Relative Labilities of the Two Types of Interchain Disulfide Bond of Rabbit γG-Immunoglobulin*

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A molecule of rabbit γG-globulin1 evidently consists of four polypeptide chains held together by interchain disulfide bonds and nonevalent interactions. Each molecule comprises a pair of similar or identical “light” chains having an approximate molecular weight of 20,000, and another pair of “heavy” chains of weight 50,000 to 55,000 (2-7). Each light chain is linked to a heavy chain and the two heavy chains are joined to one another (3, 8). The presence of 1 S-carboxymethylcysteine residue per light chain, isolated after reduction and alkylation with iodoacetate, indicates that a single interchain disulfide bond joins each light chain to a heavy chain (3). Similarly, in a large part if not all of the molecules, reduction of a single disulfide bond is sufficient to permit separation at low pH of half-molecules, each consisting of a light and a heavy chain (9, 10). This is evidently the same disulfide bond that links two “univalent” (Fab’ (11)) fragments after peptic digestion (11). To be consistent with the bond joining half-molecules,2 conditions for preferential reduction of the H-H disulfide bond, with minimal cleavage of interchain disulfide bonds linking light and heavy chains, were defined. The extent of exchange of the bond joining two heavy chains (11). The present investigation provides direct evidence that this is the case.

A study was also made of the relative labilities of the disulfide bonds linking light and heavy chains as compared with that of the bond joining half-molecules.2 Conditions for preferential reduction of the H-H disulfide bond, with minimal cleavage of L-H disulfide bonds, were defined. The extent of exchange of light and heavy chains, under the conditions used for separation and hybridization of half-molecules, was estimated. Also, it was found that after reduction of all interchain disulfide bonds, separation into half-molecule subunits occurs under milder conditions than are required for dissociation into separate light and heavy chains.

EXPERIMENTAL PROCEDURE

Materials and Methods

γG-Globulin—A γG-globulin fraction was obtained from a pool of serum from nonimmunized rabbits by three precipitations with decreasing concentrations of sodium sulfate (12) followed by passage through a column of diethylaminoethyl cellulose equilibrated with 0.0175 M phosphate buffer, pH 7.0. The product consisted only of γG-globulin by the criterion of immunoelectrophoresis, carried out with a sheep antisera containing antibodies against numerous serum components, including γ- and β-globulins.

Reduction of γG-Globulin—Reductions were carried out by procedures previously employed, i.e., with 2-mercaptoethanol hydrochloride at pH 5.0 and 37° (9) or with 2-mercaptoethanol at pH 8.2 and room temperature (3). One reduction was done with 2-mercaptoethanol at pH 8.2 and room temperature. The solvents used were 0.1 M sodium acetate, adjusted to pH 5.0 with 1 M acetic acid, or 0.3 M Tris adjusted to pH 8.2 with HCl. At pH 5, reduction was carried out for 75 min. Sodium iodoacetate was then added to a final concentration equimolar with that of the reducing agent. Mixtures were allowed to stand overnight in the refrigerator, then dialyzed for 2 days against two 4-liter portions of cold sodium chloride-boracic buffer, pH 8.0, ionic strength 0.16. Reductions at pH 8.2 were allowed to proceed for 1 hour. At the end of this period, alkylation at pH 8.2 and dialysis were carried out as described above. Decreases in pH on standing in the presence of the sodium iodoacetate were less than 0.2 unit.

The following tests were run with separate portions of each reduced, alkylated sample: (a) the degree of dissociation into half-molecules at pH 2.5; (b) the yield of light chains obtained by filtration on a column of Sephadex G-100 equilibrated with 1 M propionic acid; (c) the yield of light chains from a similar column of Sephadex G-100 equilibrated with 0.025 M NaCl, pH 2.5. S Carboxymethylcysteine analyses were carried out on light and heavy chains in one experiment, as described below.

Estimation of Degree of Dissociability into Half-molecules after Reduction—Yields of half-molecules at low pH were determined by ultracentrifugation at 50,780 rpm of reduced, alkylated samples, after dialysis against 0.025 M NaCl and acidification to pH 2.5 with 1 M hydrochloric acid (10). Double 2° sector cells with filled Epon centerpieces were used; the solvent was placed in the second compartment. The temperature during centrifugation was 20° ± 0.2°. Half-molecules of rabbit γG-globulin sediment with a velocity (Vmax) of 3S under these conditions, whereas the sedimentation coefficient of whole γG-globulin is approximately 4.2S. In partially reduced samples, the amounts of half- and whole molecules were estimated by area measurements of enlargements of the schlieren patterns. Corrections were applied for the Johnston-Ogston effect on the basis of sedimentation patterns obtained with known mixtures of half- and whole molecules (10).
Yields of Light Chains—To estimate the extent of reduction of L-H disulfide bonds, the yield of light chains from each reduced sample was determined by gel filtration at room temperature of approximately 15 mg on a column of Sephadex G-100 (1.5 \times 40 cm) equilibrated with 1 M propionic acid (13). The reduced, alkylated protein was dialyzed overnight against cold 1 M propionic acid prior to filtration.

