CHROMATOGRAPHIC STUDY OF HUMAN SERUM BY GEL FILTRATION

WALLACE V. EPSTEIN AND MARGARET TAN

The Allergy Division and the Rheumatic Disease Group, Department of Medicine, University of California School of Medicine, San Francisco, Calif. (U.S.A.)

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By means of cross-linked dextran polysaccharide, it has become possible to separate substances of different molecular size by gel filtration[1-3]. The dextran network has polar properties due to the high content of hydroxyl groups and has a low content of ionized groupings[2]. The degree of cross linkage determines the minimum size of particle excluded from the interstices of the gel grains. These are therefore able to pass through the column at a rate greater than that of molecules able to enter the grains. This method of separation is applicable to ionized as well as nonionized solutes.

Solutes passing through a gel column can be characterised by a partition coefficient (K_D)[2] between the external aqueous phase (void volume V_0) and the internal aqueous phase of the grains (V_i). The internal aqueous phase is determined as the product of the water regain of the gel and the weight of dry material used. When the total elution volume (V_e) for a solute is known, the partition coefficient may be expressed as:

$$K_D = \frac{V_e - V_o}{V_i} \qquad (1)$$

A solute of low molecular weight can freely diffuse into the grains and has a K_D of about 1. However, since part of the inner volume (V_i) is bound to the polysaccharide matrix as water of hydration, thus restricting the size of the interstitial system, small solute particles usually show a partition coefficient of about 0.8, indicating nonrestricted diffusion. Substances partially excluded from the gel grains because of molecular size have a K_D value between 0 and 1.

Studies by GELOTTE[2] and PORATH[4] have shown sorption of solutes high in aromatic amino acid, as well as basic amino acids such as histidine, when the eluant was distilled water. When the eluant contains electrolyte, this sorption phenomenon for basic amino acids disappears. These effects are ascribed to small amounts of ionized carboxylic groups in the bed material reacting with the positively charged groups of the solute[2,4]. Such materials show a K_D value of > 1.0.

The special qualities of this system make possible the rapid separation of proteins from solutes of lower molecular size, as well as from their electrolyte vehicle. At the same time this permits transfer of large molecules such as proteins from one solvent to another in a single passage through the column.

At least one further application of the unique conditions produced by gel filtration appeared possible. Implicit in the separation of large molecular weight protein from its solvent system is that the solubility of the protein in the equilibrating buffer be the same as that of the solvent used to load the column. If the proteins are less soluble in the eluant than the original solvent, the protein would be restricted in its flow down the column to the front containing the original solvent system. In a mixture of proteins of different solubilities in this regard separation would be effected. If the differential solubility is determined by an eluant of low ionic strength, in contrast to the original protein solvent of higher ionic strength, the electrolyte dilution due to diffusion into and out of the gel grains could be controlled by the addition of electrolyte to the protein mixture before loading the column.

Normal human serum provides a mixture of proteins, some of which are insoluble in media of low ionic strength (euglobulins) in contrast to those soluble in media of low ionic strength (pseudoglobulins). Certain human sera obtained from patients with diseases such as rheumatoid arthritis and macroglobulinemia are particularly rich in euglobulins, in part due to their increased macroglobulin content.

METHOD

A column, 29 cm long and 2 cm in diameter, was used for 2 ml samples of serum, and was run at room temperature.

The gel material used in the present study, Sephadex G-25, excludes materials of greater than 2000 to 3000 molecular weight from the grains, is insoluble in water and salt solutions and is stable in alkaline solutions and weak acids. The water regain is 2.7 g/g of dry weight.

Sephadex G-25, 20.7 g, was washed in distilled water and permitted to settle three times to remove fine particles. It was then poured into the column and permitted to settle by gravity. The column was equilibrated with 0.02 *M* Na phosphate buffer, pH 8, and mounted over an automatic fraction collector. The eluate for all studies was the same buffer used for initial equilibration of the column.

Determination of void volume

The void volume was determined by the application of 0.1% ferritin solution to the column and then elution with the same buffer system to be used in the protein separation. The void volume measured to the point of the first appearance of ferritin off the column was 40 ml.

Preparation of sera

Sera were from normal subjects and from patients with rheumatoid arthritis in which rheumatoid factor, a macroglobulin having the solubility characteristics of an euglobulin, could be measured by a hemagglutination system using tannic-acid treated sheep erythrocytes coated with commercial preparations of normal human γ-globulin (F-II hemagglutination test)[5].

