Reversible Dissociation of Fragment Fc of Rabbit γG-Immunoglobulin*

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SUMMARY

Crystallized Fragment Fc of rabbit γG-globulin, prepared by digestion with papain in the presence of 0.05 M L-cysteine, dissociates into half-fragments in 0.05 M NaCl at pH 2.7. Dissociation is approximately half-complete at pH 3.1. The conditions are similar to those required for dissociation of γG-globulin into half-molecules, which supports other evidence that the noncovalent interactions between heavy chains are largely localized in Fragment Fc. Schlieren patterns, molecular weights, and results of gel filtration indicate that two subfractions, approximately equal in size, are liberated at neutral pH, recombination of the half-fragments occurs spontaneously through noncovalent interaction.

After proteolysis in the presence of a low concentration of reducing agent, the resulting crystallized Fc is largely undissoziable at low pH but is rendered dissociable by further reduction and alkylation.

If alkylation is omitted, after a 1-hour or 4-hour digestion with papain and cysteine, a large proportion of the Fc fragments become undissociable after removal of the reducing agent by dialysis, owing to re-formation of an interchain disulfide bond; dissociability is restored by a second reduction.

Prolonged digestion with papain results in a decrease in the S-carboxymethylcysteine content of alkylated Fc fragments, and in loss of the capacity to reoxidize, if alkylation is omitted. A probable explanation is that the enzyme slowly attacks that region of Fragment Fc which contains the interchain disulfide bond.

Dissociation into half-molecules can be accomplished at low pH after reduction of one disulfide bond in most, if not all, γG-globulin molecules (8, 9). Circumstantial evidence indicates that this is the same bond as that which joins two univalent fragments after proteolysis with pepsin (10). Direct evidence that this disulfide bond links two heavy chains was the finding, after a minimal reduction and alkylation sufficient to permit separation of half-molecules, that the S-carboxymethylcysteine was present almost exclusively in heavy chains (9).

Fleischman, Pain, and Porter (2), Fleischman, Porter, and Pecas (11), and Olin and Edelman (12) have presented evidence, based on analysis of antigenic determinants, that fragment Fc of rabbit or human γG-globulin consists of portions of two heavy chains. Evidence for symmetry within Fragment Fc has come from the studies of Marler, Nelson, and Tanford (6), who showed that the molecular weight of reduced, alkylated Fc decreases to approximately half its initial value in the presence of 6 M guanidine hydrochloride. Also, the number of spots in a peptide map of Fc is equal to about half the number of bonds hydrolyzed by trypsin (13, 14). Thus, there is good evidence that Fragment Fc comprises two similar portions of heavy chains, held together at neutral pH after reduction by noncovalent interactions. Although the crystallizability of Fragment Fc is indicative of its relative homogeneity as compared to that of γG-immunoglobulin, there is evidence for microheterogeneity among the population of fragments (15-21).

There is some indication that the remainder of the heavy chains, which are present in the Fab fragments, do not interact strongly with one another through noncovalent bonds. Thus, proteolysis with papain or pepsin in the presence of cysteine liberates the two types of Fab fragments at neutral pH in the absence of any dissociating agent (22, 23). This evidence is not conclusive, since it is possible that there are noncovalent interactions between the two univalent fragments which are disrupted by the enzyme. On the assumption that the noncovalent interactions responsible for holding together half-molecule subunits after reduction of interchain bonds are largely present in Fragment Fc, one would expect that reduced Fragment Fc might dissociate into halves under conditions of low pH similar to those which result in the separation of half-molecules from reduced γG-immunoglobulin. The present report provides evidence that this is the case. In addition, half-fragments were found to recombine spontaneously at neutral pH. It is also

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A molecule of rabbit γG-immunoglobulin comprises two "light" chains with an approximate molecular weight of 20,000 and two "heavy" chains with a molecular weight of 55,000 (1-6). The molecule dissociates into equivalent halves in dilute salt solution at pH 2.5 after mild reduction and acidification; each half-molecule consists of one light and one heavy chain (7).