In addition, another 15-mg portion was filtered through a similar column equilibrated with 0.025 M NaCl adjusted to pH 2.5 with HCl. In this case, the protein was dialyzed against 0.025 M NaCl and the pH was adjusted to pH 5.5 prior to application to the column.

Preparation of Polypeptide Chains for S-Carboxymethylcysteine Analyses—In one of the reduced, alkylated samples the amounts of S-carboxymethylcysteine present in the light and heavy chains were determined. In this sample, the interchain disulfide bonds had not been completely reduced. However, to determine S-carboxymethylcysteine in each type of chain, it was necessary to separate the chains completely without a second alkylation, which would alter the analytical results. To do this, the reduced, alkylated γ-G-globulin was reduced a second time with 0.2 M 2-mercaptoethanol, a procedure which cleaves all of the interchain bonds (13). After reduction for 1 hour at room temperature, the protein sample (120 mg) was immediately transferred, without alkylation, to a column of Sephadex G-100 (2 \times 100 cm) equilibrated with 1 M propionic acid. The light and heavy chains were then separated in the usual manner; the yield of light chains was 25% of the weight of protein applied to the column. The light chains were further purified by passage through a 30-cm \times 15-cm column of carboxymethyl cellulose equilibrated with 0.01 M sodium acetate, pH 5.5. This procedure has been found to retain heavy chains and complexes of heavy and light chains, while permitting nearly all of the free light chains to pass through the column. Extinction coefficients at 280 nm used for light and heavy chains were those of Crumpton and Wilkinson (14). The extinction coefficient \( E_{1\text{cm}}^{1\text{cm}} \) used for Fragment Fab was 15.

Determination of S-Carboxymethylcysteine—The content of S-carboxymethylcysteine in each preparation of isolated polypeptide chains was determined with the long column (0.9 \times 150 cm) of the Spinco model 120 automatic analyzer (15); 7 to 10 mg of protein were applied. This overloaded the column with respect to most amino acids, but it does not affect the resolution or recovery (10) of S-carboxymethylcysteine, which is the first amino acid to be eluted. Protein samples were hydrolyzed for 22 hours at 110°C in constant boiling HCl prior to analysis. The method used to evacuate sample tubes, for removal of oxygen, is that of Crestfield, Moore, and Stein (16). To determine the amount of protein applied to the long column, an aliquot equal to exactly one-sixth of the volume applied to the long column was analyzed for lysine, histidine, and arginine on the short column (0.9 \times 15 cm) by the standard method. The values taken for the content of these amino acids per unit of weight of light chains, heavy chains, or whole γ-G-globulin were those of Crestfield and Wilkinson (14). The amount of protein applied to the short column was taken as the average of the three values calculated from the recoveries of lysine, arginine, and histidine. This result was multiplied by six to obtain the amount of protein applied to the long column. An additional check of the amount of protein applied to the overloaded long column was obtained by measurement of the proline peak, which was sufficiently small and well resolved for this purpose. The maximum difference between the values obtained by the two methods was 6.5%.

Digestion of γ-G-Globulin with Papain—Digestions with papain were carried out in the presence of 0.01 M L-cysteine and 0.002 M disodium ethylenediaminetetraacetate for 4 hours at 37°C by the method of Porter (17). Fragment Fab (corresponding to Porter's Fragment I) was isolated by passage through a column of carboxymethyl cellulose, equilibrated with 0.01 M sodium acetate, and adjustment to pH 5.5 with acetic acid. The volume of the column was 1 cm\(^3\) per mg of protein applied. In the first fraction, 50 to 60% of the digested protein was recovered. Analysis by the Ouchterlony method indicated that this fraction, in each instance, was free of Fragment Fe (Porter's Fragment III). Further confirmation of the nature of the fragments collected was obtained by amino acid analysis (see "Results").

Precipitations with Antiallotype Serum—Estimations of the amount of γ-G-globulin of a particular allotypic specificity, present in a mixture, were obtained by precipitations of radiolabeled Fragment Fab with monospecific antiallotype serum. After the initial precipitation, unlabeled γ-G-globulin of the appropriate allotypic specificity was added to a small portion of the supernatant, and the precipitation was repeated. This procedure was repeated until the amount of radioactivity precipitated was less than 3%. The method has been described in detail elsewhere (18).

