Characterization of the γ-Globulin Complexes Present in Certain Sera Having High Titers of Anti-γ-globulin Activity *

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It is well established that the principal anti-γ-globulin factors in the sera of patients with rheumatoid arthritis detected by such commonly employed procedures as the sensitized sheep cell agglutination and latex fixation tests are immunoglobulins of the γM (19 S γ-globulin) (2) class (3–6). These anti-γ-globulins, often referred to as rheumatoid factors, are not limited in occurrence to rheumatoid arthritis, but are sometimes found in a variety of other disease states (7–10). Extensive evidence is now available in support of the concept that these factors are indeed antibodies to various antigenic determinants on the γG (7 S γ-globulin) (2) molecule. This evidence has been reviewed by Kunkel (11, 12).

Certain sera having high titers of anti-γ-globulin activity contain significant quantities of materials sedimenting at a rate intermediate to the 7 and 19 S components (13, 14). Such sera have been encountered in patients having rheumatoid arthritis or a number of other disorders (13–15). These materials were shown to be complexes of γG that are readily dissociated in acid buffer or urea (14). It seemed possible that the complexes might represent antigen-antibody complexes of the γ-globulin-anti-γ-globulin type. This possibility was supported by the subsequent demonstration of a low molecular weight anti-γ-globulin activity in such sera (16). This activity was clearly distinguished from γM activity in that it was not sensitive to mercaptoethanol treatment. A cryoglobulin of the γG class that precipitated with aggregated γ-globulin has also been described (17).

The present study was undertaken in an effort to further characterize the γ-globulins responsible for complex formation. This followed an initial observation that fractions containing intermediate complexes precipitated aggregated γ-globulin. These fractions appeared to be free of γM anti-γ-globulins. The evidence obtained clearly supports the concept that the active γ-globulins of the complexes are in fact antibodies to γG. The technique of fragmentation with pepsin (18, 19) was particularly useful in this respect and in defining the specificity of these anti-γ-globulins.

Methods

The intermediate complex preparations were isolated from the sera of three patients (Do, Od, and Go). These sera had high titers in the various reactions for the detection of rheumatoid factors and contained moderate or large amounts of intermediate γ-globulin complexes. All three patients had rheumatoid arthritis. Euglobulin precipitates were prepared and fractionated on Sephadex G-200, using pH 4.1 sodium acetate buffer, 0.1 mole per L, as previously described (20). Column fractions containing the dissociated intermediate complexes were pooled, dialyzed against pH 7.5 sodium phosphate buffer, 0.2 ionic strength, and concentrated by ultrafiltration to a protein concentration of approximately 10 mg per ml.

Reduction by ethyl mercaptan and alkylation by iodoacetamide were by the procedures previously described (20). Samples that were reduced by mercaptoethanol

* Submitted for publication August 19, 1965; accepted December 16, 1965.

A preliminary report of this work was presented at the annual meeting of the American Rheumatism Association, Philadelphia, Pa., June 18, 1965, and appeared in abstract form (1).

This work was supported by grants TI AM 5000 and AM 03555 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, and Merck and Company, Rahway, N. J.

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were made 0.1 M concentrations with respect to 2-mercaptoethanol and allowed to stand at room temperature for 3 hours or were dialyzed against 0.1 M mercaptoethanol in pH 7.5 buffer for 16 hours. At the end of the reduction period all samples were dialyzed against 0.02 M iodoacetamide in pH 7.5 buffer and then buffer.

The preparations containing intermediate complexes were digested by pepsin at pH 4.1 according to the procedure of Nisonoff, Wissler, Lipman, and Woernley (18). The ratio of substrate to enzyme employed was 100:1. Digestion mixtures were incubated for 16 to 18 hours at 37°C. Digestion was terminated by dialysis against pH 7.5 sodium phosphate buffer, 0.2 ionic strength, in the cold. The fragments obtained by this procedure will be referred to as F(ab')2 fragments.

Ultracentrifugal analysis was carried out in a Spinco model E ultracentrifuge with double sector 12-mm cells. Plate measurements were made according to the procedures described by Schachman (21) or Markham (22). The sedimentation coefficients were corrected to the S20,w values by the accepted procedures. The partial specific volumes were assumed to be 0.73.

