RAPID SEPARATION OF SERUM MUCOPROTEINS FROM OTHER FOLIN–CIOCALTEU-POSITIVE SUBSTANCES BY MEANS OF GEL FILTRATION

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The existing methods for isolating mucoproteins from human blood are rather time-consuming and too drastic. These mucoproteins are usually prepared by fractional salting out with ammonium sulphate according to WEIMER *et al.*[1]. For analytical purposes mucoproteins can be isolated from an acid serum filtrate by means of an acid solution of phosphotungstic acid[2]; the denatured mucoprotein must then be dissolved in sodium hydroxide. For the electrophoretic separation of blood mucoproteins into fractions MP-1, MP-2 and MP-3, the filtrate must be dialysed for a long time (especially sulphosalicylic acid is very difficult to remove), and then concentrated from a large volume by freeze-drying[3,4]. During the dialysis considerable losses of mucoproteins occur[5].

The use of gel filtration with Sephadex permits a simple and less drastic isolation of blood mucoproteins. So far Sephadex, has been used for this purpose only by KOČENT[6], who separated sulphosalicylic acid serum filtrates after lyophilisation, and observed a perfect separation from the acid.

METHODS

Preparation of serum filtrates

Human blood sera were deproteinized with perchloric acid as follows: 4 ml of serum was dissolved in 15.2 ml distilled water and 12.8 ml 0.75 *M* perchloric acid was added slowly, under continual mixing. After 10 min the mixture was filtered through Whatman No. 1 filter paper.

In a number of experiments the filtrate was dialysed in a cellophane bag in running water (electrodialysis is not recommended in this case—see ref.[5]). The dialysate was then concentrated by vacuum distillation below 45°.

In the other experiments the filtrates were concentrated by dialysing against a cellophane bag containing a 30% aqueous dextran solution, which was exchanged at 8 h intervals.

Packing of the columns

8.5 g of dry Sephadex G-25 medium, 100–250 mesh (Pharmacia, Sweden) was stirred in a dilute salt solution, the suspension was packed into a tube and the column (total volume 36.9 ml) was then washed with water. 3–15 ml of the blood filtrate (concen-

trated or non-concentrated) were then slowly pipetted onto the top of the bed. As soon as the sample had entered the column, the latter was eluted with a large quantity of distilled water. It was not necessary to regulate the flow rate, which varied in the range from 28.8 to 49.2 ml/h. Distilled water was also used for the regeneration of the column. All the curves relating to Sephadex G-25 mentioned in this paper were obtained with the same column.

A column of Sephadex G-200 (140–400 mesh) was prepared in an analogous manner. For the preparation of a column with a total volume of 47.7 ml, 1.8 g of dry Sephadex was necessary. The flow rate was very low: 3.9 ml/h.

Analysis of the eluate

The effluent was collected in 3 ml amounts, 2 ml of which was used for the determination of Folin–Ciocalteu-positive substances as follows: 2 ml of a 20% sodium carbonate solution and 0.2 ml of Folin–Ciocalteu reagent (diluted 1:3) were added, and after 30 min the solution was measured in a 1 cm cell at 610 mμ against a blank. In the remaining 1 ml of the effluent the acidity was determined by titration with 0.1 N sodium hydroxide (using phenolphthalein).

Determination of the amount of protein-bound hexoses in the filtrate

For the determination an orcinol reaction was used. The orcinol reagent was prepared according to STARY *et al.*[7] and standardized against a glucose solution; the reaction was performed according to SÖRENSEN AND HAUGAARD[8]. In the first place, the total hexose concentration of the filtrate was determined. Then the mucoproteins (or mucopeptides) were precipitated with an acid solution of phosphotungstic acid, the precipitate redissolved, and the protein-bound hexoses determined. The difference in the results of the two determinations is due to the presence of free blood sugar. As in other investigations we found that under the given conditions free glucose leaves the column almost simultaneously with fraction II (see below). Determination of bound hexoses in fraction III was therefore not necessary.

Polarographic activity of the fractions

This was measured in a cobalt (III) solution (composition: 0.001 M $Co(NH_3)_6Cl_3$, 0.1 N NH_4Cl and 1 N NH_3). 5 ml of this solution was mixed with 0.5 ml of the sample, and the height of the polarographic double-wave, beginning at —800 mV was registered (at galvanometer sensitivity 1:150).

RESULTS

Fig. 1 shows the results of gel filtration on Sephadex G-25 of deproteinized perchloric acid filtrates. The upper curve shows clearly that gel filtration separates the Folin–Ciocalteu-positive substances into three fractions, designated I, II and III; the same results are obtained with the original untreated filtrate (without concentration or dialysis). The peak of the eluted perchloric acid coincides for the most part with that of fraction II. The quality of separation of the first mucoprotein fraction from the perchloric acid wave is improved considerably by previous concentration of the filtrate, although this process takes more time. In some of the sera investigated it was found that the peak of fraction II was split into two fractions.