Radioiodination—Proteins were trace labeled with \(^{131}I\) or \(^{125}I\) by the method of McFarlane (19). The number of atoms of iodine incorporated per molecule of protein was 1.7 to 2.0. Immediately after iodination, proteins were passed through a column of IR-45 anion exchange resin to remove unbound iodide. Potassium iodide was added to the eluate to a concentration of 0.02 M, and the protein was dialyzed against a large excess of cold NaCl-borate buffer, pH 8, ionic strength 0.16. More than 99% of the radioactivity in each dialyzed preparation was precipitable by 5% trichloroacetic acid. Initial specific activities were greater than 1.5 \times 10^6 cpn per mg. Samples were counted in a well-type scintillation counter. \(^{131}I\) and \(^{125}I\) were estimated in samples containing both isotopes by the use of a pulse height discriminator at two appropriate settings. Standard samples containing the individual isotopes were counted at both settings of the discriminator to determine the small contribution of each isotope at the setting used for the other. A minimum of 6000 counts was recorded for each sample. Background corrections were small.

RESULTS

Comparison of Relative Labilities of Interchain Disulfide Bonds Linking Half-molecules or Light and Heavy Chains—The yield of light chains obtained by gel filtration on Sephadex G-100, equilibrated with 1 M propionic acid, provides a measure of the extent of reduction of disulfide bonds linking light and heavy chains. The degree of reduction of interchain disulfide bonds linking half-molecules is indicated by the extent of dissociation into half-molecules in 0.025 M NaCl, pH 2.5, as shown by ultracentrifugation; the sedimentation coefficient \( s_{20, w} \) of half-molecules under these conditions is 35 (10).

The elution patterns obtained after reductions with increasing concentrations of 2-mercaptoethanol are shown in Fig. 1 (left).
Fig. 1. Effects of reductions of G-globulin with increasing concentrations of 2-mercaptoethanol at pH 8.2: A, 0.001 M; B, 0.005 M; C, 0.01 M; D, 0.03 M; E, 0.2 M. The first and second columns are elution patterns of 14 to 16 mg of protein from Sephadex G-100 in 1 M propionic acid or 0.025 M NaCl, pH 2.5. Photographs of schlieren patterns were taken after 90 min at 50,780 rpm and 20°; solvent, 0.025 M NaCl, pH 2.5; protein concentration, 6 mg per ml. The numerals are $s_{20, w}$ values.
and yields of light chains are summarized in Table I. Results of filtration of the same samples on Sephadex G-100 equilibrated with 0.025 M NaCl, pH 2.5, are indicated in Fig. 1 (center column). Photographs of schlieren patterns of the reduced, alkylated preparations, in 0.025 M NaCl, pH 2.5, are in the right column of Fig. 1. The yields of half-molecules (38 peak) are listed in Table I. These data were corrected for the Johnston-Ogston effect as described above. Results similarly obtained after reductions with increasing concentrations of 2-mercaptoethylamine are presented in Fig. 2 and summarized in Table I.

As the concentration of 2-mercaptoethanol used for reduction was increased, a corresponding increase was observed in the yield of light chains eluted from Sephadex G-100 in 1 M propionic acid (Fig. 1, Table I). After reduction with 0.2 M 2-mercaptoethanol, the yield of light chains represented 25.5% of the protein, indicating that 8% of the proteins to the column, indicating that reduction of interchain disulfide bonds between the light and heavy chains (L-H disulfide bonds) was essentially complete (3). After reduction with 0.01 M 2-mercaptoethanol, the yield of light chains was only 3% of the protein, indicating that 97% of the L-H disulfide bonds had been reduced. In contrast to this result, 46% of the protein dissociated into half-molecules in 0.025 M NaCl, pH 8.2, as indicated by the schlieren pattern obtained in the ultracentrifuge (Fig. 1, Table I). After reduction with 0.01 M 2-mercaptoethanol, nearly one-fifth of the L-H disulfide bonds were cleaved, as estimated from the yield of light chains, whereas ultracentrifugation indicated almost complete dissociation into half-molecules at pH 2.5.

The effects of reduction with 2-mercaptoethanol at pH 5 (Fig. 2, Table I) were somewhat different from those observed with 2-mercaptoethanol at pH 8.2. The concentration of reducing agent needed for an equivalent extent of dissociation of light chains or of half-molecules was higher with 2-mercaptoethanol than with 0.01 M 2-mercaptoethanol at pH 5. After reduction with 0.1 M 2-mercaptoethanol, the highest concentration tested, 56% of the light chains were liberated. (Reduction of L-H bonds is incomplete, even with 0.5 M 2-mercaptoethanol at pH 5 (9)).