Sera were either dialyzed against 1 *N* NaCl prior to gel filtration, or had NaCl added to produce a final ionic strength equivalent to 0.85.

The protein content of elution fractions was determined as extinction values at 280 mμ using a Beckman DU spectrophotometer. The electrolyte content of elution fractions was determined by conductivity measurements with values expressed as 10^{-4} mho. The electrophoretic pattern of the elution fractions was determined using an agar supporting medium on glass slides (pH 8, barbital buffer, 250 V, 30 min).

RESULTS

The pattern for the protein content and conductivity of the fractions obtained using normal human serum are shown in Fig. 1. Four peaks designated I, II, III and IV are recorded. Peak I emerges in a medium having the conductivity of the buffer used for equilibration of the column. Peak II emerges just ahead of the peak of conductivity

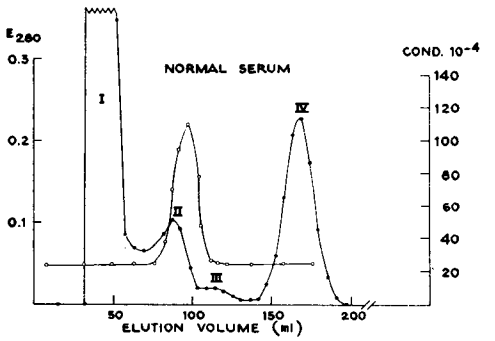

Fig. 1. Gel filtration of normal human serum. O–O–O : conductivity expressed as 10^{-4} mho; ●–●–● : optical density at 280 mμ.

but has components extending to the peak of salt content. Peaks III and IV, having K_D values of 1.4 and 2.13 respectively, are materials capable of sorption to the column matrix.

After further study by spectrophotometric analysis, peak III showed a maximum value at 255–270 mμ and peak IV at 290 mμ. This, together with failure of the material to precipitate on the addition of 10% trichloracetic acid, indicated we were dealing with predominantly nonprotein material. As shown in Fig. 2, the pattern produced by gel filtration of normal serum after dialysis of the serum against 1 *N* buffered saline, pH 8, demonstrates the dialyzable nature of the nonprotein material constituting peaks III and IV.

In order to demonstrate that the migration of the protein of peak II is determined by solubility characteristics, peak II was re-run on a Sephadex column which was equilibrated with 0.15 *M* phosphate buffer, pH 8. Elution of peak II with the same buffer revealed an elution volume similar to that of peak I (Fig. 3).

The electrophoretic pattern of the proteins found in peaks I and II of normal

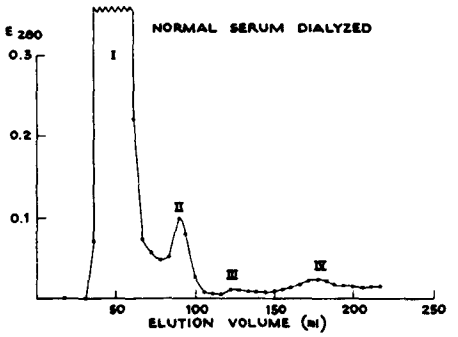

Fig. 2. Gel filtration of normal human serum after dialysis against 0.15 *M* phosphate-buffered saline, pH 8, 24 h at 4°.

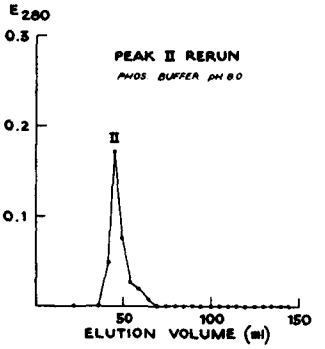

Fig. 3. Re-run of peak II obtained from Sephadex column equilibrated and eluted with 0.15 *M* phosphate-buffered saline, pH 8.