Density gradient ultracentrifugation was performed according to the general methods described by Kunkel (23). Continuous gradients of 10 to 40% sucrose prepared in either pH 7.5 sodium phosphate buffer, 0.2 ionic strength, or pH 3 glycerine-saline buffer, 0.1 ionic strength, were utilized. Centrifugation was performed by using an SW-39 rotor in the Spinco model E ultracentrifuge at 33,450 rpm for 16 hours. After centrifugation serial fractions were collected from the bottom of the tube. The activities of fractions from either the pH 3 or 7.5 gradients could be determined without prior dialysis. The buffers employed as diluents in the slide precipitin or latex fixation tests were sufficient to completely neutralize the relatively weak pH 3 buffer. Also there was no indication that the sucrose interfered with the activity measurements. In some experiments pooled fractions from the various levels of both neutral and acid gradients were analyzed after dialysis against pH 7.5 buffer. In all cases the results corresponded exactly to those obtained without prior dialysis.

 Reactivity with aggregated γ-globulin was determined by precipitin formation in capillary tubes (24) or by a simple glass slide technique. Aggregated γ-globulin was prepared by heating human Cohn Fraction II at 63°C for 10 minutes. The aggregates were isolated by preparative ultracentrifugation and resuspended in saline or pH 7.5 buffer. A concentration of about 2.5 mg per ml was used for capillary tests. A concentration of 1 mg per ml was found satisfactory for the slide test. The slide test was performed by placing one drop each of sample, pH 7.5 buffer, and aggregated γ-globulin solution in the well of a serological slide and mixing on a rotary agitator for 15 minutes. Precipitation was evaluated with the aid of low magnification. This test was particularly useful for gradient fractions. The results of either test were graded on a zero to four plus scale and were found to correspond closely.

The optimal concentrations of samples and aggregated γ-globulin for preparative scale precipitates were estimated by using the slide test, and they varied somewhat from one sample to another. Precipitates of the intermediate complex preparations from patients Do and Od were prepared by mixing equal volumes of sample (3 mg per ml in pH 7.5 buffer), aggregated γ-globulin (6 mg per ml in saline), and pH 7.5 buffer. The precipitate of the complex preparation from patient Go was prepared by mixing equal volumes of sample (5 mg per ml in pH 7.5 buffer), aggregated γ-globulin (2.5 mg per ml in saline), and pH 7.5 buffer. Precipitates of the F(ab')2 fragments of the complex preparations were prepared by mixing equal volumes of sample (approximately 6 mg per ml in pH 7.5 buffer), aggregated γ-globulin (7.5 to 10 mg per ml in saline), and pH 7.5 buffer. The mixtures were incubated at room temperature with occasional agitation for 1 hour then in the cold overnight. In all instances the precipitate began to form within a few minutes after mixing. No precipitate formed in controls prepared with buffer in place of the sample. The precipitates were washed twice by large volumes of buffered saline. The precipitates were finally suspended in a small volume pH 3 glycerine-saline buffer, 0.1 ionic strength, and then dialyzed against the same buffer. The dialysis was necessary to effect complete solution, since pH 7.5 buffer trapped in the precipitate partially neutralized the small volume of relatively weak pH 3 buffer.

The latex fixation test was performed according to the procedure of Singer and Plotz (25). Agglutination tests with sensitized sheep cells or human group O Rh-positive cells coated with Ripley incomplete anti-Rh antibodies were performed by the usual procedures (26, 19). Precipitating antisera specific for γG were produced by hyperimmunizing rabbits with human Cohn Fraction II in complete Freund's adjuvant and subsequently absorbing the antisera with F(ab')2 fragments of Cohn Fraction II. Precipitating antisera specific for γM were produced by hyperimmunizing rabbits with electrophoretically isolated Waldenstrom's macroglobulins in complete Freund's adjuvant and subsequently absorbing the antisera with human Cohn Fraction II. Precipitating antisera specific for γA prepared in the goat were commercial products.

Ouchterlonry agar diffusion studies (27) were performed in 2.0% agar in pH 8.6 barbital buffer, 0.05 ionic strength. Protein concentrations were estimated by the Folin-Ciocalteu method (28) with Cohn Fraction II as the standard.

Results

Isolation and properties. The euglobulin precipitates from the various sera contained moderate

3 The Cohn Fraction II preparations utilized in this study were the products of Mann Research Laboratories, New York, N. Y., or Lederle Laboratories, Pearl River, N. Y.

