# Fractionation of Human Plasma Macroglobulins by Gel Filtration on Pearl-Condensed Agar.* (29058)

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During recent years gel filtration has been widely used for separation of substances according to their molecular size. There now exists a range of cross-linked dextran gels (Sephadex)† that permit separation of substances with molecular weights below 400-500,000. Other gel materials effective in the same range as Sephadex, such as polyacrylamide, polyvinylethylcarbitol and polyvinylpyrrolidone have also been used as reviewed by Porath(1). Granulated agar and agarose gels have been used for separation of macromolecules such as virus and cell particles(2-6). These granulated gels, however, give low flow rates at low agar concentrations and high mesh values. This difficulty has been overcome by introduction of pearl-condensed agar gels(7,8) which allow higher flow rates even with gel particles of smaller size than those earlier used.

The fractionation of human serum proteins into 3 main fractions on Sephadex G-200 has been reported(9). This paper reports the separation of human plasma macroglobulins on a column of 3.5% pearl-condensed agar gel prepared according to Bengtsson and Philipson(7).

*Materials and methods. Serum.* Human plasma and serum samples were either used fresh or stored at −20°C.

*Gel filtration* on cross-linked dextran gel (Sephadex G-200) 200-270 mesh (U. S. sieve series) was performed at +6°C-8°C on plexiglass columns fitted with plexiglass plungers and equipped with cooling jackets. The elution was performed at a rate of 1-1.5 ml/cm²/hour in 0.1 M sodium phosphate buffer with 0.4 M NaCl and 0.02% sodium azide at pH 7.4. Further details of the technique have been reported(10).

The pearl-condensed agar gel was prepared by spraying a hot solution of 3.5% (w/w) Noble agar (Difco) into ice cold ether(7). The wet gel, collected in water, was sieved and the fraction with 100-140 mesh (U. S. sieve series) equilibrated with the buffer used in the experiments, 0.1 M sodium phosphate buffer 7.4 with 0.4 M NaCl and 0.02% sodium azide. The gel was deaerated and poured in columns with an inner diameter of 2.6 cm fitted with an adjustable plunger. Plexiglass equipment was used to avoid absorption of lipoproteins. A hydrostatic pressure of 200-300 cm $H_2O$ was then applied, a slow flow started and the gel left to sediment overnight. Columns prepared in this way had high flow rates, 5-6 ml/cm²/hour with a hydrostatic pressure of 39.5 cm which was the usual length of the column except for recycling experiments(11) when the column measured 38.1 cm. To achieve optimal separation conditions(12) the flow rate was lowered to 1.6-2.5 ml/cm²/hour by means of a peristaltic pump or hydrostatic pressure. The samples in 2-5 ml volume were applied as described elsewhere(10, 11). All experiments were carried out at room temperature.

*Protein measurements.* The protein concentration of the eluate was continuously registered as transmission at 254 mμ by means of a Uvicord absorptiometer with a 3 mm cell (AB LKB-produkter, Stockholm, Sweden) and was plotted in the diagrams as optical density on a linear scale.

*Concentration of fractions* was carried out by ultrafiltration in collodium tubes (Membranfilter AG, Göttingen, Germany).

*Immunoelectrophoresis* was performed by the Scheidegger micromethod with the following modifications. Bacto agar (Difco) in 1.5% solution in barbital-HCl buffer, pH 8.2,

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† Manufactured by AB Pharmacia, Uppsala, Sweden.

ionic strength 0.05, was poured in 1 mm thick layers on 26 × 76 mm glass slides. The sample in 0.5-3 μl was separated by electrophoresis for 60-90 minutes at a potential gradient of 6 V/cm and the pattern developed with about 75 μl of antiserum.

*Double diffusion in agar gel* was performed on glass slides covered with a 1 mm thick layer of the agar solution described above. A 1 or 2 mm × 65 mm trough was cut in the middle for the antiserum and cylindrical wells (2 or 4 mm in diameter) were arranged in rows at 3 or 4 mm distance on either side for the antigen. In other experiments 6 cylindrical wells (diameter 4 mm) were arranged around a central hole at 6 mm distance.

