Human Gamma Globulin Fractionation on Anion Exchange Cellulose Columns*

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The serum proteins designated as γ-globulins include all proteins of the slowest moving of the several major protein groups seen on serum electrophoresis conducted at alkaline pH (2). Among the γ-globulins of normal serum are proteins of differing electrophoretic mobility (3, 4), proteins with sedimentation coefficients of 6.6 S and 18 S in the ultracentrifuge (2, 3, 5, 6), many individual antibodies (5, 7), and the isohemagglutinins (8). In disease states, within the γ-globulins are also found the relatively specific serum properties identified in rheumatoid arthritis (9), Hashimoto’s thyroiditis (10, 11), lupus erythematosus (12), cold hemagglutination syndrome (13, 14), multiple myeloma (15, 16), macroglobulinemia (17) and cryoglobulinemia (18).

Study of the γ-globulins has been handicapped by the poor resolution of fractionation procedures and the amount of material or effort required to carry out each separation. The introduction by Peterson and Sober (19) of substituted cellulose ion exchangers with a high protein-binding capacity offered an opportunity to fractionate and characterize the γ-globulins. Previous studies utilizing whole serum have demonstrated the capacity of anion exchange cellulose chromatography to separate the γ-globulins into a number of regions of differing electrophoretic mobility (20, 21). However, with whole serum as a starting material, many of the chromatogram fractions containing γ-globulins also included large amounts of other serum proteins.

In the present work the γ-globulins were first separated from whole serum by electrophoretic techniques and subsequently subfractionated by anion exchange cellulose chromatography. The γ-globulin subfractions thus obtained have been characterized electrophoretically, ultracentrifugally, immunochemically, and by measurement of the hexose content. The findings demonstrate that the γ-globulins are a heterogeneous group of proteins with differing physicochemical and immunological properties, and that anion exchange cellulose chromatography is a useful means of subdividing the γ-globulins.

EXPERIMENTAL

Materials and Methods

Blood samples were obtained in the morning from fasting subjects and the serum separated after several hours at room temperature. Serum was used immediately or frozen for future use. Individual normal sera, as well as pools of three normal sera, were utilized. Ten donors, in current good health and with no history or evidence of hepatic disease, contributed the sera utilized in these studies. Each serum was normal by analytical electrophoresis and ultracentrifugation.

Protein and hexose determinations and analytical paper electrophoresis were performed on whole serum and protein fractions as described previously (21). When necessary, dilute γ-globulin solutions were dialyzed at 5° against phosphate-buffered saline (1 volume of 0.15 M sodium phosphate pH 8.0:9 volumes of 0.14 M NaCl).

Analytical ultracentrifugation was performed in a Spinco model E ultracentrifuge at 59,750 r.p.m. Samples were dialyzed thoroughly against 0.14 M NaCl before analysis. All experimentally observed sedimentation coefficients were corrected to a water basis at 20° (S20, w) and are expressed as Svedberg units of sedimentation, S = 10-13 cm per second per unit field of force.

Antisera to normal γ-globulin were produced by subcutaneous or intramuscular injection of rabbits with 5 mg of whole γ-globulin obtained by preparative electrophoresis, as described below, plus Freund’s adjuvant and a similar injection 2 weeks later. After another 2 weeks, removal of 40 ml of blood by cardiac puncture was followed by intramuscular injection of 5 mg of γ-globulin. Similar blood removals and booster immunizations were performed at weekly intervals until each rabbit was killed. All antisera samples were tested by gel-diffusion techniques (22) against γ-globulin and whole serum to determine qualitative antibody composition and by precipitin procedures (23) to determine potency.

Gel-diffusion (Ouchterlony) plates were prepared as described by O’Connor (24). Samples, 0.1 ml, were applied to each cup and diffusion allowed to proceed at room temperature or at 4°. When the cups were almost empty, another 0.1 ml of solution was added. Reaction was usually complete in 48 to 72 hours, at which time photographs were obtained.

Preparative Electrophoresis—The γ-globulins (also referred to as whole γ-globulins) were separated from serum by electrophoretic techniques. Zone electrophoresis on polyvinyl blocks was conducted by procedures modified from those suggested by Müller-Eberhard and Kunkel (4). A trough, 20 × 6 × 2.5 cm, was lined with parafilm and moistened wicks of Telfa placed at its ends. A mixture of 65 g of polyvinyl particles and 65 ml of sodium diethylbarbiturate buffer, pH 8.6 and ionic strength 0.075, was poured into a trough. Excess fluid was removed from the block by touching the wicks to a towel. A 5.0 × 0.2-cm

* A preliminary report of this study has been published (1).

1 Telfa gauze, Bauer and Black Company, Englewood, New Jersey.

2 Geon, Goodrich Chemical Company, Akron, Ohio.
The block was cut into 1.0-cm sections. Proteins were eluted from the above buffer. Electrophoretic separation was carried out in a cold room at 5° with a constant current of 20 ma applied for about 18 hours, i.e. until the color bands were well separated.