4 Serum from patient Ripley was kindly provided by Dr. Marion Waller, Medical College of Virginia, Richmond, Va.

5 Mann Research Laboratories, New York, N. Y.
or large amounts of intermediate complexes in addition to the usual 7 and 19 S materials. Separation was accomplished by gel filtration on Sephadex G-200 with pH 4.1 sodium acetate buffer. The fractionation of a euglobulin preparation from patient Go is shown in Figure 1. Under these conditions, the intermediate complexes were dissociated into their 7 S components and recovered with the 7 S materials in the second peak from the column. The materials contained in this peak, neutralized and concentrated, were utilized in this study without further purification. The 19 S materials, including the γM anti-γ-globulins, were recovered in the first peak. The elution pattern obtained with the euglobulin preparations from Patient Od were very similar to those from Go. The euglobulins from Patient Do differed in that a smaller quantity of 19 S materials was recovered. The elution patterns did not vary in an appreciable manner for different euglobulin preparations from the same patient or serum sample.

Examples of the ultracentrifugal patterns of the intermediate complex preparations, in pH 7.5 buffer, from the three patients are shown in Figure 2. Three preparations from patient Do were examined and gave essentially identical patterns. All demonstrated a small 7 S peak, a large broad 16 S peak, and a small amount of faster sedimenting materials separated poorly from the 16 S peak.

The relative amounts of materials sedimenting at 16 S or faster were 83, 85, and 86%. Two preparations from Go and one from Od were examined. All demonstrated larger 7 S peaks than the Do preparations. The relative amounts of complexes in the Go preparations were 41 and 62%. About 50% of the Od preparation sedimented as complexes. The various preparations dissociated completely in pH 4 sodium acetate buffer, 0.1 mole per L, and sedimented as a single symmetrical peak at 6 S. The sedimentation rate for human γG (Cohn Fraction II) in the pH 4 buffer was the same as that observed for the dissociated complexes. The absence of fast sedimenting components after acid dissociation demonstrated that the preparations were relatively free of γM, which does not dissociate at low pH and continues to sediment rapidly (14).

Ouchterlony agar diffusion indicated that, in addition to γG, the intermediate complex preparations contained at most trace amounts of γM and
TABLE I
Activities of preparations containing intermediate complexes isolated from euglobulins by gel filtration

<table>
<thead>
<tr>
<th>Patient</th>
<th>Preparation</th>
<th>Precipitation with aggregated γ-globulin*</th>
<th>Latex fixation titer†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do</td>
<td>I</td>
<td>+++</td>
<td>1:640</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>+++</td>
<td>1:320</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>+++</td>
<td>1:320</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>+++</td>
<td>1:1,280</td>
</tr>
<tr>
<td>Od</td>
<td>I</td>
<td>+++</td>
<td>1:320</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>+++</td>
<td>1:640</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>+++</td>
<td>1:320</td>
</tr>
<tr>
<td>Go</td>
<td>I</td>
<td>+++</td>
<td>1:160</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>+++</td>
<td>1:640</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>+++</td>
<td>1:160</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>+++</td>
<td>1:160</td>
</tr>
</tbody>
</table>

* The strengths of precipitation with aggregated γ-globulin by the samples at protein concentrations of 10 mg per ml in the capillary tube or slide tests are listed. Multiple determinations were performed on most preparations and were within 1 U of the listed values.
† The titers for samples having protein concentrations of 10 mg per ml are listed. Multiple determinations were performed on many preparations and demonstrated a maximal variation of two tubes of a serial twofold dilution.

small amounts of γA. Two preparations from patient Do, two from Go, and one from Od were each tested with one anti-γA, two anti-γM, and two anti-γG antisera. Most tests were performed at least twice, with complete agreement in all instances. When tested at 10 mg per ml, each preparation formed strong precipitin lines with only the anti-γG antiserum. No lines were formed by the Do preparations with the other antisera. The anti-

γA antiserum formed weak lines and the anti-γM antiserum formed very weak lines with the Go and Od preparations. No lines were detected with these antisera when the preparations were diluted to 1 mg per ml. The anti-γG antiserum continued to give strong precipitin lines with the diluted preparations.

