml 1 *M* Tris–HCl, pH 7.5, was introduced into the bed followed by the original buffer. The distribution of material was determined spectrophotometrically and is shown in Fig. 7. The major part of the protein-containing material was collected in the elution zone: Fraction e_1. This fraction was red-coloured while the displaced material, Fraction A, was blue.

Step 2. Fraction e_1 was concentrated to 50 ml, dialysed against 0.10 *M* Tris–HCl, pH 7.5, and chromatographed on the 2 × 39 cm column, this time equilibrated

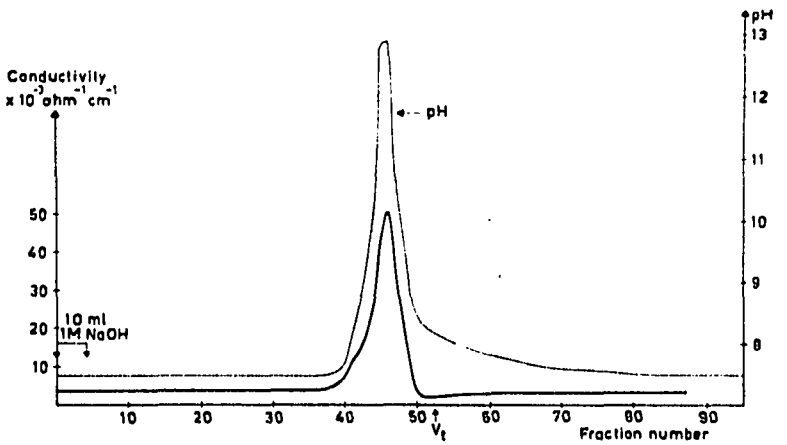

Fig. 6. Illustration of the rapid restoration of the original equilibration conditions after passage of a zone of 1 *M* NaOH (effective for displacement of residual proteins). Column: 2 × 36 cm, arginine–Sepharose 6 B. Buffer: 0.05 *M* Tris–HCl in 1 *M* glycine, pH 7.6. Flow rate: 26.5 ml/h. Fraction number: 12/h. ——, pH; ———, conductivity.

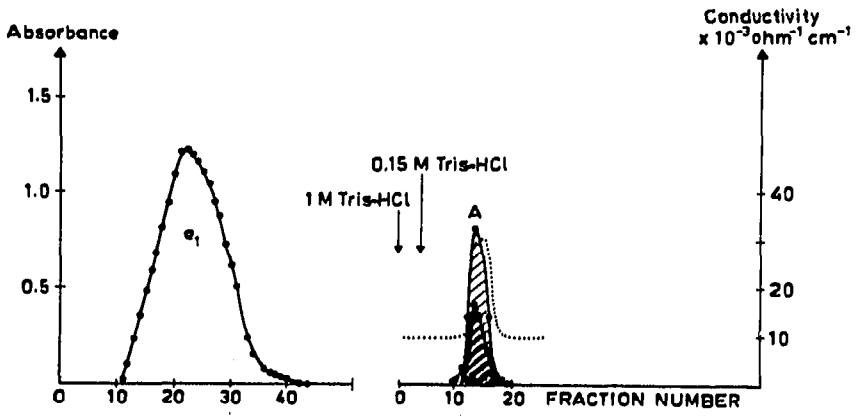

Fig. 7. Chromatogram of serum. Sample: 50 ml serum. Column: 2 × 39 cm, arginine–Sepharose 6 B. Buffer: 0.15 *M* Tris–HCl, pH 7.5. Flow rate: 28 ml/h. Fraction number: 3/h. ———, 280 nm; – – – –, 610 nm; ·····, conductivity.

with 0.10 *M* Tris-buffer. Displacement was again effected with a zone of 1 *M* buffer. Two fractions were obtained, e_2 and B (Fig. 8), the first being red, the second yellow-coloured.

Step 3. Fraction e_2 was concentrated to about 50 ml, dialysed against 0.05 *M* Tris–HCl, pH 7.5, and again chromatographed on the same column as before, but this time in 0.05 *M* Tris-buffer. As before, a broad peak appeared immediately after the passage of an effluent volume corresponding to the void volume of the column (Fraction F) (Fig. 9). This fraction was very weakly yellow-coloured and was found to consist of γ-globulins (Fig. 13). A slightly red-coloured zone passed the column at a slower rate and appeared in the eluate after passage of about four total bed volume equivalents of effluent ($V_e/V_t = 4.3$). Between these fractions D and F, less well-separated components were obtained (Fraction E).

