

Purification of Rat Gamma Globulin and the Production of a Specific Antirat Gamma Globulin Serum (34403)

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(Introduced by H. Baer)

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Several methods for the isolation of gamma globulin from human serum have been published (1-5). The product obtained from these procedures demonstrates a high degree of homogeneity. However, when the separation procedure of Baumstark *et al.* (1) was applied to rat whole serum the same degree of homogeneity was not obtained. The present paper presents a laboratory procedure for obtaining an immunoelectrophoretically pure rat 7S gamma globulin and the preparation of a monospecific antiserum to rat 7S globulin. The procedure presented here requires modifications from that used for the separation of human 7S globulin by the DEAE-Sephadex A-50 batch method (1).

I. Materials and Methods. Serum. The serum was obtained from fasting adult Sprague-Dawley rats. The rats were anesthetized with halothane (Fluothane)-ether mixture (125 ml of Fluothane + 59 ml of ether), and bled by cardiac puncture. Serum was separated by centrifugation, and stored at -20° until ready to be used.

Separation procedure. The DEAE-Sephadex A-50 gel (3.5 ± 0.5 meq/g, 40-120 μ m) was conditioned as previously reported by Baumstark *et al.* (1) with the following slight modification. The optimum buffer pH for separating 7S globulin from rat serum was determined empirically. Phosphate buffers, 0.01 M, with pH ranges of 5.0-7.5 in increments of 0.5 pH units were tried. From this preliminary assay, a buffer pH of 7.5 was found effective for the elution of a fairly clean, though not completely pure rat gamma globulin when characterized by immunoelectrophoresis.

Fifty ml of normal rat serum (undialyzed)

was added to a beaker containing 10 g of DEAE-Sephadex A-50 equilibrated in 0.01 M phosphate buffer, pH 7.5. The mixture of serum and gel was allowed to adsorb in a refrigerated room at 3° for 1.5 hr while constantly being stirred with a magnetic bar. Following the 1.5-hr adsorption period, the serum-gel mixture was transferred to two 40-ml cellulose nitrate tubes and centrifuged in a refrigerated centrifuge at 4080g for 15 min. The supernatant was decanted into a beaker containing another 10-g portion of moist gel, and again allowed to stand in the cold room while being stirred for 2.5 hr. After this (adsorption number 2) the serum-gel mixture was centrifuged once again as before, and the supernatant was decanted into a flask. If necessary, it can be stored under refrigeration at any point during the adsorption procedure. The procedure was continued with a third adsorption time of 1.5 hr, a fourth of 2.5 hr and a fifth of 1.5 hr. Even after five adsorptions the sample was not immunoelectrophoretically pure gamma globulin. Further isolation involved the use of a DEAE-Sephadex A-50 freely packed column (20 cm \times 11 mm) using a phosphate buffer, pH 7.5, as eluant.

Before the adsorbed fraction was added to the top of the column, a small filter disc was carefully placed on top of the gel. To the properly prepared column 2.0 ml of the five-times adsorbed rat serum fraction were added. After the fraction had passed through the filter disc, buffer was added slowly with a pipette until it reached a point of about 5 cm above the top of the gel. The eluted fractions were collected in 2-ml portions at a flow rate of 1 ml/min. The content of the tubes