These techniques were used for qualitative as well as semiquantitative analyses of antigens. Each or every second fraction of the eluate was tested before or after concentration. Immunoelectrophoresis and diffusion in gel experiments were recorded at 6-12 hour intervals for 48-72 hours. The slides were then washed in saline, distilled water and stained by amido black.

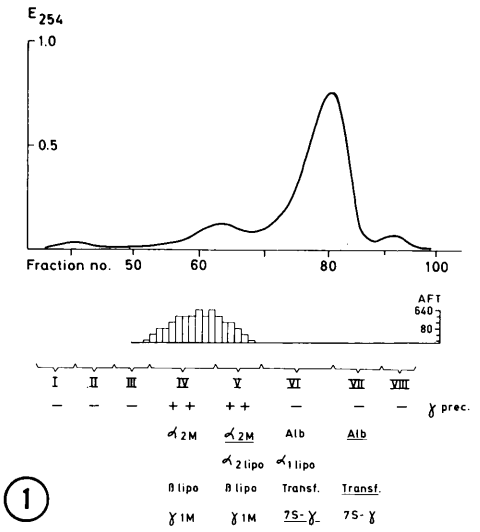
Rabbit anti-human plasma protein sera and specific rabbit antisera against human $\gamma_{1M}$, 7S γ- and $\alpha_{2M}$-globulins and against $\alpha_2$-, β- and $\alpha_1$-lipoproteins as well as fibrinogen were used. The antisera used for analysis of the low-density $\alpha_2$- and β-lipoproteins were found to cross-react. The specific antisera were absorbed and the specificity was tested by immunoelectrophoresis and agar diffusion experiments against whole human serum.

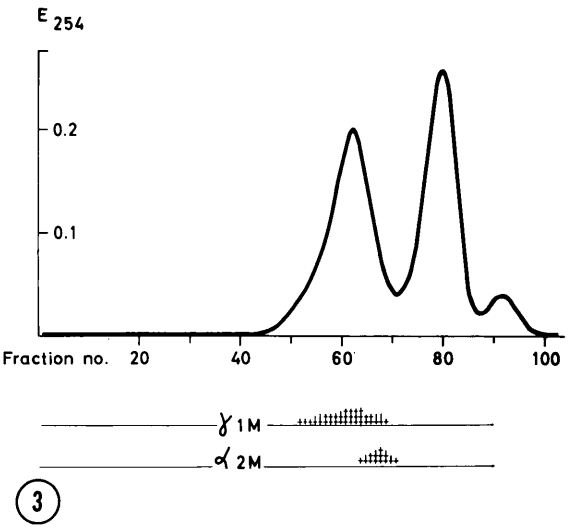
*Assay of rheumatoid factor.* This was determined by the sensitized sheep cell agglutination test (SCAT), the acryl fixation test (AFT) or by the γ-globulin precipitation test (γ-prec.) according to standard methods described in detail elsewhere(13).

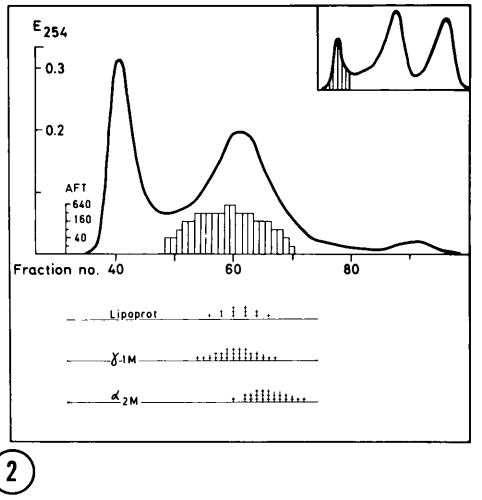
*Paper electrophoresis.* This was carried out using filter paper Whatman No. 1 and barbital buffer pH 8.6 ionic strength 0.1. The electrophoresis was run for 15 hours at a potential gradient of 4 V/cm after which the proteins were stained by amido black. The lipoproteins were stained according to Swahn (14).