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shown that digestion by papain can yield crystallizable Fragment Fc that is either dissociable or nondissociable at low pH, depending on the concentration of L-cysteine present during proteolysis. Reduction of the disulfide bond joining the half-fragments of Fragment Fc was found to be reversible. Prolonged proteolysis, however, yields a crystallizable fragment which cannot be stabilized by reoxidation; this is evidently due to removal by enzymatic digestion of the region of Fragment Fc containing the interchain disulfide bond.

EXPERIMENTAL PROCEDURE

\( \gamma G \)-Immunoglobulin—\( \gamma G \)-immunoglobulin was prepared from the pooled sera of a number of nonimmunized rabbits by two precipitations with sodium sulfate at final concentrations of 0.18 and 0.125 M per ml, followed by passage through diethylaminoethyl cellulose (24) in 0.0175 M phosphate buffer, pH 7.0. Immunoelectrophoresis with a multispecific sheep antiserum prepared against whole rabbit serum showed the presence only of \( \gamma G \)-immunoglobulin.

Papain Digestion of \( \gamma G \)-Immunoglobulin—Papain digestion was carried out at 37° in 0.1 M phosphate buffer (pH 7.0) in the presence of 0.02 M disodium ethylenediaminetetraacetate and varying concentrations of recrystallized L-cysteine. The amount of papain used was 2% of the weight of \( \gamma G \)-globulin present, and the reaction was carried out under nitrogen; in most experiments the period of digestion was 4 hours. To terminate the reaction, 0.5 M sodium iodoacetate (pH 7) was added to a molarity in excess of the reducing agent present. The final concentration was 0.1 M, except in an experiment in which 0.001 M L-cysteine was used; in this case, iodoacetate was added to a concentration of 0.01 M.

Subsequent to alkylation, the reaction vial was flushed again with nitrogen, the mixture was allowed to stand overnight in the refrigerator, and it was then dialyzed for 1 day against 4 liters of cold water. The crystals of Fragment Fc that formed were collected by centrifugation, washed twice with 0.5-M portions of cold water, then recrystallized from borate-buffered NaCl solution, pH 8.0, ionic strength 0.16. This was done by adjusting the concentration of the suspension to 6 mg per ml and heating to 51.2° in a water bath, which resulted in complete solution of the crystals. After cooling in the refrigerator, crystals that formed were collected and washed with cold 0.05 M NaCl.

In one experiment, described under "Results," the Fc was purified by gel filtration and recrystallization was omitted. Concentrations of Fragment Fc were estimated by use of the extinction coefficient \( E_{\text{280}}^{\text{m}} = 12 \) (22).

Sedimentation and Diffusion Measurements—Sedimentation velocities were measured in a Spinco model E ultracentrifuge operated at 59,750 rpm and 20° ± 0.1°. Double 2° sector, 12-mm cells with filled Epon centerpieces were used. The solvent was placed in the second compartment of the cell. Areas were measured on photographic enlargements of schlieren patterns. For correcting sedimentation coefficients to \( s_{20, w} \) the partial specific volume of Fragment Fc was taken as that of \( \gamma G \)-globulin, 0.745 (25).

Diffusion constants were determined at 20° with a synthetic boundary, double 2° sector, 12-mm cell, with the ultracentrifuge operated at a low speed (9041 rpm). The outer solution for the final dialysis was placed in the second compartment. Calculations were made by the method of Uhlenberg (26).

Labeling of Protein with Radioiodine—\( \gamma G \)-globulin and bovine pancreatic ribonuclease were labeled with \( ^{125} I \) and \( ^{131} I \), respectively, by the method of McFarlane (27), which makes use of iodine monochloride. Excess radioiodine was removed from the protein solutions by dialysis for 4 days against repeated changes of borate-buffered NaCl solution; all but the final solution also contained 0.02 M KI. Alternatively, the protein solution was passed through an Amberlite IR-45 ion exchange column, and then dialyzed. In either case, the radiolabel was more than 99% precipitable by trichloroacetic acid at a final concentration of 5%. The \( \gamma G \)-globulin contained 1.8 atoms of \( ^{125} I \) and the ribonuclease contained 3.0 atoms of \( ^{131} I \) per molecule of protein. The radioisotopes, \( ^{125} I \) and \( ^{131} I \), were estimated separately with a pulse-height analyzer and a \( \gamma \)-scintillation counter.

Gel Filtration—Cross-linked polyacrylamide gels (Bio-Gel), used for gel filtration were obtained from Bio-Rad.