Another difference between the two types of reduction is that the relative lability of disulfide bonds joining half-molecules (L-H disulfide bonds), as compared to L-H disulfide bonds, was somewhat greater with 2-mercaptoethanol as the reducing agent. Thus, after reduction with 0.005 M 2-mercaptoethanol at pH 8.2, there was essentially complete separation into half-molecules and one-fifth of the light chains were released. The concentration of 2-mercaptoethanol (pH 5) required for the same degree of release of light chains lies between 0.01 M and 0.03 M (Table I). With these concentrations of 2-mercaptoethanol, the yields of half-molecules were only 32% and 67%, respectively. It is evident, however, that the disulfide bond linking half-molecules is more labile than L-H disulfide bonds under either set of conditions.

It is possible to estimate roughly the relative labilities of the two types of bond from the yields of light chains and of half-molecules after partial reduction. The data of Table I indicate a ratio of approximately 6:1 in the case of reduction with 0.001 M 2-mercaptoethanol, since 46% of the protein dissociated into half-molecules whereas 8% of the light chains (2.5% of the total protein) were released. For reduction with 0.01 M 2-mercaptoethanol at pH 5, a similar calculation indicates that the fraction of the H-H disulfide bonds reduced is approximately 3 times as great as that of L-H bonds. It is evident that such a calculation is meaningful only after partial reduction since the ratio becomes unity when all interchain disulfide bonds are cleaved.

Localization of Disulfide Bond Linking Half-molecules—Indirect evidence suggests that the disulfide bond joining half-molecules connects two heavy chains (10). To obtain data bearing directly on this question, S-carboxymethylcysteine was determined in isolated light and heavy chains after reduction and alkylation under conditions which result in essentially complete dissociation into half-molecules but in relatively slight dissociation of light from heavy chains. As indicated in Table I, this condition is satisfied by reduction with 0.01 M 2-mercaptoethanol at pH 8.2. A separate portion of rabbit γG-globulin was therefore reduced with 0.01 M 2-mercaptoethanol and alkylated. Ultracentrifugation of a portion of this material in 0.025 M NaCl, pH 2.5, indicated essentially complete dissociation (>95%) into half-molecules (3.18). The yield of light chains on Sephadex G-100 (1 M propionic acid) was 4% or one-seventh of the maximum.

Another portion of the reduced, alkylated protein was dialyzed against Tris buffer, pH 8.2, and reduced once again with 0.2 M 2-mercaptoethanol, a procedure which results in essentially complete reduction of interchain disulfide bonds. Without further alkylation, the protein was immediately transferred to a column of Sephadex G-100, equilibrated with 1 M propionic acid, and separated into light and heavy chains. The yield of light chains was 25% of the total protein. The light chains were purified further on carboxymethyl cellulose as described under "Methodes."

S-Carboxymethylcysteine analyses of the separated chains are given in Table II. The data reflect the extent of only the first reduction (with 0.01 M 2-mercaptoethanol) since alkylation

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**Table I**

<table>
<thead>
<tr>
<th>Concentration of reducing agent</th>
<th>Yield of light chains*</th>
<th>Yield of half-molecules per cent of total protein</th>
<th>Yield of half-molecules per cent of total protein</th>
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<tbody>
<tr>
<td></td>
<td>Per cent of total protein</td>
<td>Maximum</td>
<td>Per cent of light chains</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
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<td></td>
<td></td>
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<td>0.001</td>
<td>2.5</td>
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</tr>
<tr>
<td>0.005</td>
<td>5.5</td>
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<td>&gt;97</td>
</tr>
<tr>
<td>0.01</td>
<td>5.5</td>
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<td>&gt;97</td>
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<tr>
<td>0.05</td>
<td>12.4</td>
<td>41</td>
<td>&gt;97</td>
</tr>
<tr>
<td>0.2</td>
<td>96.9</td>
<td>100</td>
<td>&gt;97</td>
</tr>
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<td>2-Mercaptoethamine</td>
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<td></td>
</tr>
<tr>
<td>0.01</td>
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<td>32</td>
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<tr>
<td>0.03</td>
<td>9.2</td>
<td>31</td>
<td>97</td>
</tr>
<tr>
<td>0.05</td>
<td>12.4</td>
<td>41</td>
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</tr>
<tr>
<td>0.1</td>
<td>16.9</td>
<td>56</td>
<td>&gt;97</td>
</tr>
</tbody>
</table>

* From a column of Sephadex G-100 equilibrated with 1 M propionic acid

† From areas in ultracentrifuge patterns; corrected for the Johnston-Ogston effect.

The extinction coefficients used for light and heavy chains were those of Crompton and Wilkinson (14).