The activities of preparations from the three patients are listed in Table I. The various preparations from all three patients gave moderately strong or strong precipitates with aggregated γ-globulin when tested at 10 mg per ml concentration. This precipitin activity could be detected at concentrations of 1 mg per ml or less. Substitution of saline for either the sample or the aggre-

γ-globulin did not result in precipitation. The preparations also agglutinated latex particles coated with Fraction II. The agglutinations were usually strong, being comparable to moderately reactive rheumatoid sera. Multiple determinations were performed on many of the preparations and varied by no more than two tubes of a serial twofold dilution. Two preparations from each of the three patients were tested and found not to agglutinate sensitized sheep cells. Several preparations from Do and Go were tested with human cells coated with incomplete anti-Rh antibody and found inactive towards this detection system, even at high protein concentrations. The single preparation from Od tested with the coated human cells was weakly positive at 10 mg per ml but negative.

TABLE II
Effect of ethyl mercaptoethanol reduction on the activities of intermediate complex preparations (Do and Go) and an isolated γM anti-γ-globulin (Jo)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>Latex fixation* µg/ml</th>
<th>Precipitation with aggregated γ-globulin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do</td>
<td>Untreated</td>
<td>31</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>31</td>
<td>+++</td>
</tr>
<tr>
<td>Go</td>
<td>Untreated</td>
<td>16</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>16</td>
<td>+++</td>
</tr>
<tr>
<td>Jo</td>
<td>Untreated</td>
<td>0.9</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>Negative at 115</td>
<td>0</td>
</tr>
</tbody>
</table>

* The protein concentrations of the maximal dilutions giving a one plus agglutination are recorded.
† The strengths of precipitation of the undiluted samples with aggregated γ-globulin in the capillary tube test are listed. The protein concentrations were 10 mg per ml each for Do and Go and 2.3 mg per ml for Jo.

TABLE III
Effect of mercaptoethanol reduction on the activities of intermediate complex preparations (Do and Od) and a typical rheumatoid arthritis (R.A.) serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>Latex fixation* µg/ml</th>
<th>Precipitation with aggregated γ-globulin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do</td>
<td>Untreated</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reduced, 3 hours</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>Od</td>
<td>Untreated</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reduced, 3 hours</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>R.A. serum</td>
<td>Untreated Reduced, 3 hours</td>
<td>1:2,560</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reduced</td>
<td>Negative at 1:20</td>
<td>0</td>
</tr>
</tbody>
</table>

* The protein concentrations of the maximal dilutions giving a one plus agglutination are listed for the complex preparations.
† The strengths of precipitation with aggregated γ-globulin in the slide test of the Do and Od samples at protein concentrations of 1 mg per ml and the R.A. serum samples at a dilution of 1:10 are listed.
most procedures, is lost after dissociation under conditions similar or identical to those used above (3, 6, 16, 20).

The distributions of the anti-γ-globulin activities among the components of the preparations were examined by density gradient ultracentrifugation. Analysis of an intermediate complex preparation from patient Do on a pH 7.5 gradient is illustrated in the upper section of Figure 3. Both the aggregated γ-globulin precipitation and latex fixation activities were associated principally with the

![Figure 3](image-url)  
**Figure 3.** Density gradient ultracentrifugal experiments showing the distribution of anti-γ-globulin activity in an intermediate complex preparation from patient Do (upper section) and an acid-dissociated sample of the same preparation (lower section). A pH 7.5 gradient was used for the experiment in the upper section and a pH 3 gradient in the lower. The gradient fractions are numbered from the bottom. Aggregated γ-globulin precipitation and latex fixation are indicated. The activity of the intermediate complex preparation was associated with the faster sedimenting materials. After dissociation of the complexes, the activity was associated with the 7 S components.

at greater dilutions. Since the γM anti-γ-globulins isolated from the same serum samples were highly active toward the coated human cells, it was not likely that the activities detected in the complex preparations were due to the presence of small amounts of γM anti-γ-globulins. This conclusion was supported by the other findings of this study.

The activities of several samples each of preparations from Do and Od and one from Go were determined after ethyl mercaptan or mercaptoethanol treatment. The results are listed in Tables II and III. In contrast to controls containing γM anti-γ-globulin activity, no significant losses of activity were observed for the intermediate complex preparations. Numerous studies have shown that the activity of γM anti-γ-globulins, as measured by

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6 The γM anti-γ-globulins from Do and Od were the subject of a previous investigation (20). The titer of the γM anti-γ-globulin from Go (2.8 mg per ml) with human red cells coated with Ripley anti-Rh antibody was 1:20,480.

