INTERACTION OF THE RHEUMATOID FACTOR WITH ANTIGEN-ANTIBODY COMPLEXES AND AGGREGATED GAMMA GLOBULIN

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The term "rheumatoid factor" has been applied to the protein component of serum which is responsible for a group of agglutination and precipitation reactions, some of which have been employed as diagnostic procedures in rheumatoid arthritis (1, 2). In these reactions this factor interacts with antibody coating red cells or organisms, with antibody in antigen-antibody precipitates, (3) or with gamma globulin itself (4). Previous studies from this laboratory (5) have indicated that direct precipitation of the rheumatoid factor with gamma globulin takes place only if the gamma globulin is in an aggregated form. In addition, some interaction with low molecular weight gamma globulin appears to occur, since the factor in serum and plasma is found as a soluble complex with 7 S gamma globulin (5, 6).

Analytical studies have indicated that the rheumatoid factor is a typical 19 S gamma globulin with chemical, physical, and immunological properties closely akin to those of known 19 S antibodies (7, 5). These observations suggest that it is an antibody. On the other hand, certain analogies to the behavior of complement suggest that it might be an unusual type of complement-like material. It is clear, however, that it is not one of the known components of the complement-properdin system (8, 3, 9).

The present studies were undertaken to characterize further the antibodies and gamma globulin with which the rheumatoid factor will interact. Reactions with soluble and insoluble aggregates of gamma globulin were observed, and evidence was obtained for a reaction with a variety of soluble antigen-antibody complexes.

Material and Methods

Rheumatoid Factor.—The sera employed in this study were obtained from four different patients who had long standing rheumatoid arthritis and concentrations of 100 to 300 mg. per cent 22 S rheumatoid factor complex in their sera (5).

Isolated rheumatoid factor was prepared by density gradient zone centrifugation (7, 5) either from euglobulin fractions or from dissolved precipitates of rheumatoid factor with aggregated gamma globulin. The precipitates were dissolved in glycine-HCl buffer, pH 3.0, and the 19 S rheumatoid factor was isolated from the sucrose density gradient solution

as described in the section on ultracentrifugation. Such preparations consisted of at least 90 per cent 19 S material. The preparations made from euglobulin fractions contained some normal 19 S proteins. Fig. 1 illustrates the ultracentrifugal pattern of a typical preparation used in this study. These preparations at a concentration of 1 mg./ml. reacted at dilutions of 1/640 to 1/5120 in the sheep cell test, and at dilutions of 1/5120 to 1/20,480 in the latex fixation test.

The various serologic tests were carried out as described previously (5). Serial 2-fold dilutions were made, and the number of the last tube giving a positive result was listed. The inhibition of the sheep cell agglutination reaction was measured by adding 0.5 ml. of inhibiting protein to 0.5 ml. of active rheumatoid serum, (usually diluted 1/10), and then carrying out serial dilutions of this mixture for the reactions with sensitized sheep cells. As controls, 0.5 ml. portions of saline in place of protein solutions were added to the same rheumatoid serum.

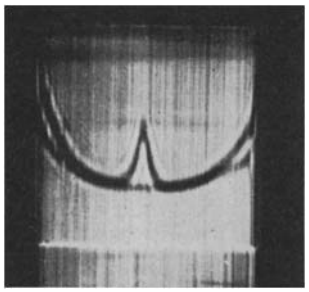

FIG. 1. Ultracentrifugal pattern of isolated rheumatoid factor. The corrected sedimentation rate was 18.8 S.

Antisera.—Rabbit antisera to human serum albumin and to 7 S gamma globulin were obtained from animals immunized subcutaneously with the antigen in Freund's adjuvant. Antiserum to crystalline ovalbumin from rabbits immunized by repeated intravenous injections of antigen was kindly supplied by Dr. Curtis Williams. Horse antiserum to pneumococcus type III was obtained from Dr. Maclyn McCarty.

The antipneumococcal and antiovalbumin sera were used in the unfractionated state. In the case of antisera to human serum albumin and human gamma globulin, the gamma globulin fractions were separated by zone electrophoresis in starch using barbital buffer pH 8.6, $\Gamma/2$ 0.1 by methods previously described (10). After elution with phosphate buffer pH 7.6, $\Gamma/2$ 0.1 or 0.15 N saline the fractions were concentrated by ultrafiltration.

Antigens.—Human 7 S gamma globulin was a Lederle fraction II preparation, lot No. C440, demonstrated to be free of 19 S gamma globulin. Ovalbumin was 2× recrystallized material obtained from Worthington Laboratories. Human serum albumin was a Squibb fraction V preparation. Pneumococcus type III polysaccharide was obtained from Dr. Walther Goebel.