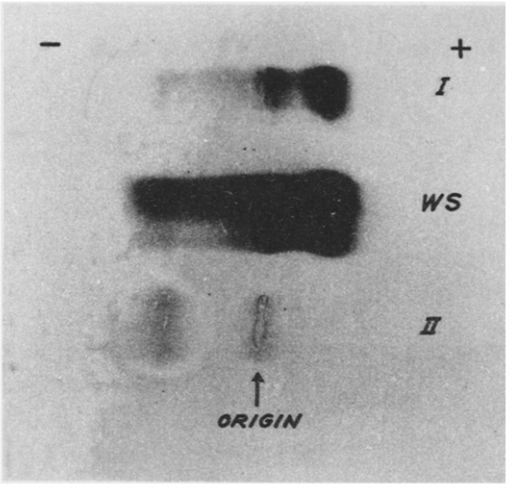

Fig. 4. Electrophoretic pattern: top, peak I; center, whole serum; bottom, peak II. γ-Globulin in peak II is in the γ_1 position.

serum are shown in Fig. 4 and show peak II to consist entirely of γ-globulin and peak I to contain the other serum proteins, together with what is presumed to be pseudoglobulin γ-globulin.

The concentration of a particular class of euglobulin γ-globulin in peak II is shown by gel filtration of sera obtained from patients having rheumatoid arthritis. The rheumatoid factor content of the elution fractions expressed as $\log_2$ reciprocal F-II hemagglutination titer is shown in Fig. 5, together with the elution and conductivity pattern.

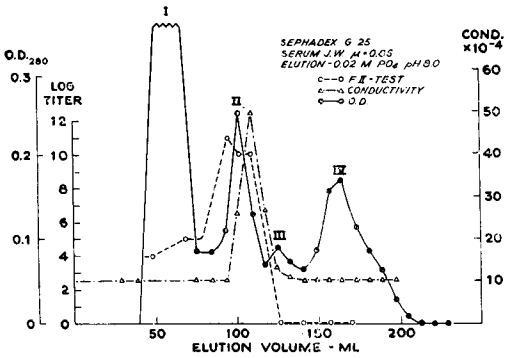

Fig. 5. Gel filtration of nondialyzed rheumatoid serum. ●–●–●: optical density; –△– – –△–: conductivity; O– – O: $\log_2$ hemagglutination titer. NaCl added to serum prior to addition to the column of final ionic strength of 0.85. Elution with 0.02 *M* phosphate-buffered saline, pH 8.

DISCUSSION

The initial application of the technique of gel filtration to the separation of materials of markedly different molecular size has been of outstanding value in accomplishing in a short time the effects of desalting by dialysis as well as the separation of mixtures of proteins, peptides and amino acids[4].

In the present study advantage is taken of the solubility characteristics of some members of the mixture of proteins found in human serum to effect separation of euglobulins and pseudoglobulins. It is apparent that the elution characteristics are not dependent on the sieve-like character of the dextran polymer since all the serum proteins far exceed the minimum size for diffusion of solute into the gel interstices. The two peaks obtained after passage of the salt front appear to be due to nonprotein dialyzable constituents of serum, probably in groups capable of partial sorption on the dextran. These are to be investigated further.

The designation of partition coefficient values as a reflection of size of particle becomes impossible when there is a differential solubility of solute in the equilibrating buffer and the solvent used to introduce the sample. Based on the characteristics of the column used in the present study the K_D for the protein of peak II in Fig. 1 would be 0.7, indicating unrestricted diffusion into the gel grains. This is certainly not true for the proteins contained in peak II. Since it has been established that peak II will be eluted with peak I when the equilibrating buffer has sufficient electrolyte

content to allow unrestricted motion through the external aqueous phase, it follows that a meaningful partition coefficient can be derived for such proteins only when the solvent furnishes no restriction to flow.

The concentration of rheumatoid factor activity in peak II confirms the euglobulin character of this class of γ-globulin. Further purification of rheumatoid macro-γ-globulin from peak II by anionic-exchange chromatography is described in another paper[6].

It would appear that the principle of gel filtration, combined with the effects of the differential solubility of the solute in the loading solvent, compared to the equilibrating and eluant solutions, allows separation of mixtures of proteins of similar size but differing solubility characteristics.

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SUMMARY

Gel filtration, using polymerized dextran granules in a chromatographic system, has been used to separate two classes of human serum proteins, both of which are unable to enter the gel matrix. The euglobulin proteins are restricted in their flow to a front containing the solvent used to load the serum on the column, while serum pseudoglobulins separate due to their solubility in the low ionic strength equilibrating buffer.

The concentration of one class of serologically measurable serum euglobulin (rheumatoid factor) has been demonstrated.

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