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NOTE

**PURIFICATION OF
RAT GAMMA GLOBULIN AND
THE PRODUCTION OF A SPECIFIC
ANTI-RAT GAMMA GLOBULIN SERUM**

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March 1969

PURIFICATION OF RAT GAMMA GLOBULIN AND THE PRODUCTION OF A
SPECIFIC ANTI-RAT GAMMA GLOBULIN SERUM

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FOREWORD

(Nontechnical summary)

Postirradiation changes in serum proteins have been noted in several species. Accurate identification of changes in the serum proteins requires a precise knowledge of the normal serum protein composition. In the past, serum proteins have been separated and named mainly on the basis of electrophoretic mobility. Other methods for characterizing serum components have been physical-chemical procedures based on the size and shape of the individual protein components and immunological techniques based on antigen-antibody reactions.

Ideally, the identification of serum components depends on the individual isolation of these components. Electrophoretic and chromatographic as well as antigen-antibody techniques can be utilized for the isolation of the several serum proteins. Chromatographic methods (for separation) and electrophoretic techniques (for identification) have been utilized in the present study to separate and characterize 7S gamma globulin. Rat serum repeatedly adsorbed with DEAE-Sephadex (a cross-linked dextran polymer) finally yielded a single protein fraction. This fraction when subjected to electrophoresis, immunoelectrophoresis as well as ultracentrifugal analysis satisfies the criteria for 7S gamma globulin (immune globulin).

Single component or homogeneous rat 7S globulin, when used to immunize rabbits, should produce an antiserum specific only for 7S globulin. Antiserum thus produced in rabbits, when tested against whole rat serum, produced reactivity against a single component, 7S gamma globulin.

ABSTRACT

A method for the isolation of rat 7S gamma globulin based on multiple adsorptions with DEAE-Sephadex A-50 has been 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 anti-rat sera confirms the homogeneity of this 7S fraction. A monospecific antiserum to rat 7S gamma globulin has been produced. This antiserum is free of reaction with all other serum components when subjected to immunoelectrophoresis against normal rat serum.

I. INTRODUCTION

Several methods have been published for the purification of gamma globulin from human serum.^{1-3, 8, 9} However, these procedures were not satisfactory for the isolation of 7S gamma globulin from whole rat serum. No method for the isolation of rat 7S gamma globulin has been published previously.

The purpose of this paper is to present a laboratory procedure for obtaining an immunoelectrophoretically pure rat 7S gamma globulin and the preparation of a mono-specific 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

II. MATERIAL AND METHODS

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

Separation Procedure. DEAE-Sephadex A-50 gel (3.5 ± 0.5 meq per gram, 40-120 μm) was conditioned as previously reported by Baumstark et al.,¹ with the following slight modification. In attempts to determine the optimum buffer pH for separating 7S globulin from rat serum, .01 M phosphate buffers 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 to be effective for the elution of a fairly clean, though not completely pure rat gamma globulin when characterized by immunoelectrophoresis.

Fifty milliliters of normal rat serum (undialyzed) were added to a beaker containing 10 g of moist DEAE-Sephadex A-50. The mixture of serum and gel was allowed to adsorb in a refrigerated room at 3°C for 1.5 hours while constantly being stirred with a magnetic bar. Following the 1.5 hours adsorption period, the serum-gel mixture was transferred to two 40-ml cellulose nitrate tubes and centrifuged in a refrigerated centrifuge at 4,080 g for 15 minutes. 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 hours. After this adsorption (adsorption number 2) the serum-gel mixture was centrifuged once again as before, and the supernatant 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 hours, a fourth of 2.5 hours and a fifth of 1.5 hours. 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 x 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 per minute. The content of the tubes corresponding to each peak was pooled, and concentrated 8-fold in Carbowax. Protein determinations were made by the method of Lowry et al.⁷

Characterization of Gamma Globulin. Immunoelectrophoresis for the characterization of the various preparations was conducted in 0.75 percent agarose media made in 0.05 M Tris buffer pH 8.6. The fractions were electrophoresed 1 hour at 300 volts using the LKB apparatus.⁶ The ultracentrifugal analyses were carried out in a Spinco analytical ultracentrifuge employing a standard 12 mm cell.