Precipitin Reactions.—These were performed in duplicate using 0.05 to 0.1 ml. of antiserum or antiserum gamma globulin. After addition of antigen, the mixture was brought to a constant final volume of 0.55 cc. with phosphate buffer or saline. After 1 hour at room temperature and 24 hours at 4°C., the precipitates were washed twice with saline or buffer at 4°C. with centrifugation at 1,000 *g* for 30 minutes between washes. Protein in the precipitates was measured by the modified Folin-Ciocalteu method (11).

Two parallel precipitin reactions were set up. After 1 hour at room temperature, 0.05 ml. of a particular rheumatoid factor preparation was added to each tube of one set, and 0.05 ml. of saline or buffer was added to the control group. Precipitates were measured after 24 hours at 4°C. No significant amounts of precipitation occurred in control tubes with antigen alone and antibody alone. A small amount of precipitate formed in control tubes containing rheumatoid factor alone, rheumatoid factor plus antigen, and rheumatoid factor plus antibody. The highest optical density value of these controls was subtracted from the optical density values for each antigen concentration before calculating the amount of protein nitrogen in the precipitin tubes.

Preparation of Soluble Antigen-Antibody Complexes.—From 8 to 20× equivalence amounts of antigen were combined with electrophoretically isolated gamma globulin containing antibody, allowed to react for 1 hour at room temperature, and were then placed at 4°C. for 24 hours to 7 days. Before use, these preparations were centrifuged at 6000 *g* for 1 hour in a Spinco model L ultracentrifuge.

Preparation of Altered Gamma Globulin.—Ten mg. of Lederle fraction II gamma globulin was placed in 1 to 2 ml. of tris buffer, 0.1 M, pH 8, which was made 6 M in urea and 0.1 M in mercaptoethanol. After 1 hour at room temperature, this preparation was dialyzed against one liter of tris buffer at 4°C. for 24 hours. In certain preparations the dialysis solution was made 0.02 M in iodacetamide.

In similar fashion preparations of gamma globulin were made using 3 M guanidine HCl alone, 6 M urea alone, and 0.1 M mercaptoethanol alone. Heated preparations were made by exposing a 10 mg./ml. solution to 63°C. for 10 minutes.

Solid gamma globulin was prepared by heating Lederle fraction II gamma globulin at a concentration of 40 mg./ml. in isotonic saline to approximately 75°C. at which temperature most of the gamma globulin precipitated. This was then washed 3 times with saline, and finally suspended in an equal volume of saline. Two additions of 0.35 ml. of such a suspension to 0.5 ml. of serum were used in the experiments shown in Table IV.

Ultracentrifugation.—Density gradient zone centrifugation was carried out in a Spinco model L machine employing the swinging bucket rotor. Protein fractions, (0.5 to 1 ml., 5 to 10 mg./ml.), were layered over a sucrose density gradient containing 37 per cent sucrose at the bottom and 10 per cent sucrose at the top. Droplet formation was minimized by slight stirring of the protein solution with the sucrose below it, and by beginning ultracentrifugation immediately after layering. Ultracentrifugation for 15 hours at 110,000 *g* resulted in sedimentation of 19 S class proteins to a position approximately three-fourths of the distance down the tube; 7 S class proteins remained in the upper portion of the tube. Isolation of the fractions from the density gradient following centrifugation was carried out in two ways; either by simple removal of samples at various levels by means of a stabilized capillary pipette, or by making a small hole in the bottom of the plastic centrifuge tube and allowing drops to collect in a fraction collector. In those cases in which aggregated gamma globulin was packed on the bottom of the tube, the hole was made in the wall just above this level. Protein analyses on these fractions furnished curves of the distribution of the separated proteins. The chief limitation of the procedure was slight contamination of upper fractions. However, it was satisfactory for the isolation and complete recovery of the 19 S rheumatoid factor. Final identification of these proteins was made by analytical ultracentrifugation, or by immunological methods (5, 12). Shorter periods of ultracentrifugation were sometimes utilized for the characterization of the size of gamma globulin aggregates. Analytical ultracentrifugation was carried out as previously described (5).

RESULTS

I. Reaction of Rheumatoid Factor with Soluble Antigen-Antibody Complexes.—In preliminary experiments, rheumatoid serum and normal serum were added to a gamma globulin–rabbit anti-human gamma globulin system in antigen excess. Rheumatoid serum caused more precipitation than comparable amounts of normal serum. Purified 19 S rheumatoid factor was used in all subsequent experiments because the different complement and gamma globulin contents in whole sera made quantitation difficult.