![Figure 4](image-url)  
**Figure 4.** Ultracentrifugal analyses of the materials precipitated by aggregated γ-globulin from intermediate complex preparations from patients Do (top), Od (middle), and Go (bottom) showing the presence of 7 S and the absence of 19 S materials. The precipitates were dissolved in pH 3 glycine-saline buffer. The patterns on the left were recorded immediately after acceleration to 52,640 rpm, and each shows a broad fast sedimenting peak due to the aggregates and a slow sedimenting peak near the meniscus. The patterns on the right were recorded 32 minutes later and each shows a single 6.5 S peak. Sedimentation proceeds from left to right.
faster sedimenting materials (i.e., the complexes). An acid-dissociated sample of the same preparation was fractionated simultaneously on a gradient prepared in pH 3 buffer. The results are illustrated in the lower section of Figure 3. Dissociation of the complexes into their 7 S components was accompanied by a corresponding decrease in the sedimentation of the activity. These observations are representative of three such studies on preparations from this patient. Comparable results were also obtained with preparations from the other two patients. No γM activity was detected in any preparation examined by this method. Control experiments, with isolated γM anti-γ-globulins or rheumatoid sera, demonstrated that this class of anti-γ-globulins would have been recovered from the bottom (tubes 2 through 8) of either gradient. These experiments demonstrated clearly that the activities were associated with the acid-dissociable complexes.

Studies on aggregated γ-globulin precipitates. The active components contained in the intermediate complex preparations were further characterized by ultracentrifugal analysis of the precipitates formed with aggregated γ-globulin. Before analysis, the precipitates were washed and dissolved in pH 3 buffer. Two preparations from patient Do and one each from Od and Go were examined by this technic. Examples of the resulting patterns are shown in Figure 4. Each demonstrated a rapidly sedimenting broad boundary due to the aggregated γ-globulin and a single component sedimenting at 7 S. No 19 S materials were detected in any of the samples analyzed.

The 7 S materials contained in dissolved precipitates were shown to be active by density gradient ultracentrifugal studies with pH 3 gradients. The results with a precipitate of a Go preparation are illustrated in Figure 5. The anti-γ-globulin activity was clearly associated with the 7 S components. Similar results were obtained with precipitates from the other two patients. The 7 S materials were also isolated from dissolved precipitates of intermediate complex preparations from patient Do by either preparatory ultracentrifugation or by gel filtration on Sephadex G-200 with the pH 3 buffer. The isolated materials, after dialysis into pH 7.5 buffer, formed intermediate complexes and precipitated with aggregated γ-globulin. The ultracentrifugal pattern was quite similar to that of the preparation before precipitation.

Properties of the F(ab')2-fragments. Antibodies of the γG class have been shown to be cleaved by pepsin, in the absence of mercaptans, into bivalent fragments (18, 19). These fragments, termed F(ab')2-fragments, sediment in the ultracentrifuge at 5 S. If the γ-globulins responsible for the complexes are antibodies of the γG

FIG. 5. DENSITY GRADIENT ULTRACENTRIFUGAL EXPERIMENT SHOWING THE DISTRIBUTIONS OF AGGREGATED γ-GLOBULIN PRECIPITATION AND LATEX FIXATION ACTIVITY IN THE MATERIALS PRECIPITATED BY AGGREGATED γ-GLOBULIN FROM AN INTERMEDIATE COMPLEX PREPARATION FROM PATIENT Go. The precipitate was dissolved in pH 3 glycine-saline buffer and the gradient prepared in the same buffer. Gradient fractions are numbered from the bottom. The results demonstrate that the slow sedimenting materials in the precipitate were active.

Fig. 6. Ultracentrifugal experiments illustrating the absence of complexes after peptic digestion of an intermediate complex preparation from patient Do and the formation of complexes by the resulting F(ab')2-fragments with human γG. Upper pattern: the pepsin-digest; middle pattern: γG control; lower pattern: after addition of γG to the pepsin-digest. The patterns were recorded after 64 minutes at 52,640 rpm. Sedimentation proceeds from left to right. All samples were in pH 7.5 sodium phosphate buffer, 0.2 ionic strength. The concentration of the intermediate complex preparation before digestion was 10 mg per ml. That of the γG in the control and mixture was 6 mg per ml.
class, it would be reasonable to expect their F(\(ab\'))\(_2\)-fragments to be active. Samples of the various intermediate complex preparations were therefore subjected to this system of degradation and the resulting fragments characterized.