Estimation of S-Carboxymethylcysteine—The content of S-carboxymethylcysteine in samples alkylated with iodoacetate was determined in the amino acid analyzer. The weight of each sample analyzed was 6 to 7 mg. This overloads the column with respect to most amino acids but does not affect the resolution or recovery of S-carboxymethylcysteine (8).

RESULTS

Proteolysis with Papain in Presence of Increasing Concentrations of Reducing Agent—In order to determine whether reduced Fc can dissociate in salt solution at low pH, Fc fragments were prepared in a series of digestions, carried out in the presence of increasing concentrations of L-cysteine. The conditions of digestion and subsequent alkylation with iodoacetate are described under "Experimental Procedure." The concentrations of L-cysteine which were used are indicated in the legend of Fig. 1. Immediately after the 4-hour incubation with enzyme and addition of iodoacetate, ultracentrifugation was carried out to determine the extent of proteolysis of the 6.2S \( \gamma G \)-globulin into 3.5S fragments. Breakdown to 3.5S was apparently complete in each case, except for the digestion in which the lowest concentration of L-cysteine was employed (0.001 M); about 10% of the faster moving component remained in this preparation. Upon dialysis against cold water, crystals of Fc formed in each experiment. Recrystallization was carried out from NaCl-borate buffer as described under "Experimental Procedure," except in the case of the crystals prepared with 0.001 M L-cysteine, which were purified as indicated below.

When examined in the ultracentrifuge in 0.05 M NaCl (pH 3.5), each of the recrystallized Fragment Fc preparations sedimented as a single peak, with the exception of that prepared in the presence of 0.001 M L-cysteine; the latter contained about 25% of a faster moving component, presumably undigested \( \gamma G \)-globulin seen in the ultracentrifuge immediately after the treatment with papain. To isolate Fragment Fc from this preparation, 100 mg were filtered through a column (2 × 100 cm) of Bio-Gel P-150 equilibrated with a solution containing 0.05 M NaCl and 0.01 M sodium acetate, adjusted to pH 4 with acetic acid. The low pH was used to minimize aggregation of Fc. The filtration resulted in separation of a minor component, which was eluted first, from the major, second peak. The last half of the second peak to be eluted was pooled, concentrated by pervaporation, and used for further tests of the properties of the fragment. Its sedimentation coefficient, \( s_{20, w} \) (pH 3.5), was 3.3S, which cor-
had a corrected sedimentation coefficient at pH 2.4 of 2.4s.

The molarities of L-cysteine, corresponding to A through F, were 0.05, 0.003, 0.006, 0.01, 0.02, and 0.05 M, respectively. Sedimentation is from left to right. Photographs were taken after 80 min (at pH 3.5) or after 176 min (at pH 2.4). Centrifugation was carried out at 39,780 rpm and 20°. The numerals are s_{20,w} values. Protein concentrations, 7 mg per ml.

responded closely with that of the major peak prior to gel filtration. During dialysis of the Fe isolated by gel filtration, crystals formed once again. However, the entire mixture was used in subsequent tests.

Schlieren patterns obtained by ultracentrifugation in 0.05 M NaCl (pH 3.5) of the various preparations of purified Fe are shown in the left column of Fig. 1. Each of the peaks was symmetrical and the sedimentation coefficients were very similar, with the exception of the sample prepared with 0.001 M L-cysteine, for which the value was slightly higher.

The right column of Fig. 1 shows the schlieren patterns of the same samples in 0.06 M NaCl at a lower pH, 2.4. After papain digestion in the presence of 0.001 M L-cysteine, the purified Fe had a corrected sedimentation coefficient at pH 2.4 of 2.4s. Corresponding to the presence of increasing concentrations of the reducing agent in the digestion mixture, increasing amounts of a slower moving component were evident in the schlieren patterns at pH 2.4. Nearly all of the Fe sedimented at the slower rate (1.7s or 1.8s) when the concentration of L-cysteine during the digestion was 0.02 M or 0.05 M. These results suggest that Fe prepared in the presence of a sufficiently high concentration of L-cysteine is dissociable at low pH, presumably because of cleavage of an interchain disulfide bond. Since the various preparations of Fe all sedimented with approximately the same velocity at pH 3.5, the dissociation evidently occurs between pH 3.5 and pH 2.4.