Fig. 2 shows the effect of adding 0.05 ml. of a purified rheumatoid factor

preparation containing 3.2 mg. protein/ml. to each tube of an ovalbumin–rabbit anti-ovalbumin system. There was a considerable increase in nitrogen values of the precipitates compared to those of the control series. This was most marked at equivalence and in antigen excess.

This procedure did not distinguish binding of rheumatoid factor by solid precipitates from binding to soluble antigen-antibody complexes. The supernates therefore were removed as quantitatively as possible from each tube in the control series. To each supernate 0.05 ml. of the same rheumatoid factor preparation was added. After 24 hours at 4°C., the amount of precipitate was measured. In Fig. 2, the amount of nitrogen in these precipitates is plotted against the antigen concentration to which each supernate corresponds. Because these points do not represent a true precipitin curve, no line is drawn connecting them.

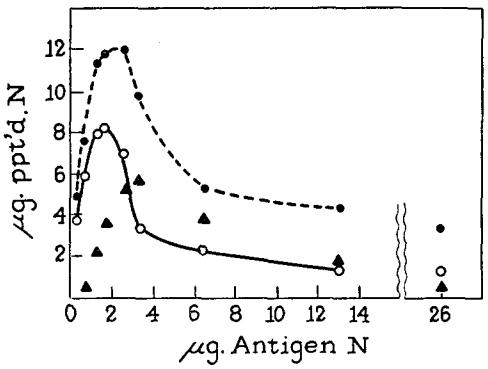

Fig. 2. The effect of rheumatoid factor on the ovalbumin-rabbit anti-ovalbumin precipitin system. O—O control precipitin reaction; ●—●– precipitin reaction with added rheumatoid factor; ▲ supernates from control curve with added rheumatoid factor.

Corresponding to the absence of soluble antigen-antibody complexes in antibody excess supernates, almost no precipitate was present in this zone. The precipitate was maximal at a point in antigen excess approximately 2× equivalence, and the values gradually fell in the region of increasing antigen excess.

When the difference between the precipitated nitrogen of the control curve, and that of the curve with added rheumatoid factor is compared to the amount of nitrogen precipitated when rheumatoid factor is added to the supernates, it is seen that the values are not the same (Fig. 2). This difference can be explained in part by the presence of antigen-antibody precipitates in the first case, and their absence in the second. Another factor that may have been responsible is the time of addition of rheumatoid factor. In the first case rheumatoid factor was added 1 hour after mixture of antigen and antibody, and in the second case it was added to 24 hour old relatively stable antigen-antibody complexes.

A further check on the reactivity of soluble antigen-antibody complexes was made using 8× equivalence ovalbumin–anti-ovalbumin soluble complexes, freed of possible suspended

particulate material by high speed centrifugation. When these complexes were layered with rheumatoid factor in capillary precipitin tubes, visible precipitation occurred within 10 minutes. Mixture of the soluble complexes with rheumatoid factor in a test tube led to the appearance of a stringy flocculant precipitate in 10 minutes.

Similar effects were observed when rheumatoid factor was added in the rabbit anti-human albumin system (Fig. 3). A rheumatoid factor preparation containing 0.81 mg. protein/ml. was used in 0.05 ml. portions per tube. The effect of rheumatoid factor in this case was greater in antigen excess than at equivalence. When 0.05 ml. of the same rheumatoid factor preparation was added to the supernates of the control curve, maximal precipitation of antigen-antibody complexes occurred at a point in antigen excess 5X equivalence.

The interaction between rheumatoid factor and the rabbit anti-human gamma globulin system also was studied (Fig. 4). Electrophoretically isolated

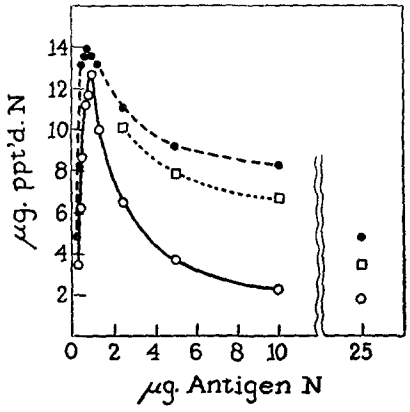

FIG. 3. The effect of rheumatoid factor on the rabbit anti-human albumin precipitin reaction. O—O- control precipitin reaction; ●—● precipitin reaction with added rheumatoid factor; □....□ precipitin reaction with added rheumatoid factor heated at 56°C. for 1 hour.