Radioimmunoelectrophoresis Technique.⁵ After the immunoelectrophoretic pattern was developed and the slides washed in saline 48 hours, ⁵⁹Fe diluted to 10 μ Ci/ml was added to the trough and allowed to stand in a covered tray 24 hours. The slides were washed in saline 48 hours 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 Antisera. Antisera were prepared in adult male rabbits, New Zealand strain, by two injections with immunoelectrophoretically purified gamma globulin. The first injection containing approximately 2 mg of immunoelectrophoretically pure rat 7S globulin incorporated in complete Freund's adjuvant⁴ 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.

III. 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 anti-rat 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.

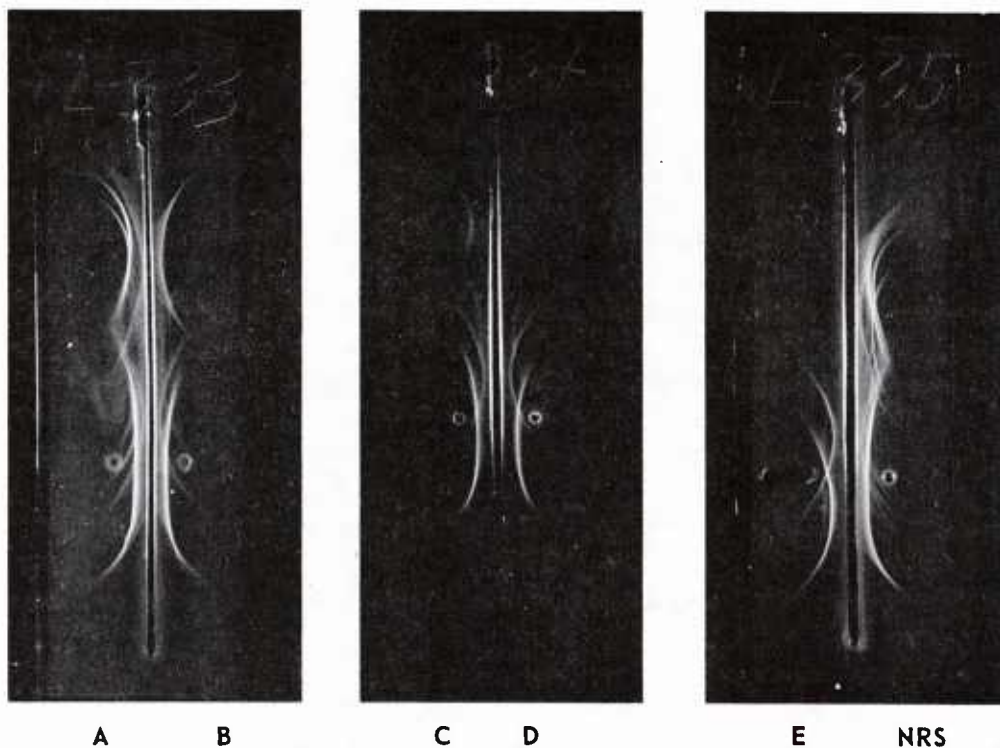


Figure 1. Immunoelectrophoretic patterns of adsorbed rat serum against rabbit anti-rat serum. Successive adsorptions (A-E) on 10 g aliquots of DEAE-Sephadex A-50. (A) 1st adsorption; (B) 2nd adsorption; (C) 3rd adsorption; (D) 4th adsorption; (E) 5th adsorption; (NRS) normal rat serum.

Two milliliters of the supernatant consisting of the 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 anti-rat whole serum.