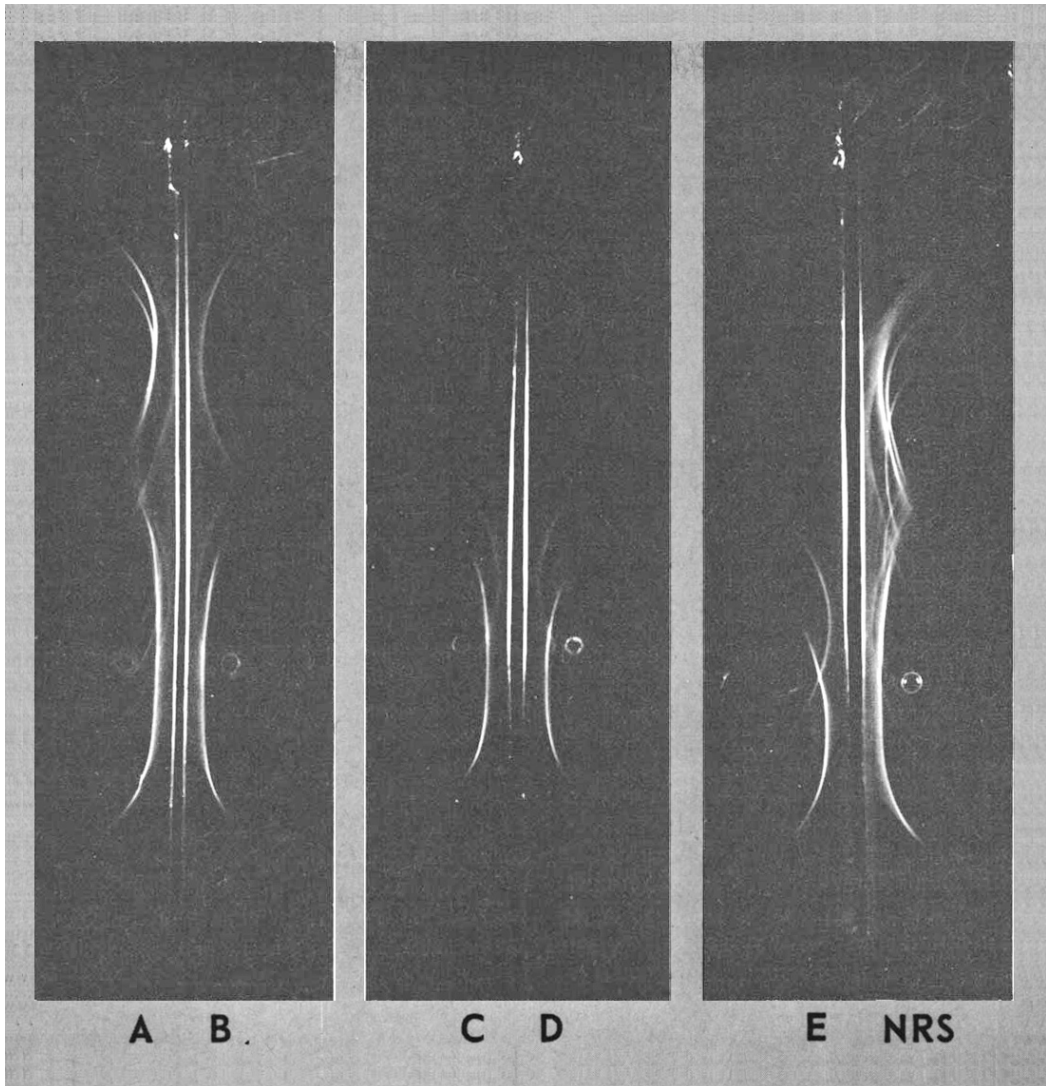


FIG. 1. Immunoelectrophoretic patterns of adsorbed rat serum against rabbit antirat serum: successive adsorptions (A-E) on 10-g aliquots of DEAE-Sephadex A-50: (A) 1st adsorption; (B) 2nd adsorption; (C) 3rd adsorption; (D) 4th adsorption; (E) 5th adsorption; (NRS) normal rat serum.

corresponding to each peak was pooled. The pooled material contained in dialysis tubing was concentrated 8-fold in polyethylene glycol 20,000,¹ a reduction to the original volume of serum. Protein determinations were made by the method of Lowry *et al.* (6).

Characterization of gamma globulin. Immunoelectrophoresis for the characterization of the various preparations was conducted in

¹ Fisher Scientific Company.

0.75% Agarose media made in 0.05 M Tris buffer pH 8.6. Fractions which had been pooled and reduced were electrophoresed 1 hr at 300 V using the LKB apparatus (7). Similarly pooled and reduced fractions with protein concentrations of 2.2 mg/ml were subjected to ultracentrifugal analysis. The ultracentrifugal analyses were carried out in a Spinco analytical ultracentrifuge employing a standard 12-mm cell.