human gamma globulin which showed no reaction with rheumatoid factor was used in these experiments. Certain difficulties arise in the interpretation of this precipitin system. Because rheumatoid factor is a 19 S gamma globulin, which reacts with antibody to gamma globulin in the zone of antibody excess, this portion of the curve cannot be compared with preceding curves. In the region of antigen excess, an exchange of rheumatoid factor with 7 S antigen gamma globulin in the complexes could occur. This seems improbable, however, since the difference between the two curves was greater in the antigen excess zone where exchange was less likely. Finally, a minor component of antibodies to 19 S gamma globulin might be present. This difficulty was avoided by using antisera to 7 S gamma globulin which had no antibodies to 19 S gamma globulin. It seems likely that interaction of rheumatoid factor with antibody complexes was involved in this system as in the others described above.

In Table I the amount of nitrogen precipitated in the horse anti-pneumococcus type III system is compared to the values obtained when 0.05 ml. of rheumatoid factor (0.9 mg./ml.) was added to the same precipitation system 1 hour after mixture of antigen and antibody. No tendency to increased pre-

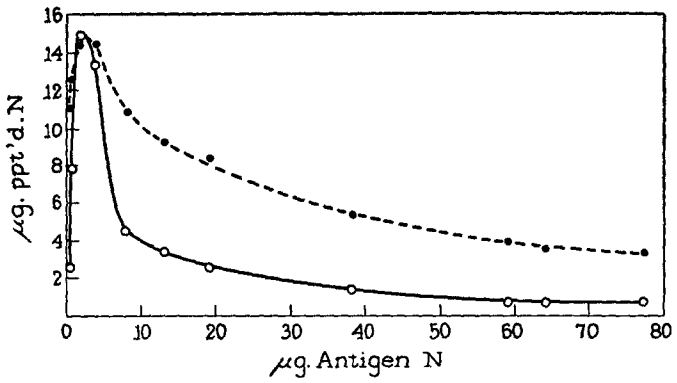

FIG. 4. The effect of rheumatoid factor on the rabbit anti-human gamma globulin precipitin reaction. ○—○ control precipitin reaction: ●—● precipitin reaction with added rheumatoid factor.

TABLE I

Nitrogen Precipitated in Horse Anti-Pneumococcus Type III Precipitin Reaction, with and without Rheumatoid Factor

Antigen	Ppt'd. N control	Ppt'd. N control +RF	Ppt'd. N control supernates + RF
μg.	μg.	μg.	μg.
0.4	8.8	8.0	0
0.8	10.1	9.0	0
1.6	9.9	9.0	
3.3	9.0	9.3	0
6.6	9.0	8.2	
13.1	8.3	7.9	0
26.2	8.0	7.1	
52.5	6.9	5.9	0
105.0	5.6	5.4	0
210.0	3.4	3.5	0

cipitation was seen. The same amounts of rheumatoid factor added to the supernates of the control series yielded no precipitate greater than that of the corresponding control containing rheumatoid factor alone.

II. Factors Involved in the Reaction with Antigen-Antibody Complexes.— Equal amounts of unheated rheumatoid factor, and rheumatoid factor heated to 56°C. for 1 hour were added to separate tubes of the anti-human albumin

system. The capacity of rheumatoid factor to precipitate antigen-antibody complexes was only slightly reduced by heating (Fig. 3). This is in contrast to effects observed with complement (14). The presence of 3×10^{-3} M ethylene dinitrilotetraacetic acid did not influence the interaction between rheumatoid factor and soluble complexes.

Prolonging the reaction time of the antigen-antibody system had little effect. Duplicate tubes, with and without rheumatoid factor were prepared, using the rabbit anti-human albumin system. After 7 days at 4°C., precipitate nitrogen values were compared to values of tubes prepared in the same way and read at 24 hours. There was no significant difference. In most instances the rheumatoid factor shortened the appearance time of antigen-antibody precipitates.