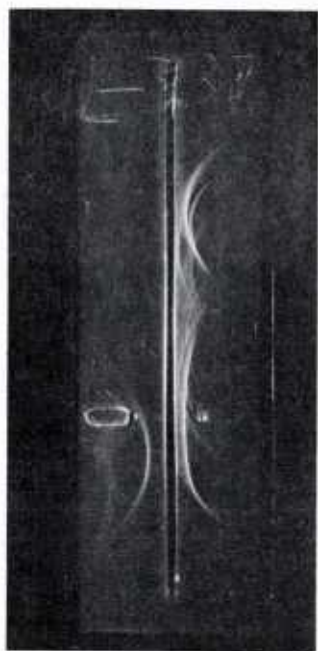


Figure 2. Comparative immunoelectrophoretic pattern of DEAE-Sephadex column fractionation of a 5X adsorbed rat serum and normal rat serum against rabbit anti-rat serum. (A) column fractionated serum; (B) normal rat serum.

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

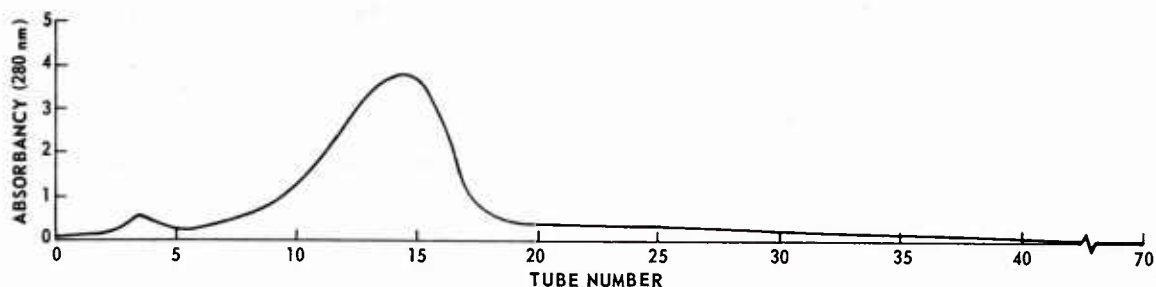


Figure 3. Elution from DEAE-Sephadex A-50 column of a 5X adsorbed rat serum in 0.01 M phosphate buffer, pH 7.5.

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

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 the antigen previously used. 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 adsorbed rat serum by employing the radioimmuno-electrophoresis technique.⁵ It was noted that ^{59}Fe would attach to the faster moving component. Since transferrin is the iron-containing constituent of the serum and will incorporate ^{59}Fe , this may indicate that the faster moving component is transferrin. Figure 6 shows the radio-autograph along with the plain immunoelectrophoretic pattern.