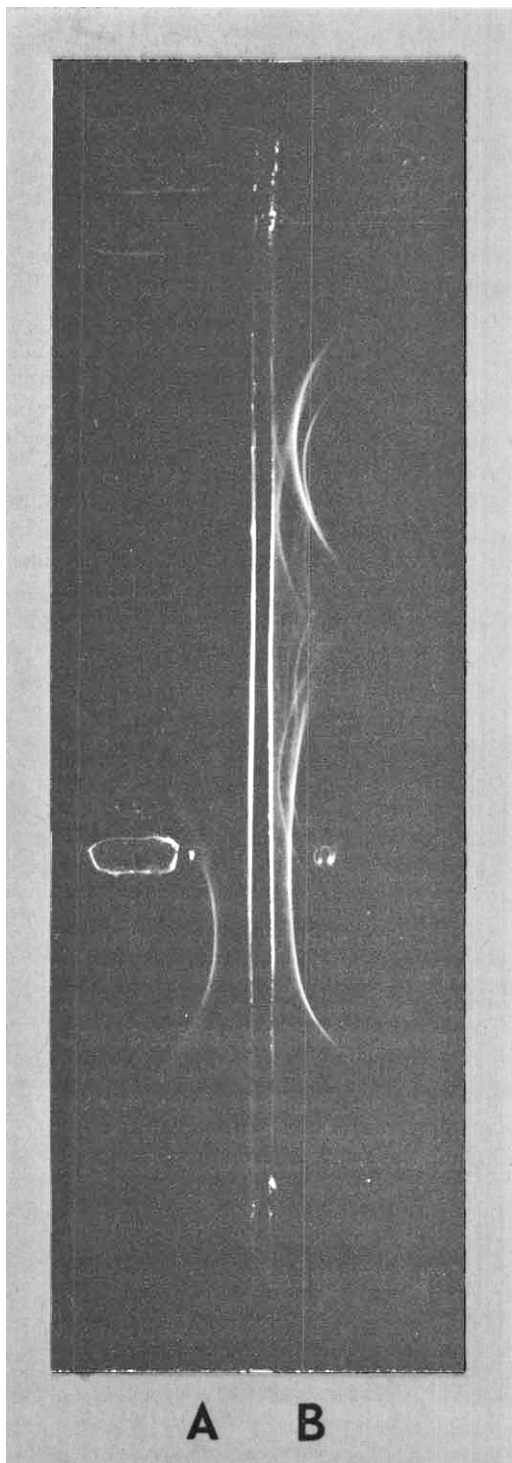


FIG. 2. Comparative immunoelectrophoretic pattern of DEAE-Sephadex column fractionation of a $5\times$

adsorbed rat serum and normal rat serum against rabbit antirat serum: (A) column fractionated serum; (B) normal rat serum.

Radioimmuno-electrophoresis technique. (8) After the immunoelectrophoretic pattern was developed and the slides were washed in saline 48 hours, ^{59}Fe as FeCl_3 , diluted to $10\ \mu\text{Ci/ml}$ was added to the trough and allowed to stand in a covered tray 24 hr. The slides were washed in saline 48 hr and then dried. The dried slides were placed in contact with X-ray film for 14 days, after which the film was processed.

Preparation of specific antiserum. Antiserum was prepared in adult male rabbits, New Zealand strain, by two injections with immunoelectrophoretically pure gamma globulin. The first injection containing approximately 2 mg of immunoelectrophoretically pure rat 7S globulin incorporated in complete Freund's adjuvant (9) was made subcutaneously in the nuchal area and all foot pads. The second injection of antigen (1.5 mg) incorporated in incomplete Freund's adjuvant was administered in the nuchal area only. The total amount of antigen received by each rabbit was 3.5 mg.