The three lower curves of Fig. 1 show the result of an experiment in which three separate portions of the effluent were collected, corresponding to the peaks of the above-mentioned three fractions. These portions were poured once more onto the same column of Sephadex G-25, and their identity ascertained. Fig. 1 (B–D) proves that gel filtration gives rise to three separate fractions with standard properties, their peaks always being localised in the same place on the elution curve.

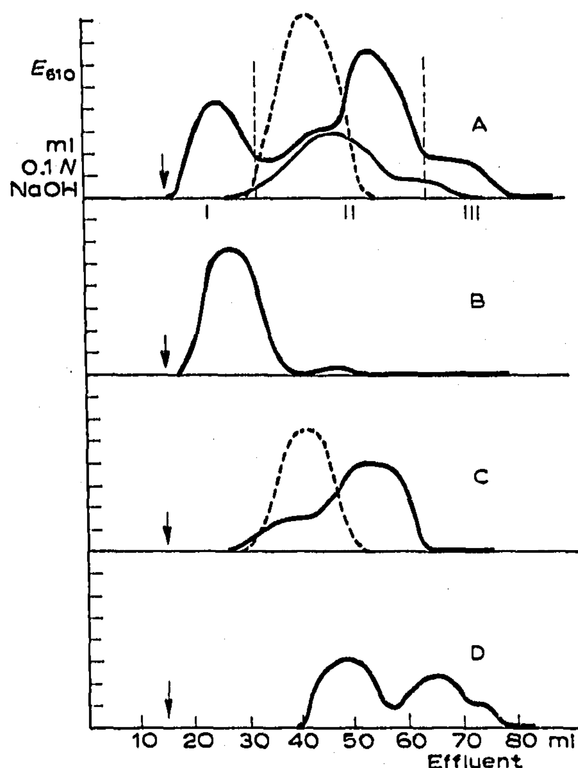

Fig. 1. Separation of the Folin–Ciocalteu-positive substances of the perchloric acid serum filtrates on Sephadex G-25 (A) and repeated gel filtration of the portions I–III (B–D). Solid line: Folin phenol reagent; dotted line: acidity; thin line: Folin colour obtained with a mixture of tyrosine, tryptophan and uric acid (see text).

Chemical and physico-chemical analysis yielded more detailed data on the composition of these fractions (Table I).

Further data on the properties of the above-mentioned three fractions were obtained from experiments in which the filtrates were concentrated and dialysed. Fig. 2 shows clearly that the values obtained with fractions II and III decrease after 24 h, and more so after 48 h, of concentration and partial dialysis. Especially curve D proves that only the macromolecular fraction I remains after total dialysis.

The conclusion can be drawn that fraction I is identical with blood mucoprotein (seromucoid, orosomucoid), whereas fraction II has the character of a mixture of glycopolypeptides and low-molecular nitrogen substances. In Fig. 1A the thin line demonstrates the result of the reaction of the Folin–Ciocalteu reagent with an artificial mixture of L-tyrosine, D,L-tryptophan and uric acid in concentrations (1.0, 1.0 and 3.5 mg/100 ml, respectively) corresponding to their ratio in normal serum; the procedure for the mixture was identical to that for serum. The results show that a considerable part of fraction II and an appreciable amount of fraction III consist

TABLE I

CHEMICAL AND PHYSICO-CHEMICAL ANALYSIS OF THE FRACTIONS

	*Hexoses**	*Coagulability with phosphotungstic acid*	*Specific polarographic activity***
Fraction I	8.23	+	0.956
Fraction II	1.49	+***	0.177
Fraction III		—	0.195

* In per cent of the total amount of the Folin–Ciocalteu-positive components.
** Ratio of the wave-height in mm and the concentration of Folin–Ciocalteu-positive substances in mg/100 ml. The activity of the total filtrate was 0.517.
*** Only after reduction of the perchloric acid concentration by filtration through a column of the anion-exchanger Anionit EGE (USSR) in OH^- form.

of these low-molecular Folin–Ciocalteu-positive components of blood serum. This fact explains the low polarographic activity of fractions II and III (see Table I). Wave III has no mucoid character; it is a mixture of peptides. These observations were supported by some further experiments. Paper electrophoresis of the isolated fraction I in barbital buffer of pH 8.6 yielded two zones (probably corresponding to mucoproteins MP-1 and MP-2 according to MEHL AND GOLDEN[3]); after dialysis, however, only one strongly marked zone remained on the electrophoregram. An