*Results.* The separating capacity of the 3.5% agar gel was first studied by filtering whole human serum. In the elution diagram (Fig. 1) 4 peaks were found with ratios of elution volume (Ve) / total gel bed volume (Vt) of 0.43, 0.66, 0.83 and 0.96 respectively. At the flow rates used these ratios were constant in repeated experiments. The eluate was divided into 8 fractions as shown in Fig. 1, concentrated and analyzed for different types of proteins by immunoelectrophoresis, agar diffusion technique and paper electrophoresis. No proteins were found in the fractions corresponding to the first peak. These fractions, however, were turbid and on examination by dark field microscopy particles of a size ranging from 0.4-2 μ were observed. The second peak was found to contain β-lipoproteins and $\gamma_{1M}$-globulins together with small amounts of $\alpha_{2M}$-globulins in the front part (fraction IV) and $\alpha_2$- and β-lipoproteins together with $\gamma_{1M}$- and $\alpha_{2M}$-globulins in the rear part (fraction V). The majority of serum protein was recovered in the third peak with maxima for the 7S γ-globulins in fraction VI and for the albumin and transferrin in fraction VII. No proteins were found in the fourth peak (Ve/Vt = 0.96) which had an elution volume close to the total volume of the gel bed and could thus be expected to contain components of small molecular size. The serum used in this experiment contained large amounts of rheumatoid factor (RF) activity; this activity was found to elute in the region of $\gamma_{1M}$-globulins.

To study the elution properties of the large molecular size proteins more closely, the same serum was fractionated on a Sephadex G-200 column. The first peak which contained the macroglobulins was collected, concentrated and subjected to gel filtration on 3.5% agar gel. As seen in Fig. 2 the first two peaks were this time more distinct and the first one much higher than in the first experiment whereas the third was almost missing. The first peak was very turbid and contained large amounts of light-scattering particles of the same size range as in the first experiment. The peaks were analyzed for proteins and lipoproteins as described above. The same results as in the first experiment were found for the lipoproteins, $\gamma_{1M}$- and $\alpha_{2M}$-globulins and RF activity but trace amounts of 7S



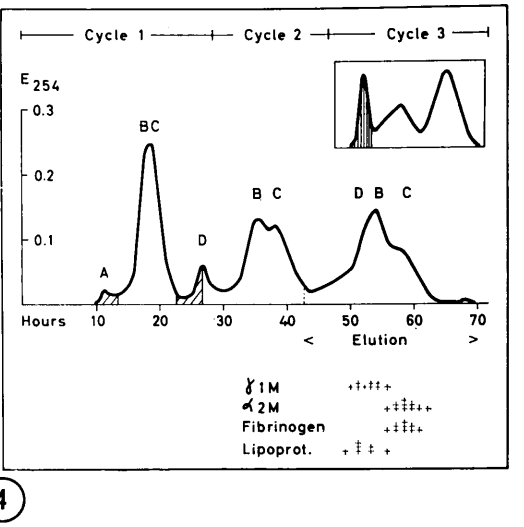




FIG. 1. Gel filtration of 2.1 ml of human serum S.K. on 3.5% pearl-condensed agar. The fraction volume was 2.2 ml. Total protein was determined as optical density at 254 mμ. Rheumatoid factor activity was determined as AFT in the eluted fractions and by γ-precipitation test in the concentrated fractions I-VIII. The maxima of different proteins in the concentrates analyzed by immuno- and paper-electrophoresis and agar diffusion technique are indicated below; underlined letters mean higher concentrations.

FIG. 2. Gel filtration on 3.5% pearl-condensed agar of the macroglobulin fraction obtained by gel filtration of 10 ml of serum S.K. on a Sephadex G-200 column (4.2 × 83.4 cm) (inserted diagram). The fraction volume was 2.2 ml. Total protein in the eluate was determined as optical density at 254 mμ and the relative concentrations of $\alpha_{2M}$-, $\gamma_{1M}$-globulins and lipoproteins in the collected fractions by agar diffusion technique. RF activity was measured as AFT.