II. Results. The pH of the serum after the removal of the clot was 8.1. When adding 50 ml of normal rat serum to 10 g of moist gel that had been previously conditioned in phosphate buffer, pH 7.5, the serum-gel mixture had a pH value of 8.0. The pH value of the serum-gel mixture remained at 8.0 during the five adsorptions while the final pH value of the extracted material, separated from the gel by centrifugation, was 7.6.

Figure 1 shows the immunoelectrophoretic pattern of the supernatant (consisting of gamma globulin) following each DEAE-Sephadex A-50 adsorption. The samples were tested against rabbit antirat serum. Note the gradual disappearance of various precipitin bands from one adsorption to the other, with the fifth and final adsorption having essentially two bands, one with a faster mobility than the other. It is suggested that the faster moving component is one of the beta globulins or transferrin.

Two ml of the supernatant consisting of the

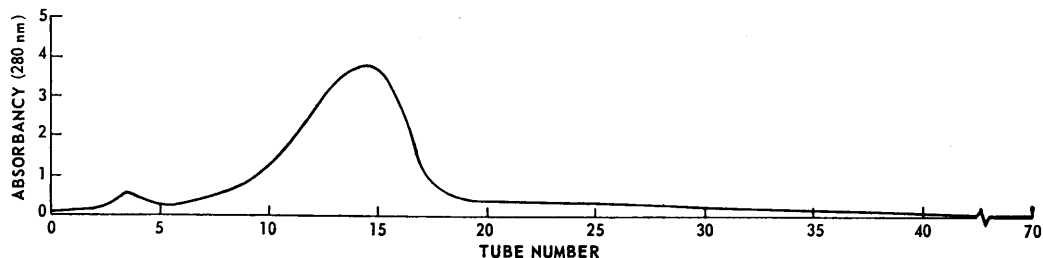


FIG. 3. Elution from DEAE-Sephadex A-50 column of a $5\times$ adsorbed rat serum in $0.01 M$ phosphate buffer, pH 7.5.

two components were put on a freely packed DEAE-Sephadex A-50 column. The eluted fractions were collected and pooled with their respective peaks. Figure 2A illustrates the immunoelectrophoretic pattern of the pooled fraction against rabbit antirat whole serum.

A diagrammatic tracing of the eluted fractions from the column is demonstrated in Fig. 3. The first peak in Fig. 3 represents a component which does not react with antirat whole serum when subjected to immunoelectrophoresis. The second peak and all of the trailing material contained rat gamma globulin, and is illustrated immunoelectrophoretically in Fig. 2. The present results indicate that the material is specific for rat gamma globulin only, and shows a high degree of homogeneity.

Examination of the purified gamma globulin at a concentration of 2.2 mg/ml in the analytical ultracentrifuge revealed a single homogeneous peak. Figure 4 shows the sedimentation pattern. The sedimentation coefficient computed from this sample was 6.0 at 20° .

To obtain a monospecific antiserum to rat gamma globulin, adult rabbits were injected initially with immunoelectrophoretically pure rat $7S$ globulin. Four weeks after the initial injection, the rabbits were given a second injection of antigen. Figure 5 shows the results obtained 7 weeks after the initial injection.

Attempts were made to characterize the faster moving component in the five-times absorbed rat serum by employing the radioimmunoelectrophoresis technique (8).

Transferrin is an iron-containing constituent of the serum and will incorporate ^{59}Fe . That

the faster moving component incorporates ^{59}Fe strongly suggests that it is transferrin. Figure 6 shows the radioautograph along with the immunoelectrophoretic pattern.

III. Discussion. The present results show that rat gamma globulin can be separated by an absorption method on DEAE-Sephadex A-50, pH 7.5, followed by elution from a freely packed column containing DEAE-Sephadex A-50, pH 7.5. When the eluted material trailing the first peak is pooled and concen-