* The value obtained with 0.2 M 2-mercaptoethanol (29.9%) was taken as the maximum.
was not carried out after the second reduction. The values for a given type of chain are subject to a maximum correction of 0.1 S-carboxymethylcysteine group per chain, since the unreduced, alkylated γG-globulin contained 0.2 S-carboxymethylcysteine residue per molecule (Table II). It is evident that nearly all of the S-carboxymethylcysteine in the unreduced, alkylated sample was present on the heavy chains. This result demonstrates that the disulfide bond linking half-molecules connects two heavy chains. The net S-carboxymethylcysteine content per mole of heavy chains was between 8 and 15 times as great as that of the light chains, thus confirming the relatively greater lability of the H-H disulfide bond. The uncertainty in the ratio is owing to lack of knowledge as to which chain, or chains, contains the S-carboxymethylcysteine present in the unreduced, alkylated γG-globulin.

If one considers this possible correction, the S-carboxymethylcysteine content of the heavy chain was 1.5 to 1.6 moles per mole. Of this number, 0.1 to 0.2 mole per mole is attributable to L-H.
Chains were taken as 144,000, 22,000, and 50,000, respectively.

A The molecular weights of γG-globulin, light chains, and heavy chains were taken as 144,000, 22,000, and 50,000, respectively.

<table>
<thead>
<tr>
<th>Alkylated preparation</th>
<th>S-Carboxymethylcysteine content</th>
<th>moles/molea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unreduced γG-globulin</td>
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<td></td>
</tr>
<tr>
<td>Reduced γG-globulin</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Light chains</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Heavy chains</td>
<td>1.6</td>
<td></td>
</tr>
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</table>

| Separation of Light Chains in 0.025 M NaCl, pH 2.5—Elution patterns on Sephadex G-100 equilibrated with 0.025 M NaCl, pH 2.5, of γG-globulin preparations reduced with increasing concentrations of 2-mercaptoethanol or 2-mercaptoethylamine are shown in Figs. 1 and 2 (center columns). In each case, the proportion of light chains separated was much less than that obtained upon filtration in 1 M propionic acid (Figs. 1 and 2, left). In addition the light chains were poorly resolved from the heavy chains. These results will be considered in the “Discussion.”

| Extent of Exchange of Light and Heavy Chains under Conditions Resulting in Dissociation into Half-molecule Subunits—As indicated above, it was possible to obtain some separation of light from heavy chains on Sephadex in 0.025 M NaCl, pH 2.5. However, ultracentrifugation of reduced γG-globulin in this solvent yields a single symmetrical peak and a molecular weight corresponding to that of a half-molecule (9, 10). The following experiment was carried out to obtain an estimate of the degree of dissociation by measuring exchange of light and heavy chains among half-molecules under conditions used previously for preparation and recombination of half-molecules.

Exchange of light and heavy chains will be abbreviated as L-H exchange and is defined as follows.

\[ L_1H_1 + L_2H_2 \rightarrow L_1H_2 + L_2H_1 \]

The test of exchange was carried out with normal γG-globulins from doubly homozygous rabbits of genotypes \( Aa'Ab', Ab'Ab' \) and \( Ab'Ab, Ab'Ab' \). The allotypic specificity, \( a' \), is associated with heavy chains, whereas \( b' \) and \( b \) are associated with light chains (20, 21). Both are localized in Fragment Fab (22-25). Exchange would result in the association of light chains of allotypic specificity, b'5, with heavy chains that were originally linked to b4 light chains. The a1,b4 γG-globulin used was labeled with \(^{131}I \); exchange should thus have resulted in the association of b5 light chains with \(^{131}I \)-labeled heavy chains.

Since Fragment Fab (Porter Fragment I or II) produced by the action of papain consists of a complete light chain and a portion of a heavy chain (3), exchange should also have been reflected in the association of b5 light chains with \(^{131}I \)-labeled protein in that portion of the heavy chain present in Fragment Fab. The amount of \(^{131}I \) precipitated by anti-b5 serum from Fragment Fab prepared by papain digestion, after separation into half-molecules and recombination, should therefore indicate the extent of exchange of light and heavy chains.

It was necessary to make use of papain fragments for the following reason. It has been found that half-molecule subunits of different allotypic specificity recombine randomly after mixing at low pH and subsequent neutralization (26). Thus, an anti-b5 serum would necessarily precipitate the \(^{131}I \) label, in the absence of any L-H exchange, owing to the combination at neutral pH of a1,b5 half-molecules with a1,b4 half-molecules. This objection, however, does not apply if Fragment Fab is examined.