FIG. 3. Gel filtration of 1.7 ml of a 1:2 dilution of a macroglobulinemic serum A.L. on 3.5% pearl-condensed agar. The fraction volume was 2.2 ml. Protein, $\alpha_{2M}$- and $\gamma_{1M}$-globulins were determined as in Fig. 2.

FIG. 4. Recycling gel filtration on 3.5% pearl-condensed agar of the large molecular size fraction obtained by gel filtration of 7 ml human plasma on a Sephadex G-200 column (4.0 × 37.3 cm) (inserted diagram). The flow rate was 8 ml/hr. Obliquely shaded areas represent parts of the eluate which were not recycled. Peak D which only contained low molecular substances was not completely eluted in cycle 1; the recycled part was eluted in cycle 3 immediately before peak B. Total protein, $\alpha_{2M}$- and $\gamma_{1M}$-globulins and lipoproteins were analyzed as in Fig. 2. Fibrinogen was determined by agar diffusion technique.

γ-globulins could be demonstrated in the first peak.

The elution properties of α and γ macroglobulins were further studied by agar gel filtration of a serum sample from a patient with macroglobulinemia. As shown in Fig 3 no peak corresponding to the first peak found in the previous experiments could be demonstrated in this case, but the second (Ve/Vt = 0.66) was much increased and contained large amounts of $\gamma_{1M}$-globulins partly separated from $\alpha_{2M}$-globulin. It may be noted that this peak and the distribution of $\gamma_{1M}$-globulins are both asymmetric which may be due to aggregates of $\gamma_{1M}$-globulins eluting in front of the main portion. Analytical ultracentrifugation of the serum revealed a high 19S component and a smaller one of 26-30S. No lipoproteins could be found when unconcentrated fractions nos. 36-74 were analyzed by agar diffusion technique.

To achieve a better separation of the macroglobulins the first peak from a Sephadex G-200 filtration of normal human plasma was concentrated and subjected to 3 cycles of recycling filtration according to the technique of Porath and Bennich(11) on the agar column. As demonstrated in Fig. 4, where A, B + C and D correspond to the first, second and fourth peaks respectively in Fig. 1, a progressive separation of the second peak into 2 peaks B and C occurred even if this separation was not complete after 3 cycles. It is also shown that peak B contained most of the lipoproteins and $\gamma_{1M}$-globulins and peak C most of the $\alpha_{2M}$-globulin and the fibrinogen. A similar experiment was also carried out with a serum with high RF activity. Fig. 5 shows that the RF activity measured as sheep cell agglutinating activity eluted in the same region as $\gamma_{1M}$-globulins and that a tendency towards 2 peaks was obtained. The separation of RF activity on gels has been reported elsewhere(13).

The first turbid peak after agar gel filtration contains large amounts of light-scattering spherical particles measuring 0.4-2 μ. When analyzed for proteins by immunoelectrophoresis and agar diffusion technique this fraction contained no proteins except for trace amounts of 7S γ-globulins in one of 4 experiments. On paper electrophorograms stained for proteins and lipids, non-migrating lipid-staining material was found, but no protein. This material was found to float when analyzed in the ultracentrifuge at a density of 1.06 g/ml. It was also found that serum samples depleted of lipoproteins(15) lacked this peak.