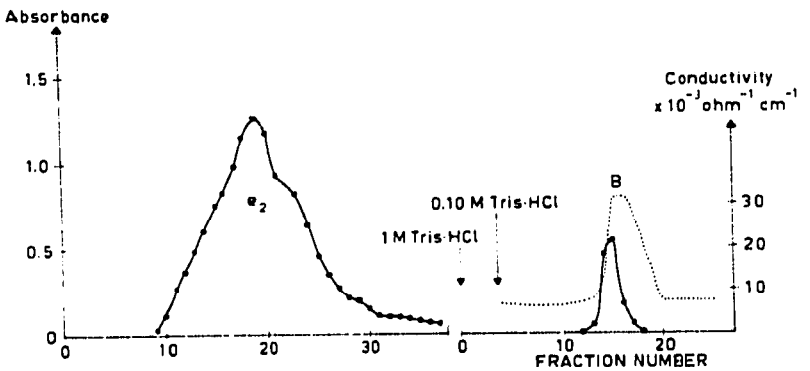

Fig. 8. Chromatogram of Fraction e_1 (Fig. 7). Sample: 50 ml concentrated Fraction e_1. Column: 2 × 39 cm, arginine–Sepharose 6 B. Buffer: 0.10 *M* Tris–HCl, pH 7.5. Flow rate: 25 ml/h. Fraction number: 3/h. ———, 280 nm; ·····, conductivity.

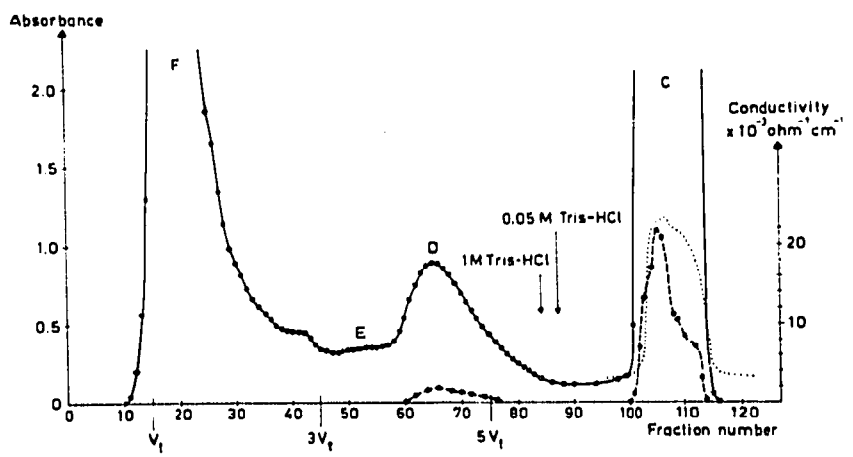

Fig. 9. Chromatogram of Fraction e_2. Sample: 50 ml concentrated Fraction e_2. Column: 2 × 39 cm, arginine–Sepharose 6 B. Buffer: 0.05 *M* Tris–HCl, pH 7.5. Flow rate: 25 ml/h. Fraction number: 3/h. ———, 280 nm; – – – –, 410 nm; ·····, conductivity.

It was noticed that the major part of the red material accumulated during the run in a concentrated zone, the speed of which was slowed down during the development with the buffer and finally stopped at a distance of 21–23 cm from the top. On displacement with 10 ml 1 *M* Tris–HCl the yellow material, distributed over most of the column, was pushed together with the red zone material and recovered in the

protein Fraction C. The column attained equilibrium against 0.05 *M* Tris–HCl immediately after the passage of the protein zone, as found by the decrease in the conductivity of the effluent.

Step 4. Fraction C was concentrated from 130 ml to 25 ml. After dialysis against 0.1 *M* imidazole–HCl in 1 *M* glycine, pH 6.3, 5 ml of the sample was transferred to a column (2 × 36 cm) of arginine–agarose equilibrated in the same buffer. As seen in Fig. 10, three components C_1, C_2, and C_3 were partly separated and a fourth component C_4 appeared just after an effluent volume corresponding to V_t. A 10-ml pulse of 1 *M* imidazole–HCl in 1 *M* glycine removed the remaining proteins (C_5). The material in Fraction C_4 was shown to be albumin by electrophoresis (Fig. 13).

Step 5. Fraction A was dialysed against 0.02 *M* imidazole–HCl in 1 *M* glycine, pH 6.3, and concentrated to 10 ml from 60 ml. 8.6 ml of the dialysate was fractionated on a 2 × 17.7 cm sulphanilic acid–agarose column in imidazole buffer. The chromatogram was developed at a rate of 26 ml per h and fractions were collected every 15 min.