Examples of the ultracentrifugal patterns of the pepsin-digested complex preparations in pH 7.5 buffer are shown in Figures 6 and 7. A minimum of two such preparations from each of the three patients were examined by this technic. The characteristic fast sedimenting complexes were absent after peptic digestion. Also, no 7 S materials were detected. The preparations contained large quantities of F(\(ab\'))\(_2\)-fragments sedimenting at 5.2 to 5.5 S. Small amounts of slower sedimenting components were also present. The effectiveness of the fragmentation procedure was also indicated by Ouchterlony agar diffusion studies on a representative preparation from each of the three patients. By using an antiserum to human Fraction II, the pepsin split complex preparations gave the expected reaction of partial identity with the untreated preparation (19).

The activities of the F(\(ab\'))\(_2\)-fragments and the corresponding untreated intermediate complex preparations are listed in Table IV. All the pepsin-digests examined (six from Do, four from Od, and two from Go) continued to precipitate strongly with aggregated \(\gamma\)-globulin. Two of the digest from each patient were tested for latex fixation activity. Although the titers were comparable to the original material, the strengths of agglutination were sometimes less. This was especially apparent for some of the Go and Od fragment preparations. Conversion of the F(\(ab\'))\(_2\)-fragments to the univalent fragments by reduction with 0.01 M cysteine and alkylation with 0.02 M iodoacetamide resulted in the expected loss of activity and decrease in sedimentation rate to 3.5 S. The F(\(ab\'))\(_2\)-fragments did not agglutinate human red cells coated with incomplete anti-Rh antibody.

Peptic digestion of the \(\gamma\)G molecule under the conditions utilized in this study results in the destruction of part of the molecule (18, 19). For human \(\gamma\)G, this part is approximately equivalent to the fast- or Fc-fragment produced by papain cleavage. The absence of complexes in the intermediate complex preparations after peptic digestion, even though the resulting fragments demonstrated anti-\(\gamma\)-globulin activity, indicated that the activity of these preparations is specific for groups in the part of the molecule destroyed by the pepsin. Further evidence for this was obtained by the formation of fast sedimenting complexes with added human \(\gamma\)G (Fraction II \(\gamma\)-globulin). The results of such an experiment with a fragment preparation from patient Do are shown in Figure 6. A large amount of complexes sedimenting at 8.6 S and a small amount sedimenting at a faster rate were present in the mixture. This was accompanied by a corresponding decrease in the quantity of 5 S fragments. The complexes were dissociated into 5 and 6 S components after dialysis into 0.1 M sodium acetate buffer, pH 4.1. Essentially identical

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**Fig. 7. Ultracentrifugal experiments illustrating the absence of complexes after peptic digestion of an intermediate complex preparation from Od and the formation of complexes by the resulting F(\(ab\'))\(_2\)-fragments with human \(\gamma\)G.** a = the pepsin-digest; b = \(\gamma\)G control; c = after addition of \(\gamma\)G to the pepsin-digest; and d = the mixture after acid dissociation. The patterns were recorded after 48 minutes at 56,100 rpm. Sedimentation proceeds from left to right. Samples a, b, and c were in pH 7.5 sodium phosphate, 0.2 ionic strength. Sample d was in 0.1 M sodium acetate, pH 4.1. The concentration of the intermediate complex preparation before digestion was 10 mg per ml. The concentration of the \(\gamma\)G in the control and mixture was 10 mg per ml.
results were obtained with a second fragment preparation from this patient.

The results of a similar experiment with an F(ab')2-fragment preparation from patient Od are shown in Figure 7. Although the results were not as dramatic as with the fragments from patient Do, the formation of complexes after the addition of γG was apparent. These sedimented at approximately 9 and 11 S and were dissociated after dialysis into the pH 4.1 buffer. The ultracentrifugal pattern of the dissociated mixture resolved into two components about equal in size sedimenting at about 5 and 6 S. These observations were confirmed by using a second fragment preparation from this patient. The results obtained when human γG was added to fragment preparations from patient Go were similar to those shown for the fragment preparation from patient Od.

The active components in two F(ab')2-fragment preparations from patient Do and one from Od were further characterized by ultracentrifugal analysis of the materials precipitated by aggregated γ-globulin (Figure 8). Before analysis, the precipitates were washed and dissolved in pH 3 buffer. In addition to the aggregates, only a single 5 S component was detected. It was not possible to examine Go fragments by this method due to the limited supply of serum from this patient. Density gradient ultracentrifugal studies of the dissolved precipitates on pH 3 gradients provided additional evidence that the anti-γ-globulin activity was associated with the 5 S materials. Similar results were obtained when a fragment preparation from patient Do was analyzed on a pH 7.5 gradient.