Further evidence for the dissociability at low pH of Fragment Fe, formed by digestion with papain in the presence of sufficient reducing agent, is indicated by the molecular weights given in Table I. As indicated above, the crystals prepared in the presence of 0.001 M L-cysteine were purified by gel filtration at pH 4.0. In order to remove any nondissociable protein, the crystals of Fragment Fe prepared in the presence of 0.05 M L-cysteine were purified, after recrystallization, by gel filtration at pH 2.4; 37 mg were passed through a column (2.5 x 125 cm) containing a mixture of approximately equal volumes of Bio-Gels P.60 and P.100. Eighty-four percent of the protein was eluted in the second of two peaks obtained; the remainder consisted of a larger component, presumably Fe that was not dissociable at pH 2.4. The major component had an average molecular weight at pH 2.4 of 24,100, as compared with the value of 52,900 obtained at pH 3.5 for recrystallized Fe prepared in the presence of the same concentration of L-cysteine (0.05 M, Table I). In contrast, Fragment Fe that resulted from the digestion carried out with 0.001 M L-cysteine had an average molecular weight of 41,500 at pH 2.4 and 48,800 at pH 3.5 (Table I). As indicated above, the sedimentation coefficients of the two preparations of Fe were similar at pH 3.5 but differed markedly at pH 2.4. The molecular weights indicate that this reflects a difference in dissociability of the two preparations at pH 2.4.

Effect of pH on Dissociability of Reduced Fragment Fe—The above data indicate that Fragment Fe prepared with papain and 0.05 M L-cysteine dissociates at pH 2.4. Fig. 2 shows the effect of pH on this preparation of Fe in greater detail. At pH 3.5 or at pH 2.4 in 0.05 M NaCl, a single peak was observed in the ultracentrifuge (Fig. 2, A and F). At intermediate pH values two components were present; there was a progressive increase in the amount of the slower component as the pH was lowered. As judged from the schlieren pattern, the dissociation was nearly half complete at pH 3.1 (Fig. 2C). If the Johnston-Ogston effect is appreciable, the degree of dissociation is actually somewhat less than the apparent value. The decrease in sedimentation coefficient of the faster component between pH 3.5 and pH 3.1 is probably due to expansion of the molecule at the lower pH. The s_{20,w} value of undissoaceable Fe decreases from 3.3s to ~2.4s as the pH is lowered from 3.5 to 2.4 (Fig. 1, Table I).

### Table I

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cysteine concentration during digestion</th>
<th>pH</th>
<th>Concentration of Fe</th>
<th>s_{20,w}</th>
<th>D_{20,4}</th>
<th>Mol wt</th>
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<tr>
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<td>3.5</td>
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<td>7.32</td>
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<td>3.36</td>
<td>6.02</td>
<td>46,300</td>
</tr>
<tr>
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<td>0.001</td>
<td>3.5</td>
<td>3.5</td>
<td>3.43</td>
<td>6.71</td>
<td>48,800</td>
</tr>
<tr>
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<td>2.4</td>
<td>3.4</td>
<td>2.56</td>
<td>5.89</td>
<td>41,500</td>
</tr>
</tbody>
</table>

* Digestions of γG-globulin were carried out with papain as described in the text.

* Refers to the pH during the molecular weight determination.

* Recombined sample, neutralized after exposure to pH 2.4; the pH was then lowered to 3.5 for the determination of molecular weight.
Recombination of Half-fragments of Fc at Higher pH—To determine whether the dissociation at pH 2.4 of reduced Fc is reversible, 11 mg of recrystallized Fragment Fc, made by proteolysis with 2% by weight of papain in the presence of 0.05 M L-cysteine, were dissolved in 0.05 M NaCl, and adjusted to pH 2.4. After being allowed to stand for 2 hours in the refrigerator, a portion was tested by ultracentrifugation. A symmetrical 1.5S peak formed, indicative of dissociation into half-fragments (Fig. 3A). The remainder of the solution of Fragment Fc, in 0.05 M NaCl (pH 2.4), was allowed to stand at 20°C for a period of time equal to that occupied by ultracentrifugation. This was done to ensure that conditions which obtained in the ultracentrifuge also applied to the rest of the sample. To this solution, 4 mM NaCl was then added to a final concentration of 0.4 M. The solution was dialyzed against cold 0.16 M NaCl, and then against NaCl-borate buffer (pH 8, ionic strength 0.16). During the dialysis, crystals formed; however, portions of the entire contents of the dialysis bag were utilized in further studies. The suspension was concentrated by pervaporation and finally dialyzed against 0.05 M NaCl and adjusted to pH 3.5. The product migrated as a single 3.5S peak in the ultracentrifuge at a concentration of 7.5 mg per ml (Fig. 3B). The sedimentation coefficient was 3.38S at 3.5 mg per ml and the average molecular weight was 40,300 (Table 1), indicating that recombination of the half-fragments had occurred.