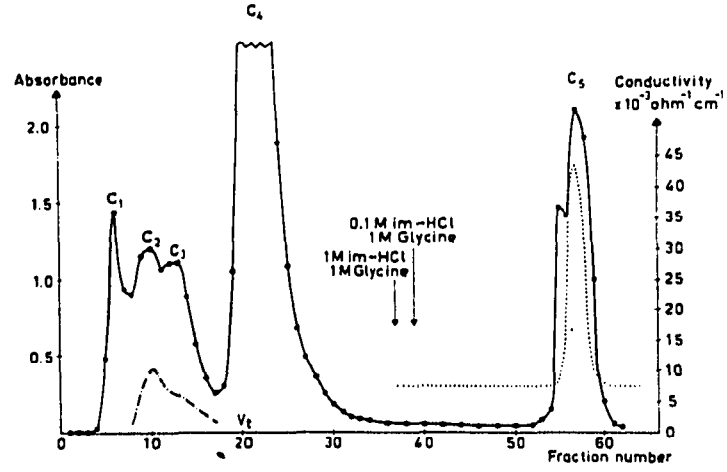

Fig. 10. Chromatogram of Fraction C. Sample: 5 ml concentrated Fraction C. Column: 2 × 36.3 cm, arginine–Sepharose 6 B. Buffer: 0.1 *M* imidazole–HCl, 1 *M* glycine, pH 6.3. Flow rate: 24 ml/h. Fraction number: 4/h. ———, 280 nm; – · – · –, 410 nm; · · · · · ·, conductivity.

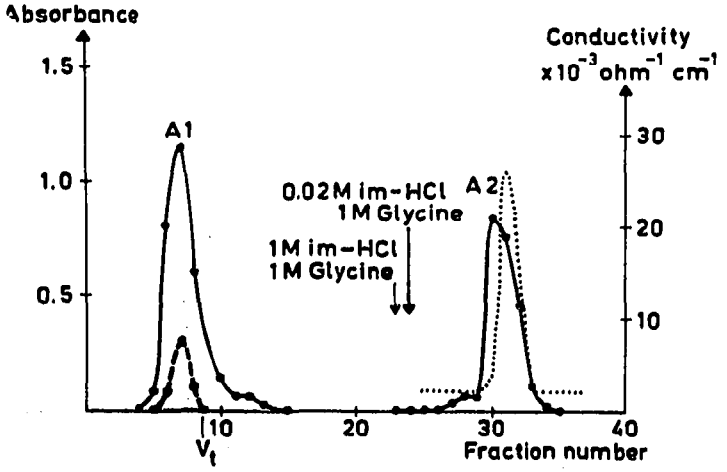

Fig. 11. Chromatogram of Fraction A. Sample: 8.6 ml concentrated Fraction A. Column: 2 × 17.7 cm, sulphonic acid-Sepharose 6 B. Buffer: 0.02 *M* imidazole–HCl, 1 *M* glycine, pH 6.3. Flow rate: 26 ml/h. Fraction number: 4/h. ———, 280 nm; – – – –, 610 nm; · · · · · ·, conductivity.

When 150 ml of buffer had passed, a pulse of 10 ml 1 *M* imidazole–HCl in 1 *M* glycine was introduced followed by the original buffer. Absorbance was measured at 280 and 610 nm as well as the conductivity of the fractions around the displaced material peak. As seen in Fig. 11 two well-separated peaks (Fraction A_1 and A_2) were obtained.

The blue material of Fraction A, ceruloplasmin, moved without retention and was collected in Fraction A_1. The electropherograms of A_1 and A_2 are shown in Fig. 14.

Step 6. Fraction B was concentrated from 32 ml to 8 ml and dialysed against 0.02 *M* imidazole, 1 *M* glycine (pH 6.3). 3 ml of this solution was introduced into the column referred to in step 5 and the chromatogram was developed in the same way (Fig. 12). The electropherograms of the eluted and displaced fractions are shown in Fig. 15.