### Discussion

Preparations that demonstrated large or moderate amounts of intermediate complexes by ultracentrifugal analysis were found to precipitate aggregated γ-globulin and agglutinate Fraction II-coated latex particles. Density gradient ultra-
centrifugation demonstrated that the activity was associated principally with the fast sedimenting materials in the preparations. A variety of evidence indicated that the activity was not due to the presence of γM anti-γ-globulins, but was in fact due to a γG anti-γ-globulin. The preparations were found to be essentially free of γM by both analytical and density gradient ultracentrifugal analyses after acid dissociation. Only 7 S materials were detected. Gamma M would have been detected readily since, unlike intermediate complexes, they are not dissociated at low pH and continue to sediment rapidly (14). Ouchterlony agar diffusion demonstrated at most trace amounts of γM. Only γG was detected in significant amounts. The γM anti-γ-globulins from the sera studied were highly reactive towards sensitized human cells, whereas the complex preparations were not. The presence of sufficient γM anti-γ-globulin to give the activities detected would have most certainly resulted in significant activity towards the sensitized cells. The activity of the complex preparations also differed from that of typical γM anti-γ-globulins in that it was not sensitive to treatment by mercaptans.

Direct evidence that the activity was indeed associated with the 7 S components of the complexes was obtained by density gradient ultracentrifugal studies of acid-dissociated preparations. These studies failed to show the presence of γM (19 S) activity. Only 7 S activity was detected. Control studies demonstrated that γM anti-γ-globulin activity would have been easily detected by this technique. Similarly, analytical ultracentrifugal analyses of the materials precipitated by aggregated γ-globulin, dissolved in pH 3 buffer, revealed only large quantities of 7 S materials. These materials, isolated from the dissolved precipitates, were shown to be active and to form complexes.

The results of the peptic digestion studies were consistent with those expected for a typical γG-antibody (18, 19). After digestion, the preparations sedimented at 5 S and continued to react in the tests for anti-γ-globulin activity. The activity was shown by a number of studies to be associated with the 5 S fragments and not undigested material. The absence of detectable amounts of components sedimenting faster than 5 S indicated a minimum of undigested material. The distribution of activity in a pepsin-digest was observed to correspond to the 5 S materials by density gradient ultracentrifugation. Analytical ultracentrifugal analyses of the materials precipitated by aggregated γ-globulin from pepsin-digests demonstrated only 5 S components. The fragments eluted from the precipitates were shown to be active by density gradient ultracentrifugation. The activity of the pepsin-digests differed from that of the untreated preparations in that it was sensitive to treatment by mercaptans. This sensitivity is consistent with that demonstrated for the 5 S fragments produced from antibodies by peptic digestion (18). The specificity of these γG anti-γ-globulins was also evident from the digestion studies. The absence of complexes in the pepsin-digests at neutral pH indicated that the group or groups that react with the active sites to form complexes were destroyed. This was further indicated by the formation of complexes with unaltered γG.

From the results of these studies it is apparent that the γG factors responsible for the formation of intermediate complexes are antibodies specific for one or more groups on the part of the γG molecule destroyed by peptic digestion. Intermediate complexes are therefore formed by the binding of γG to the active sites of γG anti-γ-globulins. Some evidence that the intermediate complexes represent antigen-antibody complexes was also obtained by Kunkel, Müller-Eberhard, Fudenberg, and Tomasi (14). It was found that the addition of γG to sera or euglobulin fractions produced marked effects on the ultracentrifugal distribution of the complexes. Those complexes sedimenting at 10 S were increased at the expense of those sedimenting at faster rates. The effect of the excess γG suggested the action of excess antigen on antigen-antibody complexes. In contrast to the present study, efforts by these workers to demonstrate an anti-γ-globulin activity for the complexes by various serological tests were not successful. The reason for this is not apparent. However, in a later study, Chodirker and Tomasi (16) demonstrated an anti-γ-globulin activity by the latex fixation test in fractions that contained intermediate complexes, but were free of γM. These fractions were isolated from sera by ion exchange chromatography or density gradient ultracentrifugation. In contrast to control rheumatoid sera, considerable activity remained in sera containing
complexes after mercaptoethanol treatment. The active components and the specificity were not characterized further. A cryoglobulin of the γG class that had the properties of the intermediate complexes and precipitated with aggregated γ-globulin has also been reported (17). The activity was not sensitive to mercaptoethanol treatment and was shown to be associated with the 7 S components.