The trough was carefully removed, the excess fluid was evaporated from the block with a warm air stream, and the block was cut into 1.0-cm sections. 

Proteins were eluted from the block sections by displacement filtration, with centrifugation at 300 X g for 4 minutes in small sintered glass funnels after the addition of Veronal buffer or saline solution. This procedure was repeated once to rinse the polyvinyl medium. The eluents from each section were combined, adjusted to a constant volume, and the protein content estimated by biuret procedures (Fig. 1) or by determining the optical density at 284 μm in a Beckman DU spectrophotometer. This wave length was suggested by Brattsen (3) in order to reduce the optical density due to barbiturate buffer while retaining as much as possible of the protein optical density. Electrophoretic separation of the γ-globulins from serum was also carried out at 5° in a Spinco model CP continuous flow paper electrophoresis cell utilizing normal serum diluted in the ratio of 1 volume of serum to 2 volumes of sodium diethylbarbiturate buffer of pH 8.6 and 0.03 ionic strength. The diluted serum was applied to the curtain at a rate of 0.8 ml per hour and exposed to a constant current of 35 ma. The effluent from the curtain was collected from 32 delivery tips into individual tubes which were changed every 12 or 24 hours by means of an automatic fraction collector. The protein content of each tube was estimated by determining the optical density at 284 μm.

The γ-globulin electrophoretic region was considered to be composed of all the proteins migrating less rapidly than the β-lipoproteins, which appeared to be the slowest migrating of the β-globulins. This protein location was determined by qualitatively ascertaining the lipid distribution in the electrophoretic fractions by applying aliquote of the fractions to paper strips and staining with Oil Red O dye (25). γ-Globulin pools were prepared by combining the content of all tubes in the γ region that did not contain lipid (Fig. 1, Sections 2 to 7), thus most of the region intermediate between the β- and γ-protein peaks was included within the γ-globulin pool. On ultracentrifugal analysis, 90 to 95% of these pools were proteins with a sedimentation coefficient of 6.6 S, and the remainder of the proteins had a sedimentation coefficient of 18 S (cf. Fig. 5). No components of 9.5 S were seen.

Anion Exchange Chromatography—Electrophoretically prepared γ-globulins equilibrated by dialysis with the initial buffer were applied to 30 X 1.0-cm (internal diameter) columns prepared as described previously (21). The columns contained 2 g of diethylaminoethyl (DEAE) cellulose3 (19) in equilibrium with diethylbarbiturate buffer of pH 8.6 and 0.03 ionic strength. The starting buffer, 90 ml, was placed in a mixing chamber prepared from a flat bottomed cylinder with 4.7-cm internal diameter. This was connected by means of a siphon to a 50-ml Erlenmeyer flask reservoir containing 35 ml of 0.30 μm phosphate buffer pH 8. 

The columns contained 2 g of diethylaminoethyl (DEAE) cellulose3 (19) in equilibrium with the initial buffer. For most of the work the initial buffer was 0.02 M potassium phosphate buffer pH 8. Phosphate buffer solutions were prepared as the potassium, sodium, or tris(hydroxymethyl)aminomethane salts. An elution gradient of increasing molarity was established, maintaining pH 8, with a total elution volume of 150 ml. Starting buffer, 90 ml, was placed in a mixing chamber prepared from a flat bottomed cylinder with 4.7-cm internal diameter. This was connected by means of a siphon to a 50-ml Erlenmeyer flask reservoir containing 35 ml of 0.30 μm phosphate buffer pH 8. 

A magnetic stirrer (21). The eluting solution was pumped through the column at a rate of 15 ml per hour and effluent fractions of 3.3 ml were collected. The optical density of each fraction was measured at 280 μm. Recovery of the γ-globulins applied to the column was 90 to 100% complete, as determined by optical density measurements at 280 μm.

The bottoms of the two containers were level and mixing was assured by a magnetic stirrer (21). The eluting solution was pumped through the column at a rate of 15 ml per hour and effluent fractions of 3.3 ml were collected. The optical density of each fraction was measured at 280 μm. Recovery of the γ-globulins applied to the column was 90 to 100% complete, as determined by optical density measurements at 280 μm.

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RESULTS

A representative normal γ-globulin chromatogram is illustrated in Fig. 2. γ-Globulins were eluted continuously throughout most of the chromatogram. For purposes of analysis the eluted γ-globulins were grouped into five fractions composing, respectively: 5 to 15% of the elution volume, Fraction 1; 15 to 25%, Fraction 2; 25 to 45%, Fraction 3; 45 to 58%, Fraction 4; and 58 to 75%, Fraction 5. However, it should be remembered that the elution of γ-globulins composing the first four groups and part of the fifth was continuous, and the division into five fractions was a convenient expedient.