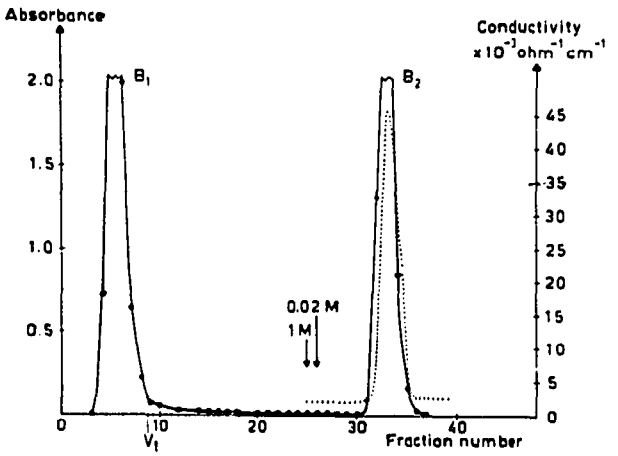

Fig. 12. Chromatogram of Fraction B. Sample: 3-ml concentrated Fraction B. Column: 2 × 17.7 cm, sulphonic acid–Sepharose 6 B. Buffer: 0.02 *M* imidazole–HCl; 1 *M* glycine, pH 6.3. Flow rate: 26 ml/h. Fraction number: 4/h. ———, 280 nm; ·····, conductivity.

DISCUSSION

The fractionation of plasma proteins with dipolar ion exchangers is based primarily on adsorption governed by electrostatic forces. In addition molecular sieving plays a major role, and sometimes ion exclusion may be a contributing factor. Of importance for the chromatographic behaviour are: (1) the structure and spatial distribution of the amphoteric groups responsible for the adsorption, (2) matrix density and (3) the nature of the medium. Extensive investigations are needed to clarify the interrelationships between these factors and thus provide the knowledge necessary for optimising the fractionation conditions for particular separation problems. From the limited experience so far, only crude judgements can be made.

Arginine–agarose acts as an anion exchanger and consequently the serum proteins will be eluted in the same or nearly the same order as on DEAE-cellulose or DEAE-Sephadex. At variance with the former, but like the latter, molecular sieving is superimposed upon ionic adsorption. The fact that the bed volume is not subjected to such large alterations as in the case of DEAE Sephadex is a significant advantage.

Alkali degrades native agarose. If agarose is sufficiently cross-linked, however, it is more stable and resists hot concentrated alkali (but not very strong acid)[7]. Dipolar ion exchangers based on cross-linked agarose, as those described in this paper, can therefore be generated or sterilised with alkali. Activation with the epoxy method simultaneously crosslinks the agarose such that if optimum conditions are employed the product will be alkali-stable yet not greatly altered with respect to its permeability. With arginine–agarose there is a risk of hydrolysis of the guanidino group upon prolonged contact with strong alkali. It is therefore important that the regeneration is accomplished by pulse elution with a rapidly moving zone of alkali (Fig. 6).

The adsorption capacity for plasma proteins is high for arginine–agarose at the degree of substitution, the pH values and the ionic strengths used here. In the experiments referred to in steps 1–3, 50 ml of serum was introduced into a column having a total volume of about 125 ml. With retained separation efficiency as in the experiment in steps 1, 2, 5, and 6, it should be possible to charge a bed with a sample volume of about 0.5 V_t and with good margin utilise only 1.5–2.0 V_t per fractionation cycle. Beds as large as 100–1000 l can be operated judging from experience with Sephadex. Large-scale plasma fractionation with agarose dipole ion exchangers employing pulse elution should therefore be possible.

A more effective separation can be obtained in the elution step at low ionic strength. The components of the elution Fraction e_1 (Fig. 7) emerge from the column in a fairly narrow zone, whereas the γ-globulins are separated from haptoglobin in step 3. In step 4 (Fig. 10) serum albumin is found in a retarded zone well separated from the other components of Fraction C. As the elution volume somewhat exceeds V_t, it is reasonable to assume that ionic adsorption is superimposed upon molecular sieving. (In a couple of experiments with another specimen of serum the albumin was divided in two peaks.)

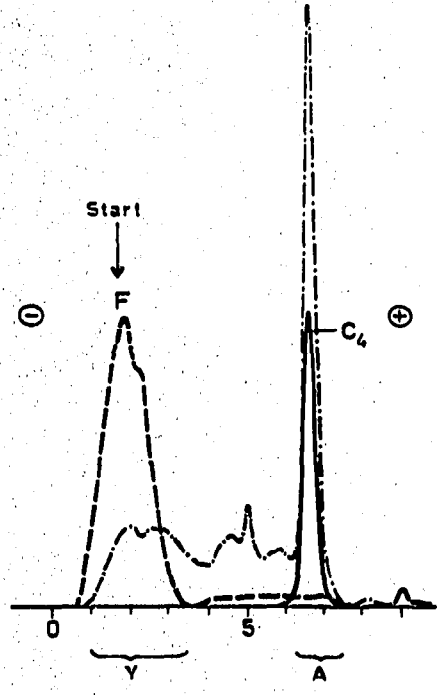

Fig. 13. Superimposed zone electropherograms. –·–·–, serum; – – – –, Fraction F; ————, Fraction C_4. Further details in the text.