The classical γM anti-γ-globulins also react with antigenic determinants in the part of the γG molecule destroyed by peptic digestion (19, 29). Since this part of the molecule appears to contain several such determinants (12), the exact relationships of the specificities of the two classes of anti-γ-globulins will require additional studies. The difference in the capacity of the γM and γG anti-γ-globulins from the same patient to agglutinate sensitized red cells suggests that the specificities are not identical. Since the γM anti-γ-globulins in individual sera can be heterogeneous with respect to reactivity in various test systems (30), it is possible that some molecules of corresponding specificity exist. The possibility also exists that this may reflect differences in avidity or agglutinating efficiency. Such differences have been demonstrated for other γM and γG antibodies (31). Inhibition as a factor in the failure of the γG anti-γ-globulins to agglutinate sensitized cells seems unlikely since the F(ab')2-fragments of the preparations also failed to agglutinate sensitized human cells. The potentially inhibitory part of the γG molecule was destroyed by the digestion procedure (19).

The accumulated evidence from this and other laboratories indicates that the anti-γ-globulins responsible for intermediate complex formation are γG counterparts to the classical γM anti-γ-globulins. The existence of such counterparts constitutes additional evidence that the rheumatoid factors represent an immune response to some form of γG. The existence of both γG and γM anti-γ-globulins of similar specificities is consistent with the known molecular heterogeneity of antibodies. The simultaneous presence of activity in all three major classes of immunoglobulins has been demonstrated for a variety of human antibodies, including antinuclear (32) and antithyroglobulin (33) factors. Also pertinent in this respect is the reported identification of γA anti-γ-globulin factors that are reactive with human and rabbit γ-globulin (34). The existence of human γG anti-γ-globulins is also consistent with the production of both 7 S and 19 S anti-γ-globulin factors in rabbits immunized with altered autologous γ-globulin (35). The production of both γM and γG antibodies in response to a single antigen commonly occurs in a variety of species, including humans (36-39). The γM antibodies have been shown to be the first antibodies produced in response to an antigenic stimulus. The γG antibodies usually appear later and frequently persist over a longer period of time. Often repeated or larger doses of antigen are required to produce γG antibodies. The incidence and distribution of the intermediate complexes appears compatible with these experimental data concerning the relationships of the γG and γM antibodies. Although exact information is not available, it is known that complexes are found in large quantities in only a limited number of patients and are usually associated with high titers of γM anti-γ-globulin activity (14).

A group of γG anti-γ-globulins that are specific for antigenic determinants in human γG revealed by peptic digestion have been described (19). These factors do not form complexes and are clearly distinguished from the anti-γ-globulins responsible for intermediate complex formation in their specificity and serological activity. These factors agglutinate red cells coated with the Fab or F(ab')2-fragments of an incomplete anti-Rh antibody but do not react in the common tests for rheumatoid factor activity.

Summary

Preparations containing significant amounts of γ-globulin complexes of the intermediate type were isolated from the sera of three patients with rheumatoid arthritis. The preparations precipitated heat-aggregated γ-globulin and agglutinated latex particles coated with Fraction II γ-globulin. Evidence from a variety of studies demonstrated that the anti-γ-globulin activities were not due to the presence of small amounts of γM (19 S) anti-γ-globulins, but were associated with the γG (7 S) components of the complexes.

The complex preparations were degraded by peptic digestion to fragments that sedimented at 5 S. Complexes and 7 S materials were not pres-
ent after digestion. The fragments demonstrated anti-γ-globulin activities similar to those of the original preparations and were found to form complexes with unaltered human γG.

The results provide evidence that the γG factors responsible for the formation of the intermediate complexes are antibodies that are specific for groups on the part of the γG molecule destroyed by peptic digestion. This group of anti-γ-globulins appears to represent γG counterparts to the classical γM anti-γ-globulins (19S rheumatoid factors).

Acknowledgments

The author wishes to thank Dr. Henry G. Kunkel for his helpful advice and Mrs. Mary Nell Beard for her capable technical assistance.

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