On rechromatography, each fraction was eluted in the same characteristic region of the chromatogram (Fig. 3). Fraction 2 contained a small amount of the preceding fraction, presumably due to spreading of a portion of the first fraction as it passed through the column.

The chromatographic γ-globulin distribution has been found to be dependent on the molarity of the initial buffer. In Fig. 4 are illustrated the chromatograms obtained with the same γ-globulin preparation chromatographed with starting (and equilibrating) buffers of 0.020 M, 0.010 M and 0.005 M phosphate concentration. A marked and progressive shift of γ-globulin from the Fraction 1 to Fraction 3 region is seen as the molarity is reduced. This change occurred to the same degree whether the potassium, sodium, or tris(hydroxymethyl)aminomethane forms of phosphate buffers, pH 8, were used. Confirmatory evidence that Fraction 1 contributes the γ-globulin obtained in the Fraction 3 of a lower starting molarity was obtained by rechromatography. When Fraction 1 was obtained from a column with an 0.020 M phosphate initial buffer and was subsequently rechromatographed with the use of 0.005 M phosphate initial buffer, much of the protein was eluted in Region 3 (Fig. 4, lowermost graph). On comparison of the whole γ-globulin chromatograms illustrated in Fig. 4, it is seen that with buffers of lower phosphate concentrations the quantity of protein eluted in Region 2 was little altered; the protein in Region 3 showed the greatest increase; whereas Region 4 increased moderately, and the quantity of protein in Region 5 was not altered.

The change of γ-globulin distribution from one region of the chromatogram to another upon lowering the initial buffer molarity was considered to be due to an increased capacity of the anion exchange cellulose to bind γ-globulins and/or an alteration in certain of the γ-globulins under these conditions. It is probable that a specific group of γ-globulins adheres to the adsorbent at the lower buffer molarity, but will not adhere or remain adherent in solutions of higher ionic strength. The shift does not seem to be due to an association of γ-globulin molecules into larger aggregates. Ultracentrifugal analyses of Region 3, obtained after chromatography starting with 0.005 M phosphate buffers, were made in the eluting solution, and after dialysis against 0.005 M phosphate buffer pH 8 and, also, after dialysis against 0.14 M NaCl solutions. These analyses revealed only molecules with sedimentation coefficients of approximately 0.0 S.

The progressive increase in the size of Fraction 3 with serial lowering of initial buffer molarity indicated that a spectrum of proteins was contained in Fraction 1. A stepwise lowering of buffer molarity could be utilized for further fractionation of this group of γ-globulins.

Another consideration in selecting the phosphate molarity of the initial chromatography buffer is the increasing insolubility of normal γ-globulins with decreasing solvent molarity. In 0.020 M phosphate buffers, pH 8, approximately 1 to 4% of the NaCl solutions. These analyses revealed only molecules with sedimentation coefficients of approximately 0.0 S.

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proteins became insoluble and were precipitated by centrifugation before application to the column. However, with 0.005 M phosphate buffers, the loss was 5 to 10%. In sera obtained in disease, γ-globulin losses on equilibration with 0.005 M phosphate solutions, pH 8, may be much greater. For this reason the chromatographic fractionation of γ-globulins was conducted with initial buffers of pH 8 and 0.02 M phosphate concentration as the best compromise.

**Characteristics of γ-Globulin Fractions**

**Physicochemical**—Electrophoretic characterization of the γ-globulin fractions revealed a progressive increase in the electrophoretic mobility of the proteins comprising Fractions 1 to 4 (Fig. 5). moving boundary electrophoresis of normal serum chromatogram fractions was performed by Sober, Gutter, Wyckoff and Peterson (20). Although the conditions of chromatography are somewhat different from those used here, the sequence of γ-globulin elution is believed to be similar. The boundary electrophoresis observations are in agreement with the relative mobility differences noted on paper electrophoretic analyses here.

**Immunological**—Electrophoretically prepared γ-globulins when tested by the gel-diffusion technique against rabbit antihuman γ-globulin serum consistently showed 2 precipitin lines (Fig. 6, upper). One of these lines was straight and the other concave toward the γ-globulin cup. Korngold and Van Leeuwen (26) have postulated that a straight precipitin line between antigen and antibody cups is indicative of an antigen of about the same molecular weight as the antibody. Similarly, a precipitin line concave toward the antigen cup is indicative of a greater molecular weight for the antigen. Support for this interpretation is illustrated in Fig. 6 where two purified γ-globulin preparations, containing only normal 6.8 S components in the first instance, and in the second only 18 S macroglobulins prepared from the sera of a patient with Waldenström's macroglobulinemia, reacted with rabbit antinormal human γ-globulin serum on each side of the normal whole γ-globulin preparation. It is seen that the concave precipitin line of the whole γ-globulin demonstrates a reaction of identity (22) with the similar concave line of the 18 S macroglobulin. Also, the straight precipitin line reacts with the corresponding line formed by the known 6.8 S γ-globulins. These findings demonstrate that γ-globulins possess at least two antigenic properties, one characteristic of the 18 S macroglobulins, the other characteristic of the 6.8 S globulins. This is in agreement with the observations of Franklin and Kunkel (27).