The electrophoretic analyses reveal that sharp group separations were obtained. Fraction F consists exclusively of γ-globulins and Fraction C_4 contains only albumin (Fig. 13).

With the exception of Fractions F and C_4, all preparations are electrophoretically heterogeneous. Further purification should be possible by gradient elution or displacement chromatography on arginine–agarose. Fractions A and B were further purified on a dipolar ion exchanger containing more strongly acidic groups, *viz.* sulphanilic acid–agarose. On this adsorbent in 1 *M* glycine, 0.02 *M* imidazole–HCl, two subfractions were obtained in each case. The electrophoretic analysis showed that a sharp group separation had also occurred in these experiments (Figs. 14 and 15). For isolation of the various substances in the subfractions, further purification steps would be necessary. On sulphanilic acid–agarose, the salt concentration pulses move as compact protein-eluting zones.

The results so far obtained demonstrate that the dipolar ion exchangers offer an alternative to the adsorbents now in common use for protein chromatography. We feel, however, that the potentialities of these new types of adsorbents have as yet not been fully realised.

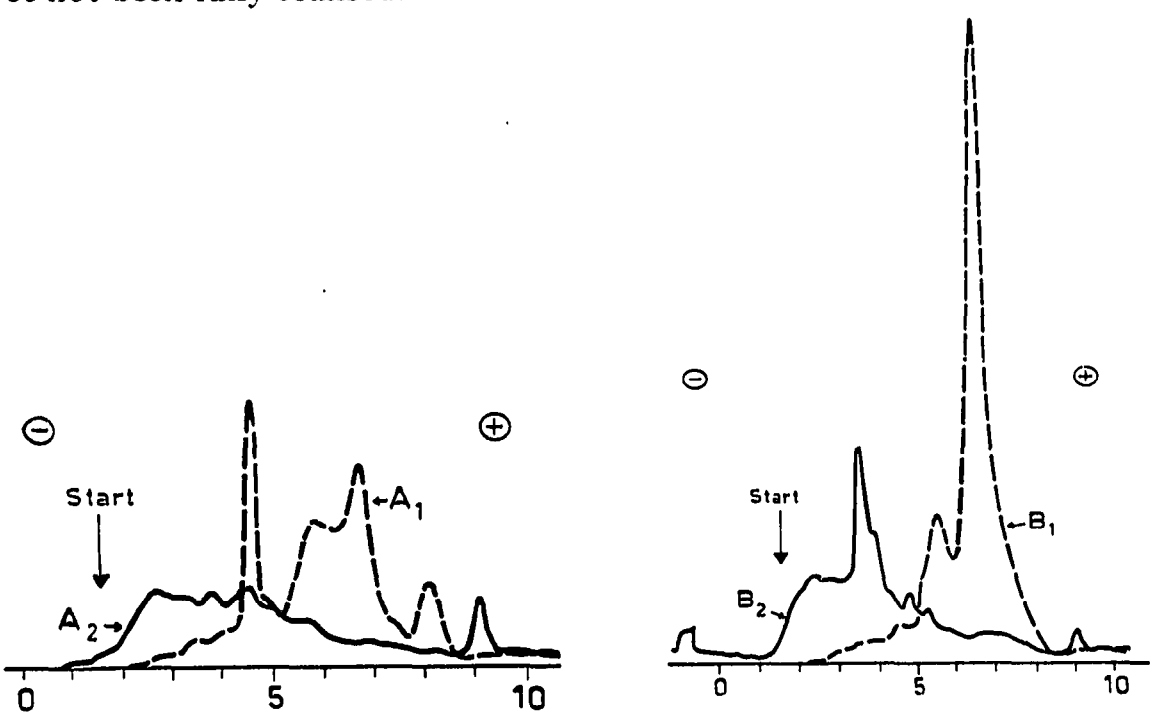

Fig. 14. Superimposed zone electropherograms. – – – –, Fraction A_1; ———, Fraction A_2.

Fig. 15. Superimposed zone electropherograms. – – – –, Fraction B_1; ———, Fraction B_2.

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