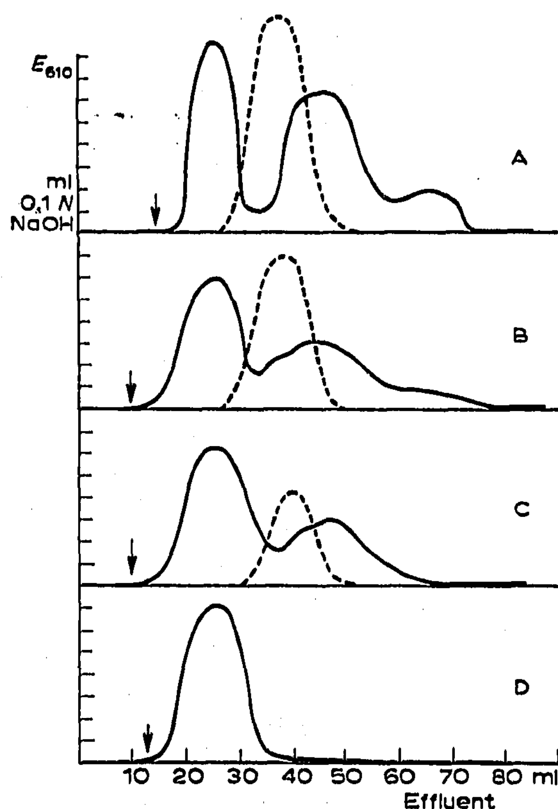

Fig. 2. Influence of concentration and dialysis on the separation of the Folin–Ciocalteu-positive substances from the perchloric acid serum filtrates on Sephadex G-25. A = result without concentration of the filtrate; B = after 24 h concentration against 30 % dextran; C = the same after 48 h; D = result after 24 h dialysis against water.

attempt was then made to find out whether fraction I could be separated in a similar fashion by means of repeated gel filtration on Sephadex G-200 (see Fig. 3). Also in this case the single mucoprotein fraction divided to give at least two distinct peaks.

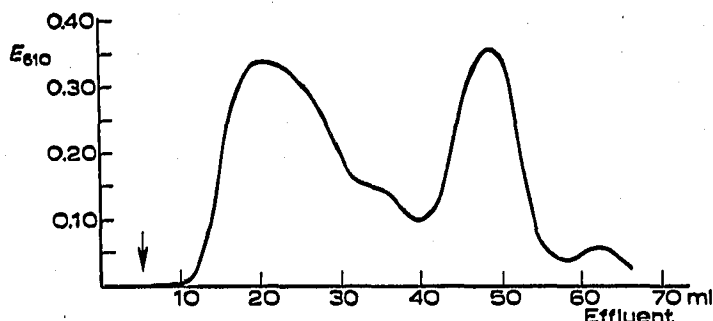

Fig. 3. Separation of the mucoprotein fraction I on a column of Sephadex G-200. 5 ml of fraction I solution was poured on to the column.

Because the second main peak obtained in this separation has the same position as in the case of the separation of the whole filtrate on Sephadex G-25, it may be assumed that the second peak could be formed from fraction I by hydrolysis or depolymerisation.

Seromucoid present in crude state in fraction I can be purified by means of some of the known preparative methods. Salting out with ammonium sulphate according to WEIMER *et al.*[1] proved to be successful.

Fractions II and III were further investigated after preliminary preparation and concentration by vacuum distillation below 45°. Desalting of the fraction II, which could not be done by dialysis, caused some difficulty. We therefore chose another procedure: the solution of fraction II was filtered through a cation-exchange column of Dowex 50 W (X2, 200–400 mesh) in H-form; perchloric acid and uncharged substances passed through, whereas amino acids and peptides were retained. These were later eluted with 6 % ammonia, which was then removed from the solution by evaporation. A small residue was finally investigated by paper chromatography. Also adsorption from the acid solution on cellulose powder (Schleicher & Schüll No. 449a) or on aluminium oxide followed by elution with ammonia was successful.

DISCUSSION

The results obtained show that deproteinized filtrates of blood serum contain not only mucoprotein, but also two further fractions of peptide nature, which are of considerable importance. This may explain the fact that results of mucoprotein estimation by the polarographic filtrate reaction (according to BRDIČKA), and results of the estimation of mucoprotein tyrosine or peptide linkage are not strictly comparable, although according to other authors[9,10], they are, as a rule, correlated.

The above-mentioned results are not identical with those obtained in the separation of sulphosalicylic acid serum filtrates on diethylaminoethylcellulose[5].

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SUMMARY

Gel filtration on Sephadex G-25 permits a rapid and relatively mild isolation of serum mucoproteins, directly from deproteinized perchloric acid filtrates of blood sera, even without previous concentration and neutralisation. During the elution mucoproteins (fraction I) separate from a fraction of glycopolypeptide character (fraction II) and from a third, easily dialysable fraction of peptides (fraction III). The peak of the deproteinizing agents and of low-molecular Folin–Ciocalteu-positive substances coincides with the peak of polypeptides (fraction II). The isolated mucoproteins of fraction I can be separated by paper electrophoresis at pH 8.6 to give two subfractions; on Sephadex G-200 at least two subfractions are also detectable. By the method described it is possible to perform a fractional separation of mucoproteins with a small amount of serum.

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