The amount of precipitate obtained when varying amounts of a solution containing a given antigen-antibody complex were mixed with rheumatoid

TABLE II

Variation in Nitrogen Precipitated by Rheumatoid Factor with Varying Amounts of Protein from the Human Gamma Globulin–Rabbit Anti-Human Gamma Globulin System at 20× Equivalence

Protein N	Ppt'd N
μg.	μg.
240	5.0
160	3.4
120	2.5
80	2.2

factor is shown in Table II. The solution was diluted with buffer to the values for total protein nitrogen content shown. A 0.4 ml. volume of each dilution was mixed with 0.05 ml. of a rheumatoid factor preparation (1.4 mg./ml.), the mixture was stored for 24 hours at 4°C., and precipitate nitrogen was determined. An approximately linear relationship is shown between the amount of precipitate nitrogen and the amount of nitrogen in the solutions containing the antigen-antibody complexes. These experiments were difficult to interpret in view of the indeterminate amounts of reactive antigen-antibody complex in the solutions. Attempts to obtain complete precipitin curves using antigen-antibody complex as "antigen" and rheumatoid factor as "antibody" were unsuccessful for technical reasons.

III. The Size of Reactive Complexes.—The minimum size of gamma globulin complexes with which rheumatoid factor will react and precipitate is difficult to determine. Several attempts were made to define a range below which no precipitation with rheumatoid factor would occur, employing the method of density gradient centrifugation.

A solution containing 7 S gamma globulin–anti-gamma globulin complexes at 10× equivalence was layered over a sucrose density gradient, and centrifuged at 110,000 *g* for 12 hours. Various layers were then tested for precipitation with rheumatoid factor, in capillary tubes and test tubes. No reaction was observed with layers below a sedimentation rate of approximately 20 S, and the main reaction was with fractions sedimenting more rapidly than 20 S. Another experiment under slightly different conditions also indicated that large size antigen-antibody complexes were involved and that there was no reaction at the level expected for limiting complexes of bivalent antibodies.

IV. Reaction of Rheumatoid Factor with Gamma Globulin Aggregates.—It was previously established (5) that heating gamma globulin from 62–65°C. for 10 minutes produced gamma globulin solutions that reacted strongly with rheumatoid sera in a direct precipitin reaction. Evidence was obtained that the increased reactivity was due to aggregates of gamma globulin. Other procedures, such as treatment with alkali, and euglobulin formation, as well as simple aging, also produced reactive gamma globulin. The possibility arose that the more classical methods of unfolding and denaturing proteins might produce more reactive gamma globulin, and perhaps some material of low molecular weight that would cause precipitation.

Table III illustrates results with some of the methods tested. The combination of urea and mercaptoethanol produced extremely active preparations, in some instances more active than the heating procedures. This activity was largely blocked by iodacetamide, which stops disulfide bridge formation. Guanidine also produced very active preparations. A second method of testing these γ-globulin preparations was by their inhibitory capacity in the sensitized sheep cell agglutination reaction. Fraction II γ-globulin is known to inhibit this reaction strongly. However it was found in the present study that electrophoretically isolated γ-globulin showed little or no inhibitory capacity. Following treatment with urea and mercaptoethanol or heating to 63°C. a marked increase in inhibitory activity was observed (Table III).

Zone ultracentrifugation in a sucrose density gradient demonstrated that the activity in each of these preparations of human gamma globulin was due primarily to aggregated material with an *s* rate greater than 20 S (Table IV). No precipitating and little or no sheep cell inhibiting activity was encountered with material with an *s* rate of 7 S—the *s* rate of the major portion of the gamma globulin even after these procedures. Concentrated preparations of aggregated gamma globulin of considerably higher activity per unit of nitrogen were readily obtained by short periods of ultracentrifugation either with or without the density gradient.

Rabbit gamma globulin could also be made reactive by the urea-mercaptoethanol procedure, and less consistently by the heating method. The precipitin activity with rheumatoid sera was always weaker than for human gamma globulin. Inhibition of the sheep cell reaction, however, was greater than for human gamma globulin. Gamma globulin freshly prepared from rabbit serum

by electrophoresis always showed considerable inhibition of the sheep cell reaction, in contrast to similar preparations of human gamma globulin. Rabbit

TABLE III

Effect of Various Procedures on the Reactivity of Human Gamma Globulin with Rheumatoid Sera

Human γ-globulin* 10 mg./ml.	Precipitin reaction with various sera			Sheep cell inhibition‡	
Treatment	Normal H.K.	Rheumatoid E.P.	Rheumatoid M.S.	Electrophoretic γ-globulin I	Electrophoretic γ-globulin II
Intact	0	0	0	1	0
Heated 63°	0	++++	++++	6	6
Urea	0	+	Tr		
Mercaptoethanol	0	++			
Urea + mercaptoethanol	0	++++	++++	6	7
Urea + mercaptoethanol + iodacetamide	0	Tr	Tr		
Guanidine	0	++++	++++		

* Led Fr. II No. C 440.