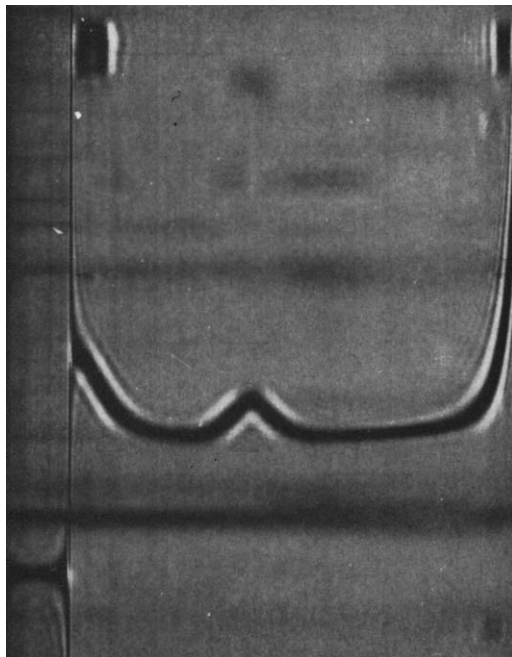


FIG. 4. Ultracentrifugal pattern of adsorbed and column fractionated rat serum: protein concentration, 2.2 mg/ml in $0.01 M$ phosphate buffer, pH 7.5; speed: $59,780 \text{ rpm}$ at 20° ; photograph taken 61 min after indicated rotor speed; S_{20} value = 6.0 .

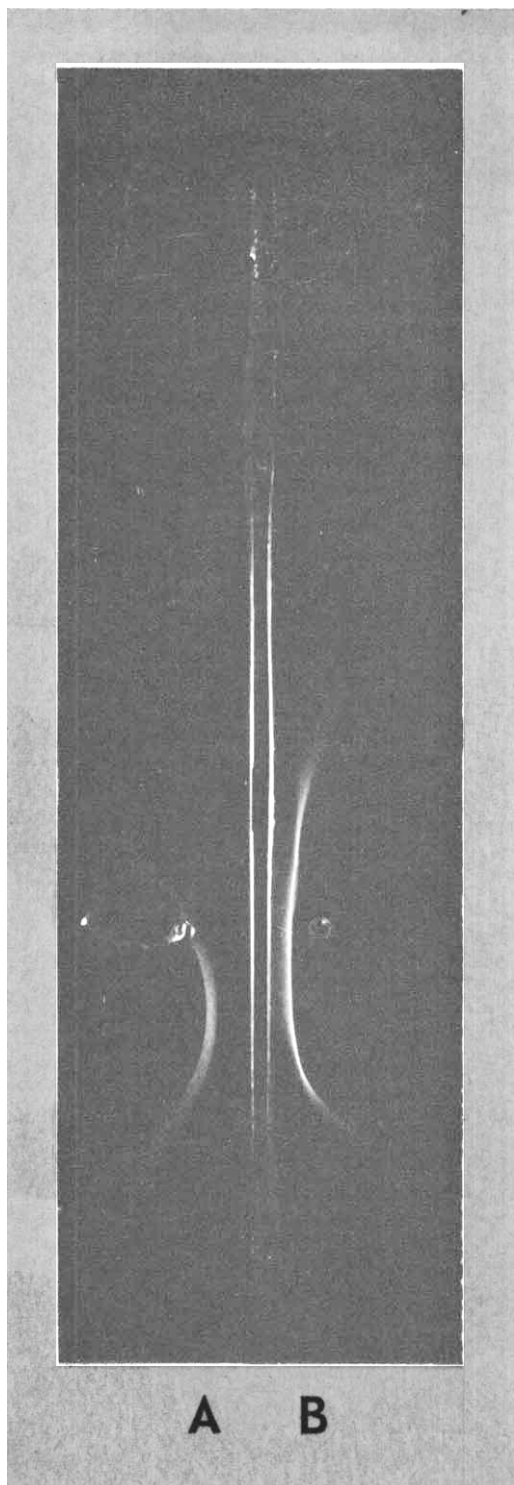


FIG. 5. Immunoelectrophoretic pattern of rat 7S globulin and normal rat serum against rabbit antirat

7S globulin: (A) 7S preparation (5 \times adsorbed column fraction); (B) normal rat serum.

trated in polyethylene glycol and characterized by immunoelectrophoresis against normal rat serum, a single precipitin band occurs only in the gamma globulin region. This indicates that the preparation is monospecific for gamma globulin. That the eluted material is a single component is confirmed when the resulting antiserum gives a single band against normal rat serum (Fig. 5).