This experiment was repeated with another sample of recrystallized Fragment Fc, with virtually identical results. The molecular weight after recombination was 49,100 at a concentration of 7.5 mg per ml. When a solution of this recombined sample was again exposed to pH 2.4, a symmetrical 1.8S peak formed, which indicates that the recombination of half-fragments is largely reversible at low pH. Since sulfhydryl groups had been inactivated after reduction, the association and dissociation evidently involve noncovalent interactions.

Reoxidation of Disulfide Bond Linking Half-fragments of Recrystallized Fragment Fc—To determine whether the disulfide bond linking half-fragments of Fc can reoxidize spontaneously after removal of the reducing agent, a comparison was made between an alkylated and an unalkylated sample. y-Globulin (647 mg) was allowed to react with 6.5 mg of papain in the presence of 0.05 M L-cysteine and 0.002 M EDTA. After 2 hours of digestion, a portion of the mixture was alkylated with iodoacetate, added to a final concentration of 0.1 M. Ultracentrifugation, carried out at this point, indicated that the breakdown into 3.5S fragments was essentially complete. The alkylated sample was allowed to stand in the refrigerator overnight under nitrogen and then was dialyzed against 4 liters of cold water. The yield of crystals of Fc was 25% of the weight of y-Globulin that had been digested and alkylated. Recrystallization was carried out as described under “Experimental Procedure.”

A second portion of the papain-treated y-Globulin was not alkylated but was dialyzed against cold water immediately after proteolysis. Dialysis was carried out in 1-inch dialysis tubing, and the outer solution (4 liters) was changed three times over a 4-hour period. Dialysis was then allowed to proceed overnight against a fourth portion of water. The yield of crystals was 30% of the weight of the protein that had been dialyzed without alkylation. The Fc fragments were washed with distilled water, then recrystallized.

The results of ultracentrifugation in 0.05 M NaCl (pH 2.4) of the crystals of Fc obtained from the alkylated and unalkylated digests are shown in Fig. 4. In agreement with results described above, the major portion of the alkylated preparation migrated as a 1.8S peak, characteristic of half-fragments of Fc (Fig. 4A). In contrast, the unalkylated preparation contained a large proportion of faster moving material (Fig. 4B); the major component had a sedimentation coefficient of 2.5S, a value close to that of Fc prepared in the presence of very low concentrations of cysteine (Fig. 1A). The slower component in this preparation migrated with approximately the same velocity as that of the alkylated Fc (Fig. 4A). These results are consistent with the view that dialysis without prior alkylation permitted reoxidation, in a large proportion of the fragments, of the disulfide bond linkings.

Fig. 2. The effect of pH on sedimentation of recrystallized Fc prepared by digestion with papain in the presence of 0.05 M L-cysteine. A through F correspond to pH values of 3.5, 3.2, 3.1, 3.0, 2.7, and 2.4, respectively; NaCl concentration, 0.05 M. Sedimentation (from left to right) for 80 min (left column) or 160 min (right) at 59,780 rpm and 20°C. The numerals are S values. Protein concentrations, 7 mg per ml.