‡ Total inhibition would be 7 (expressed as tube No. of serial dilutions).

TABLE IV

Activity of Various Fractions of Gamma Globulin, Separated by Zone Ultracentrifugation, in Precipitin Test with Rheumatoid Factor, and in Inhibition of the Sensitized Sheep Cell Agglutination Reaction

Fr. No.	Approximate s rate	Precipitin test*			Sheep cell inhibition‡				
		γ-globulin preparations			γ-globulin preparations				
		Human Fr. II 63°	Human Fr. II urea mercaptoethanol	Rabbit urea mercaptoethanol	Human Fr. II.	Human Fr. II 63°	Human Fr. II urea mercaptoethanol	Rabbit electrophoretic γ-globulin	Rabbit electrophoretic γ-globulin urea mercaptoethanol
I		0	0	0	0			1	0
II§	7 S	0	0	0	0	1	0	4	3
III		0	0	0	0			4	3
IV	19 S	0	0	0	0	1	1	4	3
V§	>20 S	++++	++++	++	4	6	6	4	7

* Carried out with rheumatoid serum E.P.

‡ Total inhibition = 7.

§ Fractions II and V were usually tested at a concentration of 5 mg./ml.

gamma globulin both in the native state and in the treated preparations, also showed significant inhibitory activity in the 7 S fraction as well as in the high molecular weight fractions (Table IV). Relatively large concentrations of gamma globulin were necessary to bring out these effects, (at least 3 mg./ml.).

Inhibition by aggregated rabbit and human gamma globulin, on the other hand, occurred at far lower concentrations.

V. Adsorption on Solid Gamma Globulin.—Human gamma globulin which was precipitated and denatured by heating, was found to adsorb the rheumatoid factor from various sera. Such gamma globulin preparations were completely insoluble, and did not go back into solution in different aqueous buffers. Two additions of solid material to rheumatoid serum usually removed much of the activity in the latex fixation, precipitation, and sensitized sheep cell agglutination tests (Table V). In some instances, slight activity remained in the serum, and the extent of removal of activity was partly dependent on the solid

TABLE V

Activity of Various Rheumatoid Sera before and after Adsorption with Solid Gamma Globulin; and Activity of Acid Eluates

	Precipitin reaction	Latex fixation*	Sensitized sheep cell agglutination*
Serum E.P.			
Before	++++	9	8
After	0	0	0
Serum R.P.			
Before	++++	9	9
After	+	5	2
Serum P.R.			
Before	++++	13	12
After	0	5	0
Eluate E.P.			
0.4 mg./ml.	++++	7	7
Eluate P.R.			
0.6 mg./ml.	++++	9	7

* Numbers refer to the positive numbers in serial dilutions starting with 1/20 dilution

gamma globulin employed. The activity which was removed from serum could be readily eluted from the gamma globulin, following washing, by means of urea or acid buffers. Dialysis to remove urea or acid usually caused some precipitation, but highly active preparations could still be recovered. Analytical ultracentrifugation indicated that these preparations consisted of approximately 70 per cent 19 S material.

VI. Composition of the Rheumatoid Factor-Altered Gamma Globulin Precipitate.—Further evidence that aggregated gamma globulin was involved in the precipitation reactions with rheumatoid factor was obtained from ultracentrifugal analyses of the composition of the specific precipitates. The precipitates made from serum, following thorough washing with saline, dissolved readily in glycine-HCl buffers as well as in 6 M urea. Analytical ultracentrifugal

analyses of the dissolved precipitates showed three major peaks. The details of these investigations will be published separately (13), but in general these peaks represented three classes of proteins—one group with an *s* rate of approximately 7 S, a second with a sharp peak of approximately 19 S, and a third heterogeneous group of considerably higher *s* rate but with the major portion sedimenting at approximately 40 S. The latter group represented the aggregates used to obtain the precipitate.

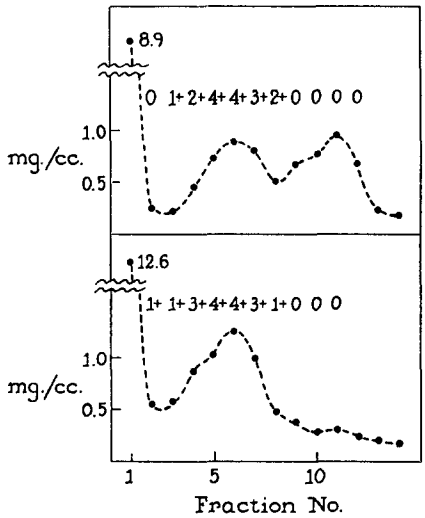

FIG. 5. Curves representing protein concentration in different fractions of dissolved specific precipitates of rheumatoid factor separated by zone centrifugation. The upper curve of precipitates from whole serum shows a 7 S peak in tube 11; the lower curve from isolated rheumatoid factor shows very little 7 S protein. The activity of the fractions in the gamma globulin precipitin test is indicated above the curves and shows the position of the rheumatoid factor. The peak in tube 1 represents aggregated gamma globulin.