Attempts were made to separate the 7S globulin from rat serum by using the batch two-times adsorption method as used in human 7S separation [Baumstark *et al.* (1)]. However, successful separation of 7S gamma globulin free of transferrin components could

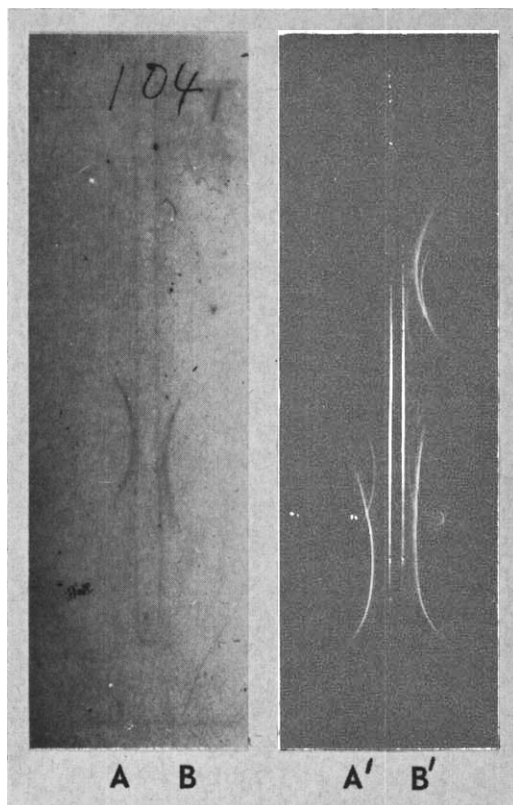


FIG. 6. Radioimmuno-electrophoretic pattern of the 5 \times adsorbed fraction against rabbit antirat serum showing the presence of transferrin: (left) radioautograph; (right) immunoelectrophoretic pattern; (A), (A') 5 \times adsorbed rat serum; (B), (B') normal rat serum.

not be obtained despite modifications in: (i) buffer pH values; (ii) adsorption time; and (iii) gel to serum ratios. On most separations the fractions were composed of other serum components in addition to transferrin.

The transferrin was finally separated from the 7S globulin when a portion of the five-times adsorbed fraction was put on a DEAE-Sephadex A-50 freely packed column. During the 140-min elution time the transferrin component was apparently not eluted from the column, because it could not be detected immunoelectrophoretically, nor was it recognized in the antiserum.

From these methods, it is possible to obtain approximately 5 mg of immunoelectrophoretically pure rat gamma globulin from 2 ml of the five-times adsorbed rat serum fraction. Due to the number of adsorptions and passages through the column it is evident that a great deal of loss occurs. Baumstark *et al.* (1) reported that 600 mg of immunoelectrophoretically pure gamma globulin could be isolated within 3 hr from 50 ml of undialyzed human serum. The difficulty in isolating greater yields from rat serum may be a function of species differences.

IV. Conclusions. A method for the isolation of rat 7S gamma globulin based on multiple adsorptions with DEAE-Sephadex A-50 was presented. The separation of rat 7S globulin requires a procedure modified from the one used for humans in terms of the buffer pH, and the number of adsorptions. Ultracentrifugal

analysis indicates the resulting fraction is a single component. In addition, immunoelectrophoresis of the fraction when tested against antirat serum confirms the homogeneity of this 7S fraction. A monospecific antiserum to rat 7S gamma globulin was produced. This antiserum is free of reaction with all other serum components when subjected to immunoelectrophoresis against normal rat serum.

The authors wish to express their appreciation to J. S. Finlayson and A. M. Young, Laboratory of Blood and Blood Products, National Institutes of Health, Bethesda, Maryland, for performing the ultracentrifugal analysis.

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Received July 7, 1969. P.S.E.B.M., 1970, Vol. 133.