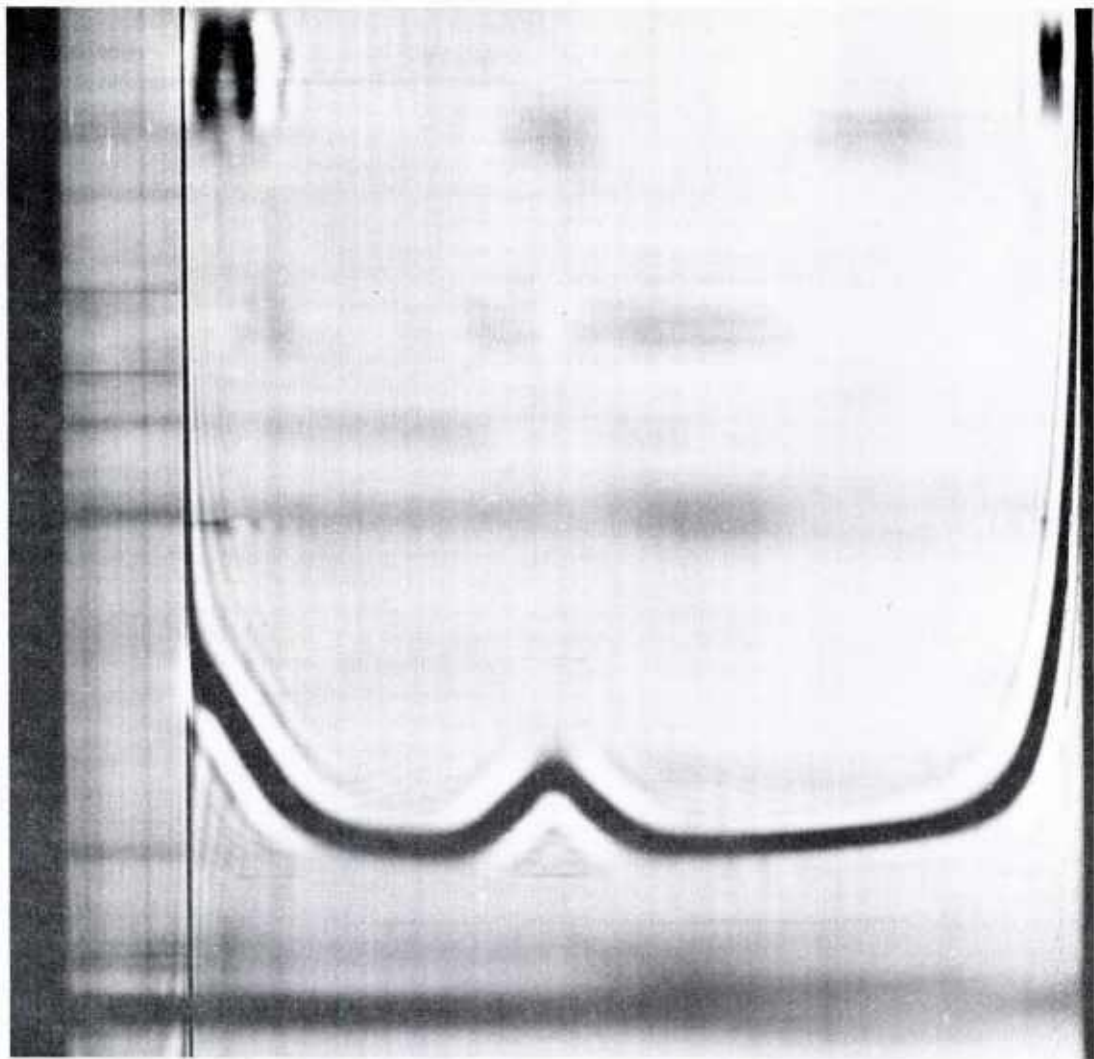


Figure 4. Ultracentrifugal pattern of adsorbed and column fractionated rat serum. Protein concentration, 2.2 mg/ml in 0.01 M phosphate buffer, pH 7.5. Speed: 59,780 rpm at 20°C. Photograph taken 61 min after indicated rotor speed. S_{20} value = 6.0.



A B

Figure 5. Immunoelectrophoretic pattern of rat 7S globulin and normal rat serum against rabbit anti-rat 7S globulin.

(A) 7S preparation (5X adsorbed column fraction);
(B) normal rat serum.



A B



A¹ B¹

Figure 6. Radioimmunolectrophoretic pattern of the 5X adsorbed fraction against rabbit anti-rat serum showing the presence of transferrin.

Left -- radioautograph. Right -- immunoelectrophoretic pattern.

(A), (A¹) 5X adsorbed rat serum; (B), (B¹) normal rat serum.

IV. DISCUSSION

The results presented here show that rat gamma globulin can be separated by an adsorption 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 concentrated in Carbowax 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 (Figure 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 not be obtained despite modifications in: (1) buffer pH values; (2) adsorption time; and (3) 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 minutes 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 hours from 50 ml undialyzed human serum. The difficulty in isolating greater yields from rat serum may be a function of species differences.

V. CONCLUSIONS

A method for the isolation of rat 7S gamma globulin based on multiple adsorptions with DEAE-Sephadex A-50 has been 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 anti-rat sera confirms the homogeneity of this 7S fraction. A monospecific antiserum to rat 7S gamma globulin has been produced. This antiserum is free of reaction with all other serum components when subjected to immunoelectrophoresis against normal rat serum.

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13. ABSTRACT A method for the isolation of rat 7S gamma globulin based on multiple adsorptions with DEAE-Sephadex A-50 has been 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 anti-rat sera confirms the homogeneity of this 7S fraction. A monospecific anti-serum to rat 7S gamma globulin has been produced. This antiserum is free of reaction with all other serum components when subjected to immunoelectrophoresis against normal rat serum.			

14. KEY WORDS	LINK A		LINK B		LINK C	
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