The two γG-globulin preparations, from doubly homozygous rabbits of genotypes \( Aa'Ab', Ab'Ab' \) and \( Ab'Ab, Ab'Ab' \), were trace labeled with \(^{131}I \) and \(^{131}I \), respectively. A portion of each labeled protein was reduced with 0.1 M 2-mercaptoethylamine at pH 5 and alkylated as described above. After dialysis against 0.025 M NaCl and acidification to pH 2.4, more than 95% of each reduced protein migrated with a velocity (\( s_{20w} \)) of 3.08, characteristic of half-molecules. Ultracentrifugation was carried out at 10°, and the remainder of each protein solution was maintained at the same temperature. After standing for 2 hours at pH 2.4, a mixture of the two reduced γG-globulins was prepared; the weight ratio of a1,b5 to a1,b4 was 10:1. Sodium chloride was added to a final concentration of 0.4 M to the mixture, which was dialyzed against 4 liters of cold 0.2 M NaCl overnight, then against 0.1 M phosphate buffer, pH 7.5. At the time the mixture was prepared, NaCl was also added to separate portions of each reduced γG-globulin preparation; these portions were dialyzed separately in the same manner as the mixture, then mixed at pH 7.5 in a 10:1 weight ratio (a1,b5 to a1,b4). Each of the two mixtures, prepared at low pH and neutral pH (18 and 10 mg, respectively) was digested with papain as described under “Methods.” Protein concentrations during digestion were 11 and 12 mg per ml. After digestion, no residual 68 S protein was observed in the ultracentrifuge. After overnight dialysis against a 4-liter portion of 0.02 M sodium acetate, the papain fragments were fractionated on carboxymethyl cellulose columns (1 cm² per mg of protein), equilibrated with 0.01 M sodium acetate, pH 5.5. The protein comprising the first peak, which contains Fragment Fab, was pooled and dialyzed against sodium chloride-borate buffer, pH 8.0, ionic strength 0.16. Approximately 60% of the protein applied to the column was recovered in this pool. Ouchterlony analysis of both preparations of papain fragments showed no contamination by Fragment Fe. The amino acid compositions agreed closely with that of Fragment Fab. Expressed as moles per cent, the averages of the relative differences between values for individual amino acids and that of Fragment Fab of pooled normal γG-globulin (27) were 4.9% and 5.0%, for the 10:1 mixture of b4 and b5 γG-globulins prepared at low pH or at neutral pH, respectively. The corresponding average differences, when compared with values for Fragment Fe (17), were approximately 40% for each preparation.

A aliquots of the solution of Fragment Fab (40 μg in 0.1 ml) were allowed to react with 0.4 ml of a nonspecific rabbit antiserum containing precipitating antibody specific for rabbit γG-immunoglobulin of allotype b5. The mixtures were incubated...
associated into half-molecules in 0.025 M KCl, pH 2.5. X-Carboxy-
chains. In contrast, more than 95% of the reduced protein dis-
corresponding to the release of about one-seventh of the light
The yield was 4% of the total protein applied to the column,
sequent to this reduction only a small proportion of the light
chains was separable on Sephadex G-100 in 1 M propionic acid.

It was necessary as a control to show that part of the 125I
label in the a1,b4 molecule was actually present in that part of
the heavy chain (Fragment Fd) which is localized in Fragment Fab. A portion of the a1,b4 yG-globulin was trace labeled and Fragment Fd was prepared by the method of Fleishman, Porter, and Press (3). The ratios of its specific activity to that of the untreated labeled yG-globulin and of Fragment Fab were 1.0 and 1.1, respectively, indicating that a large fraction of the radiolabel of the protein was in Fragment Fd.

**DISCUSSION**

Previous studies have indicated that reduction of a single
disulfide bond suffices to permit subsequent separation of half-
molecule subunits in a large proportion, if not all, of the rabbit yG-globulin molecules (10). Strong circumstantial evidence identifies this disulfide bond as the same as that which joins two fragments of type Fab’ in the 4.6S product of peptic digestion (11). Earlier data have also suggested that the bond connects two heavy chains. Thus, each light chain separated from reduced, alkylated yG-globulin contains one S-carboxymethyl-
cysteine residue; this indicates that a light chain is connected to
the remainder of the molecule through a single disulfide bond
(3, 14). Also, as there is no release of subunits without prior reduction, each polypeptide chain must be connected to
at least one other chain through a disulfide linkage. Given this
condition and the fact that each light chain is joined to the
molecule through one disulfide bond it seemed necessary to postulate that the bond joining half-molecules links two heavy chains (10).