Fig. 3. Recombination of dissociated Fragment Fc. A, sedimentation pattern at pH 2.4 of Fc prepared by digestion in the presence of 0.05 M L-cysteine; B, sedimentation of the same protein after neutralization and adjustment to pH 3.5. NaN03 concentration, 0.05 M. Sedimentation is from left to right. Photographs were taken after 96 min at 59,780 rpm and 20°C. The numerals are S values. Protein concentrations, 7.5 mg per ml.
Fig. 4. Reduction and reoxidation of Fe. The solvent in each case was 0.05 M NaCl (pH 2.4). A, Fe isolated from a digest treated with iodoacetate immediately after digestion; B, Fe from the same digest as A, dialyzed after digestion (without alkylation); C, Sample B reduced with 0.05 M L-cysteine and alkylated; D, Fe prepared by digestion in the presence of 0.001 M L-cysteine followed by alkylation; E, Sample D after reduction with 0.05 M L-cysteine, followed by a second alkylation. Sedimentation subsequent dialysis.

The relationship between absorbance and radioactivity is not linear for the γG-globulin peak. This is attributable to a low value for radioactivity obtained in one tube, corresponding to the highest optical density value in the γG-globulin peak.
Effect of Prolonged Digestion on Reoxidizability and S-Carboxymethylcysteine Content of Fragment Fc—To determine the effect of the duration of proteolytic digestion on re-formation of the interchain disulfide bond of Fragment Fc, samples of γG-globulin were digested for 1 and 10 hours, respectively, at 37°C with 1% by weight of papain in the presence of 0.05 M L-cysteine and 0.002 M EDTA. To one portion of each mixture, sodium iodoacetate was then added to a final concentration of 0.1 M; both the alkylated and unalkylated portions were dialyzed repeatedly against cold water. Crystals, which formed in each of the four mixtures, were purified by recrystallization. The extent of dissociation into half-fragments in 0.05 M NaCl (pH 2.4), was then estimated by ultracentrifugation. The relative amounts of 1.8S component, corresponding to half-fragments, are shown in Table II.

Fragment Fc obtained from the alkylated 1-hour digest was 88% dissociable at low pH; dissociation of Fragment Fc from the alkylated 10-hour digest was essentially complete. The unalkylated fragments derived from the 1-hour digest reoxidized on dialysis, as shown by the failure of a large proportion to dissociate into half-fragments at pH 2.4. In contrast, the extent of reoxidation after 10 hours of digestion was much lower; only 9% of the protein was nondissociable, as judged from the schlieren pattern. The data are approximate since they are not corrected for the Johnston-Ogston effect.

These results suggested the possibility that prolonged digestion results in removal of the region of Fragment Fc containing the interchain disulfide bond. To investigate this possibility further, the amounts of S-carboxymethylcysteine in crystals of Fragment Fc isolated after dialysis were determined and are shown in Table II. It is evident that the S-carboxymethylcysteine content was substantially lower after 10 hours of digestion than after 1 hour.

To obtain more detailed information, another series of experiments was carried out. Digestions were allowed to proceed for 1, 2, 4, and 10 hours prior to alkylation with iodoacetate. The results of S-carboxymethylcysteine determinations are shown in the last four lines of Table II. The decrease observed with increasing time of digestion supports the view that papain attacks the region of Fragment Fc which contains the interchain disulfide bond.

The S-carboxymethylcysteine content in Fragment Fc isolated from the 1-hour digest (Experiment 1, Table II) appears to be slightly low, on the basis of the degree of dissociability noted when dialysis was omitted (29%, Table II); i.e., 77% was not dissociable. The value of 1.2 moles per mole suggests a maximum reoxidizability of 60%. However, about 12% of the alkylated portion failed to dissociate, indicating that reduction was not complete. The sum, 60% + 12%, nearly accounts for the amount of undissociable protein estimated by ultracentrifugation, but suggests that there is little reduction of any other disulfide bonds in Fragment Fc. This possibility is supported by the low values of S-carboxymethylcysteine content in alkylated samples isolated after more prolonged digestion.

**DISCUSSION**

The results indicate that Fragment Fc of rabbit γG-globulin, prepared in the presence of a sufficiently high concentration of L-cysteine, dissociates into half-fragments in 0.05 M NaCl (pH 2.4). Supporting evidence includes the molecular weight data (Table I), the symmetry of the schlieren peak in the ultracentrifuge at pH 2.4 (Figs. 1 and 2), and the elution pattern obtained on gel filtration at pH 2.4 (Fig. 5). The column used for gel filtration was shown to be capable of resolving dissociated Fc from both smaller and larger molecules (radiolabeled ribonuclease and γG-globulin). Between 85 and 90% of the dissociated Fc was eluted as a symmetrical peak. Marler et al. (6) have previously shown that reduced Fc dissociates into half-fragments in 6 M guanidine hydrochloride. Also, peptide-mapping studies of Fc have suggested the presence of two symmetrical subfragments (13, 14), which evidently correspond to the two portions of heavy chains which constitute the fragment (2, 11, 12).