Density gradient ultracentrifugation gave similar results. Fig. 5 shows the results of a typical experiment in the density gradient. The heavier fractions are in the early tube numbers. The peak in tube 1 was the pellet that contained all materials with an *s* rate greater than approximately 30 S. The peak in tube 6 represented the active 19 S rheumatoid factor as proven both by activity analyses, as well as by examination in the analytical ultracentrifuge (Fig. 1). This was the standard procedure for isolating the rheumatoid factor. The peak in tube 11 had an *s* rate of approximately 7 S. This peak was barely detectable in the lower curve which represents a precipitate obtained from previously isolated rheumatoid factor, and was only evident when the precipitate was made from whole serum, or from euglobulin fractions. These results suggested that the rheumatoid factor precipitated as a complex when aggregated gamma

globulin was added to whole serum. The ratio of aggregated gamma globulin to rheumatoid factor was quite variable in these experiments.

DISCUSSION

The present observations on the interaction of the rheumatoid factor with antigen-antibody complexes throughout the region of antigen excess, extend the observations of Vaughan (3) on the adsorption of rheumatoid factor to antigen-antibody precipitates. Evidence for interaction with soluble complexes in the region of far antigen excess was obtained. In this region the proportion of "limiting complexes" should be high. All the evidence obtained, however, indicated that only large complexes with an *s* rate greater than 20 S were involved. These complexes appeared similar in size to the soluble aggregates of gamma globulin which precipitate with rheumatoid factor.

The binding of rheumatoid factor to soluble antigen-antibody complexes appeared to depend upon many variables. Among these were the concentration and purity of the rheumatoid factor, the size of the antigen-antibody complexes, the proportions of reactive constituents, and the time of addition of rheumatoid factor to the precipitin system. No reaction between rheumatoid factor and the horse anti-pneumococcal system was observed. This is in keeping with the finding that horse anti-egg albumin precipitates did not bind rheumatoid factor (3). The reaction with human soluble antigen-antibody complexes has not been investigated, but such a process may be important in detailing the role of rheumatoid factor in some of the manifestations of rheumatoid arthritis.

In some respects, the behavior of rheumatoid factor with soluble antigen-antibody complexes and immune precipitates is similar to the reported behavior of complement (14, 15). Weigle and Maurer (16), in a study of the binding of complement to soluble rabbit antigen-antibody complexes in antigen excess, propose two hypotheses to account for this effect. (*a*) Complement fixed to complexes leads to an increase in weight, or neutralization of charge, with subsequent precipitation. (*b*) Complement is multivalent to antigen or antibody, and could link complexes together to form precipitates. Whether rheumatoid factor interacts with antigen-antibody complexes in either of these ways is not known. Hill and Osler (17) noted that antibody which aggregates and flocculates readily, shows the greatest complement-fixing potency. Furthermore, Olhagen (18) showed that aggregated gamma globulin is responsible for anticomplementary effects. This suggests the possibility of an interaction of complement with gamma globulin aggregates similar to that noted with rheumatoid factor. Further studies of complement in these directions appear indicated.

In the past, some authors (19, 20) have suggested that rheumatoid factor may be identified with one of the components of complement. Singer (8) has

performed experiments to show that rheumatoid factor cannot be identified with any of the known components. It is known (14) that heating complement to 56° destroys its capacity to fix to antigen-antibody complexes. Such heating has only slight effects on binding of rheumatoid factor to antigen-antibody complexes or to immune precipitates.

Soluble aggregates of gamma globulin which precipitated readily with rheumatoid factor could be prepared in numerous ways, such as heating to 62–65°C., treatment with the combination of urea and mercaptoethanol, and treatment with guanidine. Short periods of ultracentrifugation furnished concentrated preparations of aggregated gamma globulin that were considerably more reactive per unit of nitrogen. No evidence was obtained for any precipitating activity or significant inhibition activity in the sensitized sheep cell test of non-aggregated human gamma globulin of the native 7 S class. This is in line with the lack of reaction with antigen-antibody complexes of low molecular weight. Similar observations on the important role of aggregated gamma globulin have been made by Christian and Ragan (21).