The effects of reduction with 0.01 M 2-mercaptoethanol at pH
6.2, followed by alkylation and S-carboxymethylcysteine analyses, provided direct evidence in support of this hypothesis. Subsequent to this reduction only a small proportion of the light chains was separable on Sephadex G-100 in 1 M propionic acid. The yield was 4% of the total protein applied to the column, corresponding to the release of about one-seventh of the light chains. In contrast, more than 95% of the reduced protein disassociated into half-molecules in 0.025 M NaCl, pH 2.5. S-Carboxymethylcysteine analysis of the separated chains (Table II) showed that nearly all of the sulfhydryl groups liberated under these conditions are present on the heavy chains. In this experiment, the chains were separated by reducing a second time with a concentration of 2-mercaptoethanol (0.2 M) sufficient for essentially complete reduction of interchain bonds, and then by isolating light and heavy chains without a second alkylation. The S-carboxymethylcysteine content of the light chains was 0.1 to 0.2 group per chain, with the uncertainty deriving from the value, 0.2 group per molecule, obtained with alkylated, unreduced yG-globulin; that of the isolated heavy chains was 1.5 to 1.6 groups per chain. Thus, under conditions of reduction resulting in a substantial degree of dissociation into half-molecules without appreciable release of free light chains, nearly all of the sulf-
hydryl groups formed by the reduction were found on the heavy chains. It is apparent, therefore, that the disulfide bond linking half-molecules joins two heavy chains.

The results of the same experiment are also in quantitative accord with the previous finding (10) that reduction of a single disulfide bond is sufficient to permit dissociation of half-molecule subunits at low pH. As indicated above, there were 1.5 to 1.6 S-carboxymethylcysteine residues on each heavy chain. Of this number, 0.1 or 0.2 is attributable to L-H disulfide bonds, which gives a net value of 1.3 to 1.5 for the number of residues derived from disulfide bonds joining half-molecules. Since there was essentially complete dissociation into half-molecules at low pH, the results are consistent with the premise that disso-
ciation can occur after reduction of 1 disulfide bond per molecule. The result, 1.3 to 1.5—SH groups per molecule, is a maximum since slight reduction of the 5 or 6 intrachain bonds present in each heavy chain may have occurred.

In view of their finding that 5 disulfide bonds are reducible in the absence of urea or detergent, Fleishman, Porter, and Press (3) proposed earlier that there are 3 interchain disulfide bonds linking the heavy chains; i.e. that all of the disulfide bonds cleaved were interchain. This was based on the fact that intra-chain bonds in a number of proteins are not cleaved by sulfite or mercaptan under such conditions (29, 30). Several exceptions to this general rule have been noted (31). In addition, Schur and Christian (32) were able to cleave as many as 12 disulfide bonds in sheep yG-globulin with a low concentration of 2-mer-
captoethanol in neutral buffer. In view of the general structural similarity of the yG-globulins of various species, it seems highly improbable that all 12 are interchain bonds. From their data and ours it would appear that some of the intrachain disulfide bonds in rabbit yG-globulin, of which there are approximately 17, are susceptible to attack under the conditions ordinarily used for reduction of interchain bonds.

The experiments discussed above indicate a greater degree of selectivity of reduction of the H-H disulfide bond than had been noted in our previous studies. In the earlier work (reduction with 2-mercaptoethyamine at pH 5), it was found that up to 70% of the yG-globulin could be dissociated into half-molecules after reduction of 1.2 disulfide bond for each dissociable molecule (10). However, dissociation of the remaining 30% of the popu-
lated required reduction of substantially more than 1 disulfide bond per molecule. Possible explanations offered (10, 11) were (a) that in part of the population of molecules more than 1 disulfide bond per molecule joins the half-molecule subunits; (b) that only 1 such bond might be present in each molecule, but that its lability, relative to that of other disulfide bonds varies; or (c) that the reduction is reversible and a high con-
centration of reducing agent is needed to bring it to completion. In the present investigation, essentially complete breakdown into half-molecules was effected after reduction of 1.3 to 1.5 disulfide bond per molecule.

The difference in the two sets of findings is attributable to the
between the fragments of Oige Fab'. This argument in itself is disrupted by the action of pepsin (and presumably papain). Not conclusive since it is possible that noncovalent bonds are actually present in this region of the native molecule but that they are disrupted by the action of pepsin (and presumably papain).

A second type of evidence derives from the fact that various proteolytic enzymes liberate 3.5S fragments, which are relatively resistant to further enzymatic digestion. The disappearance of 3.5S fragments to further degradation during the proteolysis indicates that there are certain regions of the molecule which are particularly susceptible to the action of enzymes. This was attributed (17, 33, 34) to the existence of a loose or unfolded structure in certain parts of the molecule, which provide points of attack for proteolytic enzymes. Evidence suggestive of a "hinge" point in the 3S fragment formed by peptic digestion, presumably located near the H-H disulfide bond, was derived from Feinstein and Rowe from electron photomicrographs (35). In addition, after proteolysis with pepsin, this disulfide bond joins the two univalent fragments; however, after treatment with papain a disulfide bond appears to be present in Fragment Fe (6). These data suggest (11) that the two enzymes cleave on opposite "sides" of the disulfide bond and that this bond may therefore lie in the unfolded portion of the molecule which is highly susceptible to proteolysis.