The conditions required for dissociation of reduced Fc are similar to those previously noted for dissociation of reduced γG-globulin into half-molecules. In 0.1 M NaCl, dissociation into half-molecules is about 50% complete at pH 2.7 (7). In the present investigation, in which the salt concentration was 0.05 M, about half the Fc fragments dissociated at pH 3.1 (Fig. 2); progressive dissociation was noted as the pH was lowered from 3.5 to 2.7. Samples were allowed to stand for 2 hours in the refrigerator at low pH prior to ultracentrifugation at 20°C. The fact that the dissociation of Fc or of reduced γG-globulin occurs under similar conditions is consistent with other data, mentioned in the introductory section, which suggest that most of the noncovalent interactions linking heavy chains are in the region of Fc.

Either dissociable or nondissociable Fc fragments can be prepared, depending on the concentration of reducing agent present in the digestion mixture. Most of the Fc failed to dissociate at pH 2.4 when digestion had been carried out in the presence of 0.001 M L-cysteine; this was indicated by the molecular weight (31,500) and sedimentation coefficient at pH 2.4 (2.4S). After digestion in the presence of 0.05 M L-cysteine, the molecular weight of the isolated Fc at pH 2.4 was 34,100 and the sedimentation coefficient was 1.8S. Both the dissociable and nondissociable forms of Fc are crystallizable. Under the microscope the two types of crystal are similar in appearance.

That this difference in molecular weight at pH 2.4 was due to...

**Table II**

Effect of period of proteolysis of γG-globulin on sulphydryl content and reoxidizability of fragment Fc

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Duration of digestion</th>
<th>S-Carboxymethylcysteine content</th>
<th>Dissociability of alkylated Fc</th>
<th>Dissociability of unalkylated Fc</th>
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<td>1 hour</td>
<td>1.2</td>
<td>88%</td>
<td>23%</td>
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<tr>
<td></td>
<td>10 hours</td>
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<td>91%</td>
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<tr>
<td>2</td>
<td>1 hour</td>
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<td>23%</td>
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<td></td>
<td>2 hours</td>
<td>1.1</td>
<td>&gt;95%</td>
<td>91%</td>
</tr>
<tr>
<td></td>
<td>4 hours</td>
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<td>91%</td>
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<tr>
<td></td>
<td>10 hours</td>
<td>0.5</td>
<td>&gt;95%</td>
<td>91%</td>
</tr>
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</table>

a Proteolysis with papain was carried out at 37°C as described in the text.
b In recrystallized Fragment Fc isolated from the alkylated mixture.
c Estimated from the sedimentation pattern at pH 2.4 as described in the text.
d Refers to recrystallized Fc that was isolated after dialysis without alkylation.
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failure to effect sufficient reduction of disulfide bonds in the presence of lower concentration of L-cysteine is indicated by the result of a second reduction, with 0.05 M L-cysteine, of the Fc isolated from the digest prepared with 0.001 M L-cysteine. The sedimentation coefficient at pH 2.4 decreased from 2.48 to 1.88, a value characteristic of half-fragments of Fc (Table I).

With increasing amounts of L-cysteine in the papain digestion mixture, increasing dissociability into half-fragments at pH 2.4 was noted (Fig. 1). When the cysteine concentration was 0.006 M, the extent of dissociation into 1.88 fragments at pH 2.4 was about 50%.

Previous studies have indicated that reduction of a single disulfide bond suffices to permit subsequent separation of $\gamma$G-globulin into half-molecules (8, 9). It would appear that this disulfide bond is present in the region of the Fc fragment produced by papain. Marler et al. (6) have previously demonstrated the requirement for reduction of Fc for subsequent dissociation in guanidine hydrochloride solution.