The immunoechemical findings when multiple chromatographic γ-globulin subdivisions were tested against rabbit antinormal human γ-globulin sera are illustrated in Fig. 6, lower. The chromatographic γ-globulin Fractions 1 to 4 seemed to have a single antigenic component which was shared in common by these fractions. Although not illustrated here, when these fractions were tested in hexagonally arranged cups about a central antiserum cup, a single straight precipitin line was seen with each fraction which showed a reaction of identity with the chromatographically adjacent γ-globulins. The proteins in Fraction 5, in addition, demonstrated a strong precipitin line that was
conceivable that these proteins are formed in individual and differing plasma cells. This has been suggested by Askonas et al. (36) who found that amino acids...
were incorporated into several rabbit y-globulin fractions at different rates by the spleen, lymph nodes, and bone marrow. It is further possible that different y-globulins, once out of the plasma cell, may not have the same metabolic status. If the turnover times of the various y-globulins are found to differ, the use in metabolic studies of y-globulin pools without subdivision might miss significant variations, either normally or in disease.

It will be of particular interest to determine if the chromatographically discernible y-globulin subgroups are similarly or differently affected by pathological states such as hepatic cirrhosis, chronic infections, the collagen diseases, leukemias and lymphomas, multiple myeloma and macroglobulinemia, and other disorders in which marked alteration in the total amount of y-globulin may occur.

Considerable attention has been devoted to the question of whether the large amounts of homogeneous serum protein seen in multiple myeloma are qualitatively or quantitatively abnormal. However, the isolated, apparently homogeneous, serum myeloma proteins have been compared with and shown to differ from the heterogeneous group of normal y-globulins which may be composed of hundreds or thousands of individual y-globulins. Thus the evidence currently available cannot be regarded as conclusively demonstrating that the myeloma proteins are abnormal. It would be unrealistic to insist that the homogeneous y-globulins found in certain diseases should be compared with isolated individual normal y-globulins. Nevertheless, the implications of extensive heterogeneity within the normal y-globulins does bear upon the problem of relating a single protein found in a disease to the proteins normally present.

Further subdivision of the normal y-globulins beyond that obtained in the present report seems to be feasible. Fraction 1, for instance, can be further fractionated by rechromatography on anion exchange cellulose columns utilizing a starting buffer of lower ionic strength. Refractionation on columns of the cation exchanger carboxymethyl cellulose can be used (37). The use of such additional procedures together with anion exchange chromatography should facilitate the detailed study of y-globulins in normal and disease states.

**SUMMARY**

1. y-Globulin fractionation by means of anion exchange diethylaminoethyl (DEAE) cellulose chromatography has been performed on electrophoretically prepared normal y-globulin pools. Chromatographic procedures have been modified so that the y-globulins of as little as 1 mL of serum can be satisfactorily subdivided.

2. The fractions of y-globulin obtained by anion exchange cellulose chromatography have been considered in 5 groups for purposes of analysis and comparison. Fractions 1 to 4, which contained only proteins with sedimentation coefficients (s2o, w) of 6.6 S on ultracentrifugal analysis, were composed of y-globulins with progressively increasing electrophoretic mobility and increasing carbohydrate content (from 1.1 to 2.3% hexose). Immunochemical testing revealed Fractions 1 to 4 to have antigenic properties in common. Fraction 5, containing all of the 18 S y-macroglobulins, had the highest hexose content (5.0%). Fraction 5 also possessed distinctive antigenic characteristics, compatible with the presence of the 18 S proteins in this fraction.

3. The heterogeneity of the y-globulins was emphasized. The y-globulins with sedimentation coefficients of 18 S, normally comprising about 5 to 10% of the total y-globulins, were readily distinguished from the smaller (6.6 S) y-globulins by their distinctive ultracentrifugal and immunochemical properties, a relatively high hexose content, and characteristic physiological activities.

4. The y-globulins with sedimentation coefficients of 6.6 S, that normally comprise 90 to 95% of the y-globulins, were distinguished by an anion exchange chromatographic distribution indicating the existence of a spectrum of molecules. The differing electrophoretic mobility, chromatographic behavior, hexose content, and distribution of physiological activities of the chromatogram fractions support the view that these are a spectrum of y-globulin molecules. The ultracentrifugal and immunochemical findings indicate that these molecules share, however, similar size and antigenic properties. The implications of these observations are discussed.

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