Vaughan and Waller (22) have recently obtained evidence from ultrafiltration experiments that low molecular weight human gamma globulin shows inhibitory activity in the sensitized red cell system in which red cells are coated with human Rh antibody. The present observations indicate that 7 S rabbit gamma globulin inhibits a similar system where red cells are coated with rabbit antibody. It appears that species specificity is involved, and is brought out in the reaction with native low molecular weight gamma globulins.

Considerable data have been accumulated (5, 6) demonstrating that the rheumatoid factor is a 19 S gamma globulin which exists in serum and plasma in a soluble state complexed to 7 S gamma globulin, but the question of whether the latter represents non-specific gamma globulin, or a specific component in this spectrum of proteins, is not entirely answered. Some preliminary experiments have indicated that isolated 19 S rheumatoid factor combines with normal fraction II gamma globulin to give a soluble complex, but this has not always been the case. It has been more readily demonstrated that native complex which is dissociated in urea recombines following removal of the urea. These results demonstrate further that an affinity exists between rheumatoid factor and low molecular weight gamma globulin, although aggregates are necessary for precipitation.

Little difference is detectable between the reactions of isolated 19 S rheumatoid factor and the native complexed rheumatoid factor. Aggregated gamma globulin precipitates with both. In the case of the native rheumatoid factor the entire complex appears to precipitate with the aggregates, suggesting that the aggregates do not displace the 7 S gamma globulin that already is present but interact at a different site on the 19 S rheumatoid factor molecule. Evidence for such a combined precipitation has been obtained from analyses in

the ultracentrifuge of urea- and acid-dissolved precipitates of rheumatoid factor and aggregated gamma globulin. This has been confirmed through the use of I^{131}-labelled aggregated gamma globulin (13), and it appears clear that considerable 7 S gamma globulin is precipitated in the reaction between rheumatoid factor complex and aggregated gamma globulin. Some evidence has also been obtained of interchange between 7 S gamma globulin in the rheumatoid factor complex and labelled 7 S gamma globulin added to the system. Just how 7 S gamma globulin can inhibit the rheumatoid factor reactions, when the factor is already coated with 7 S gamma globulin in the native complex, remains unclear.

The present observations add little to settle the question of whether the rheumatoid factor is an antibody to gamma globulin, or a complement-like substance. The reactivity with rabbit gamma globulin, which is in general weaker than with human gamma globulin, is not against the antibody hypothesis. Maurer (23) has demonstrated cross-reaction between rabbit and human gamma globulin. The additional possibility raised by the present experiments is that the rheumatoid factor might represent an antibody to antigen-antibody complexes, perhaps related to the type proposed by Najjar and Fisher (24). If this is the case, gamma globulin is only part of the antigen which then gives rise to a group of secondary antibodies which will react with gamma globulin. A search for the other portion of the hypothetical antigen-antibody complex giving rise to the rheumatoid factor might yield considerable further information.

SUMMARY

The effect of highly purified rheumatoid factor on the precipitin reactions of various antigen-antibody systems was determined. The amount of nitrogen precipitated was increased over a broad range when the factor was added to ovalbumin, human albumin, or human gamma globulin, and the corresponding rabbit antibodies. In the zone of antigen excess, soluble antigen-antibody complexes were precipitated by rheumatoid factor.

Soluble aggregates of human and rabbit gamma globulin, produced by heating at 63°C., treatment with urea plus mercaptoethanol or treatment with guanidine, also precipitated with rheumatoid factor. Ultracentrifugal analysis of dissolved specific precipitates showed the presence of aggregated gamma globulin. The sedimentation rate of reactive aggregates was greater than 20 S, and concentrated preparations free of the non-reactive 7 S gamma globulin could be prepared by various procedures of zone centrifugation. These aggregates showed a high inhibitory capacity in the sensitized sheep cell agglutination reaction.

Solid gamma globulin, prepared by heat denaturation, also selectively adsorbed the rheumatoid factor, and removed or decreased the activity in the

various precipitation and agglutination reactions. Elution of highly purified active preparations from the solid gamma globulin could be carried out with urea or acid buffers.

Evidence for interaction between rheumatoid factor and low molecular weight gamma globulin without precipitation, was also obtained. This interaction appears to occur in the circulation of patients with rheumatoid arthritis.

The question of whether the rheumatoid factor represents an antibody to gamma globulin was discussed. Points of similarity to the behavior of complement also were cited.

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