The reduction of the interchain disulfide bond which occurs during digestion with papain in the presence of 0.05 M L-cysteine is evidently reversible. When excess iodoacetate was added immediately after digestion, the sedimentation coefficient of isolated Fc at pH 2.4, 1.88, was indicative of dissociation into half-fragments (Fig. 4). In contrast, when alkylation was omitted and reoxidation was permitted to occur by dialysis at neutral pH, about 70% of the isolated Fc sedimented at pH 2.4 with a velocity characteristic of undissociated Fc (2.48); the sedimentation velocity of the remaining 30% approximated that of dissociated Fc. This result suggests that reoxidation occurred during dialysis. Further evidence was the finding that the sedimentation coefficient decreased to 1.88 after a second reduction of the non-alkylated Fc with 0.05 M L-cysteine, followed by alkylation and acidification to pH 2.4. The capability for spontaneous reoxidation of the disulfide bond linking half-molecules and of the bonds joining light and heavy chains has been noted previously (28, 29).

The noncovalent interactions which link the two half-fragments of Fc are disrupted at pH 2.4, but are largely restored at neutral pH. Thus, when a solution of half-fragments of reduced, alkylated Fc was allowed to recombine by raising the pH to neutrality, the molecular weight determined at pH 3.5 was found to be 46,000, as compared with the value 24,100 for reduced Fc at pH 2.4. The molecular weight after recombination was measured at pH 3.5 rather than at neutral pH because of the tendency of Fc to aggregate at the higher pH.

Two types of evidence indicate that prolonged digestion with papain results in gradual removal of the region of Fragment Fc which contains the interchain disulfide bond. First, the S-carboxymethylcysteine content of crystals of Fragment Fc isolated after digestion and alkylation decreased with increasing time of digestion (Table II). Second, when alkylation was omitted and the reducing agent was removed by dialysis after digestion with papain, the crystals isolated from a 10-hour digest were largely dissociable at low pH, in contrast to those isolated from a 1- or 4-hour digest (Table II, Fig. 4). Thus, stabilization through

Fig. 5. Gel filtration on a mixed bed of Bio-Gels P-60 and P-100; eluent, 0.05 M NaCl (pH 2.4). The mixture contained 18 mg of purified Fc prepared by digestion with papain in the presence of 0.05 M L-cysteine, 4 mg of $^{131}$I-labeled $\gamma$G-globulin, and 4 mg of $^{131}$I-labeled ribonuclease. •, optical density at 280 nm; ○, counts per min of $^{131}$I; ⌊, counts per min of $^{131}$I. ---, optical density corrected for the contribution of the $\gamma$G-globulin or ribonuclease, as estimated from the radioactivity measurements.
The presence of this disulfide bond after prolonged treatment with detergent does not, therefore, seem to provide evidence for a second interchain disulfide bond in the native molecule.

Another argument put forth in support of two disulfide bonds is Cebra's finding that insoluble papain yields a 58 fragment comprising two halves linked by a disulfide bond (33), and that the Fe fragment, which is distinct from this 58 piece, also contains an interchain bond linking segments of two heavy chains (6). However, as noted later in their discussion, separation of the 58 fragment after treatment with insoluble papain occurred only after 16 hours of exposure to detergent and was inhibited by sulfhydryl reagents, which indicates that intramolecular disulfide interchange was occurring during liberation of the 58 fragment. The presence of this disulfide bond after prolonged treatment with detergent does not, therefore, seem to provide evidence for a second interchain disulfide bond in the native molecule.

Addendum—In a recently published paper, Utsumi and Karush (32) proposed a model in which two interchain disulfide bonds, in close juxtaposition, link the heavy chains. Separation of half-molecules at low pH subsequent to reduction of a single disulfide bond is attributed to disulfide interchange. This seems very improbable since the separation occurs after inactivation of the heavy chains occurs preferentially in the region of the interchain disulfide bond (cf. References 10 and 31).

It proved to be very difficult to free Fe formed in the presence of 0.001 M L-cysteine from residual undigested γ-globulin by recrystallization, or even to effect a significant degree of purification by this method. It was necessary to resort to gel filtration to isolate the Fe. In view of the high degree of solubility of γ-globulin at neutral pH, it would appear that it can co-crystallize with Fragment Fe.

References

Reversible Dissociation of Fragment Fc of Rabbit γG-Immunoglobulin
F. P. Inman and A. Nisonoff


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