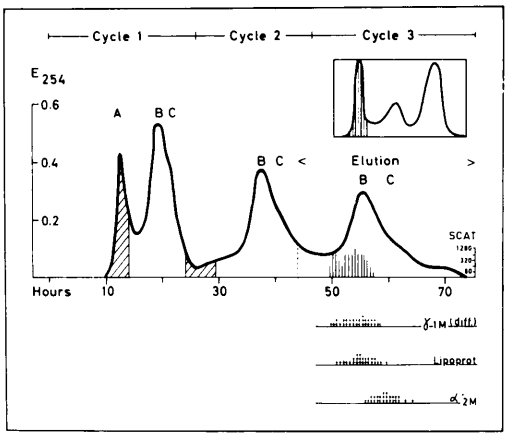



FIG. 5. Recycling gel filtration on 3.5% pearl-condensed agar of the macroglobulin fraction obtained by gel filtration on a column Sephadex G-200 (4.2 × 71.7 cm) of 12 ml human serum C.W. The flow rate was 8 ml/hr. Obliquely shaded areas represent parts of the eluate which were not recycled. Rheumatoid factor was measured as SCAT. Total protein in the eluate, $\alpha_{2M}$- and $\gamma_{1M}$-globulins and lipoproteins were determined as in Fig. 2.

The sera fractionated in experiments reported in Fig. 1-3 and 5 were stored at −20°C and thawed only once before use. In the experiment reported in Fig. 4 and in other control experiments fresh plasma or serum samples were separated. Chylomicrons, $\beta_1$, $\alpha_2$ and $\alpha_1$ lipoproteins were found to have the same relative elution volumes as when samples frozen and thawed once were fractionated.

To detect possible adsorption effects the lipoproteins of 3 sera were pre-stained by Sudan Black or Nile Blue. No visible color remained in the gel after filtration. It may also be noted that one and the same gel bed could be used for several months in more than 20 experiments without obvious changes in flow property or separation capacity.

*Discussion.* The agar used in this study contains about 0.33% (w/w) sulphur mainly as sulphate groups on the agaropectin(16).

To minimize adsorption effects as well as protein-protein interactions a buffer with high ionic strength was used(1,12). No adsorption of plasma proteins to the agar gel was observed.

The results obtained have demonstrated the separating potentiality of the method used. The lipoproteins were separated in 3-4 components. The first peak with Ve/Vt = 0.43 consisted of large size lipid components of low density (less than 1.05 g/ml) which did not migrate in paper electrophoresis. This material is regarded as chylomicrons. In the second peak (Ve/Vt = 0.66) $\beta$ and $\alpha_2$ lipoproteins were partially separated. The first part of the third peak (Ve/Vt = 0.83) was also found to contain $\alpha_1$ lipoprotein. These findings regarding chylomicrons and lipoproteins are in accordance with other reports concerning their size and behavior (17). Some of the sera separated had been frozen before use. This may have inferred degradation of the low density lipoproteins (18). In experiments with fresh samples, however, the same principal elution pattern of the lipoproteins was obtained.

In one out of four experiments trace amounts of 7S $\gamma$-globulins were found in the first peak. It may be noted in this connection that 7S $\gamma$-globulins can form complexes with lipoproteins(19).

In recycling experiments it was found that $\gamma_{1M}$-globulins can be partially separated from $\alpha_{2M}$-globulin and fibrinogen. The elution curve of $\gamma_{1M}$-globulins is often found to be asymmetric, *i.e.*, some material elutes before the main part. This became conspicuous when sera containing high amounts of macroglobulin or rheumatoid factor were fractionated. These results have been interpreted as a separation of $\gamma_{1M}$-globulins of various molecular sizes, *e.g.* 19S $\gamma_{1M}$-globulins and aggregates with higher sedimentation rates found in sera of patients with macroglobulinemia and in patients with rheumatoid activity(20).

The separation of these macromolecules and of other plasma proteins on 3.5% agar are in accordance with the results of earlier investigators(2-6) regarding the separation properties of agar columns.

The method used here is simple and permits fractionation in both analytical and preparative scale. Other concentrations of agar may be used in order to enhance the separation of selected substances.

*Summary.* Columns of 3.5% pearl-condensed agar gel have been used for separation of plasma macroglobulins. The lipoproteins were resolved into 3-4 fractions and $\gamma_{1M}$-globulins was partially separated from the $\alpha_{2M}$-globulin and fibrinogen.

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