The concept that the lability of a disulfide bond is directly related to the extent of re-enforcement by noncovalent interactions has been proposed by Cecil (29) on the basis of the fact that interchain disulfide bonds are in general more labile than intrachain bonds. This is attributable to the greater increase in entropy which accompanies the cleavage of a disulfide bond in the absence of strong supporting noncovalent interactions. In the case of γG-globulin, the relative labilities of the H-H and H-L bonds are reflected in the ease of separation of subunits subsequent to reduction of the bonds. After reduction, half-molecules dissociate in 0.025 M NaCl, pH 2.5, conditions which result in poor dissociability of light from heavy chains.

Dissociation of half-molecules into their component light and heavy chains in dilute NaCl solution, pH 2.5, is not readily achieved even after reduction of a significant proportion of L-H disulfide bonds. This is indicated first by the symmetry of the 3S peak obtained in the ultracentrifuge after reduction with 0.1 M 2-mercaptoethanol and the fact that the measured molecular weight is that of a half-molecule (9). In the present experiments, there was little exchange at pH 2.5 of light and heavy chains among reduced γG-globulins differing in allotype at the b locus (which controls antigenic determinants on the light chains), but identical at the a locus (which controls determinants on heavy chains (20, 21)). The mixture prepared to determine the extent of exchange at low pH comprised γG-globulins of allotypes a1, b5 and a1, b4, with the former present in 10-fold excess. The conditions used were those previously employed in experiments involving "hybridization" of half-molecules (36). Each γG-globulin preparation was reduced with 0.1 M 2-mercaptoethanol and the mixture was prepared in 0.025 M NaCl, pH 2.5. After neutralization, the extent of exchange was found to be very small. Less than 2% of the heavy chains (corrected for the value 2.3% obtained with the control), originally associated with a1, b4 γG-globulin chains, were found in association with the b5 chains after neutralization. The use of a 10-fold excess of a1, b5 globulin provided great sensitivity. If random exchange had occurred, 90% of the a4 chains should have become associated with light chains from the a1, b6 preparation. The method used required the measure-
sociation into half-molecules was achieved after reduction of X-Carboxymethylcysteine analyses provided direct evidence by 2-mercaptoethylamine at the highest concentration tested.

Although this experiment was necessarily carried out with γG-globulins of different allotypes, it seems probable that similar results would be obtained with γG-globulins of the same allotype, since the heavy chains in the two preparations were of the same allotypic specificity and should presumably have similar combining affinities. However, the absence of exchange might be attributable to the failure of γG-globulin of either allotypic specificity to dissociate into component light and heavy chains under the conditions of the experiment. The results therefore do not exclude the possibility that one but not both of the γG-globulins is dissociable at pH 2.5. In view of the close similarity of light chains of allotypic specificity b4 and b5 with respect to amino acid composition (28) and the fact that the heavy chains possessed the same specificity, this possibility appears somewhat unlikely.

The evidence presented indicates that there is only a slight degree of dissociation into light and heavy chains in 0.025 M NaCl, pH 2.5. Some separation of the two chains was noted on Sephadex G-100 under these conditions. However, the yield of light chains was considerably lower than that obtained in 1 M propionic acid, and the resolution of the two types of chains was comparatively poor. This was especially true after reduction with 2-mercaptoethanol (Fig. 2). The amounts of protein, the volumes of protein solution, and the degree of dissociation into light and heavy chains in 0.025 M NaCl, pH 2.5, the symmetry of the schlieren peak in the ultracentrifuge under these conditions, and data on molecular weights of half-molecules (9) suggest that there is only slight dissociation. However, this might result in appreciable separa-

milder conditions than H-L separation. This, together with other data, suggests that the relatively great lability of the H-H disulfide bond may be due to the absence of strong stabilizing noncovalent interactions between the two heavy chains in the vicinity of the bond. Little exchange of light chains was observed under conditions utilized for separation and recombination of half-molecules.

SUMMARY

Labilities of disulfide bonds linking light and heavy chains (L-H bonds) were compared to the lability of the bond which must be reduced to permit dissociation into half-molecules (H-H bond). Reductions were carried out with increasing concentrations of 2-mercaptoethanol at pH 8.2 or with 2-mercaptoethanol at pH 5. With low concentrations of either reducing agent, the number of H-H bonds cleaved was several times as great as the number of H-L bonds. The relative lability of the H-H bond is somewhat greater during reduction with 2-mercaptoethanol at pH 8.2 than with 2-mercaptoethyl-

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Relative Labilities of the Two Types of Interchain Disulfide Bond of Rabbit γ G-Immunoglobulin

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