Reduction and Reoxidation of a Critical Disulfide Bond in the Rabbit Antibody Molecule*

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Treatment of 0.5-S rabbit antibody with pepsin removes a large inactive fragment. The residual portion of the molecule, which is still bivalent, has a molecular weight of 106,000 and a sedimentation coefficient of 4.6 to 5.0 S (1, 2). Reduction of a single, highly labile disulfide bond cleaves this fragment approximately in half (2, 3). The resulting 3.5-S fragments are univalent and inhibit specific precipitation (1). Upon reoxidation 5-S protein is formed once again; and if specifically purified antibody was the starting material, most of the original precipitating activity is restored (1). Antibody of mixed specificity can be prepared by reducing and reoxidizing a mixture of antibodies specific for two different antigens (5, 6). The univalent fragments formed by the action of pepsin and a reducing agent are similar in many properties to those produced by proteolysis with papain (1, 4, 7).

The two univalent fragments of an individual molecule appear to be similar. Evidence for this is the fact that they migrate as a single 3.5-S peak in the ultracentrifuge (7) and are eluted together from carboxymethyl cellulose (8). In addition, fingerprinting studies by Gitlin and Mcllr (9) suggest that the number of peptides liberated from γ-globulin is approximately half the number of peptide bonds broken, which is consistent with the possibility of symmetry within the molecule. Fabey and Askonas (10) have shown that the electrophoretic mobilities on starch gel of the active fragments of individual molecules of mouse γ-globulin are similar.

Five additional disulfide bonds can be cleaved in univalent fragments, formed by treatment with pepsin and reducing agent, without change in sedimentation coefficient or appreciable loss of specific activity (11, 12). When such fragments are allowed to recombine only 5-S protein is formed (11, 12). The absence of products of larger molecular size indicates that only the sulfhydryl groups liberated from the disulfide bond linking the univalent fragments are available for recombination among fragments. Retention of specific activity and molecular size after reduction was subsequently reported by Grossberg, Stelos, and Pressman (13), who investigated the univalent fragments produced by pepsin treatment with papain.

In a preliminary communication (14), it was reported that the critical disulfide bond which links the univalent fragments after pepsin treatment can also be reduced readily in the untreated antibody molecule. There was no change in sedimentation coefficient on reduction, but subsequent treatment with pepsin yielded 3.5-S univalent fragments rather than the 5-S bivalent product. This fact made it possible to investigate in detail the reduction and reoxidation of this disulfide bond in the antibody molecule, before any treatment with enzyme. The results of the study are reported here.

EXPERIMENTAL PROCEDURE

Immunization—Methods used for the preparation of antiserum in rabbits have been described (4). A γ-globulin preparation was made from the pooled serum of several animals, hyperimmunized by inoculation with ovalbumin, by three precipitations with decreasing concentrations of sodium sulfate (15), followed by passage through a column of diethylaminoethyl cellulose (16) in 0.0175 M phosphate buffer, pH 6.9; the column was first equilibrated with the same buffer. The product migrated as a single peak in the ultracentrifuge (g20, w = 6.1 S), and showed only one band, characteristic of γ-globulin, when tested by paper electrophoresis. Twenty-eight per cent of the protein was precipitable by an optimal amount of ovalbumin.

Other Materials—Pepsin and chicken ovalbumin were obtained as twice crystallized preparations from the Worthington Biochemical Corporation; CMB⁰ was purchased from the Nutritional Biochemicals Corporation and MEA from the California Corporation for Biochemical Research. Reagent grade L-cysteine was obtained as the free amino acid from the Cyclo Chemical Corporation. It was recrystallized from hot water, dried under vacuum, and stored in a number of containers that were opened only once. It reacted stoichiometrically with CMB (see below).

Precipitin Reactions—Precipitating mixtures of antigen and antibody were allowed to stand in the refrigerator for 5 days. Reactions were carried out at neutral pH in mixtures of sodium chloride and sodium acetate. The ionic strength was 0.13 to 0.14 and the total volume 0.3 to 0.4 ml. Amounts of protein in the washed precipitates were estimated by dissolving in 1.0 ml of 0.02 N sodium hydroxide and determining the absorbancy at 280 nm (4). Amounts of antigen in precipitates at equivalence were estimated from the antigen-antibody ratio in the region of antibody excess, on the assumption of complete precipitation of antigen in this region. Since the extinction coefficient of ovalbumin is approximately 40% as great as that of rabbit γ-globulin, and the amount present in precipitates in the region of antibody

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The abbreviations used are: CMB, sodium salt of p-chloromercuribenzoic acid; MEA, 2-mercaptoethylamine hydrochloride.
Reduction and Reoxidation of a Critical Disulfide Bond

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Reduction of rabbit antiovalbumin γ-globulin with increasing concentrations of MEA, followed by peptic digestion

<table>
<thead>
<tr>
<th>Concentration of MEA used</th>
<th>-SH groups liberated per molecule protein</th>
<th>Recovery of protein</th>
<th>5-S protein formed on peptic digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>mole/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>82%</td>
<td>%</td>
</tr>
<tr>
<td>0.01</td>
<td>1.1</td>
<td>83%</td>
<td>70 (4.6 S)</td>
</tr>
<tr>
<td>0.025</td>
<td>2.2</td>
<td>85%</td>
<td>65 (4.6 S)</td>
</tr>
<tr>
<td>0.05</td>
<td>5.3</td>
<td>83%</td>
<td>&lt;5x</td>
</tr>
<tr>
<td>0.10</td>
<td>6.6</td>
<td>85%</td>
<td>&lt;5x</td>
</tr>
</tbody>
</table>

* Details of the reduction and proteolysis are given in the text.

+ After peptic digestion and dialysis. Values are based on the amounts of protein treated with pepsin.

+ Determined by measurement of areas from enlargements of schlieren patterns obtained after 80 minutes at 59,780 r.p.m. The per cent value is based on the nondialyzable protein. Values are based on the number of slower moving material.

+ Molecular weight of the protein was taken as 150,000. The value, 0.4—SH groups per molecule, was obtained for unreduced protein and was subtracted from the values for the reduced samples.

+ Excess is approximately 7% by weight, these corrections were made.

In tests for inhibition of precipitation by fragments of antibody, the untreated antibody was added to reaction mixtures last.

**Titration of —SH Groups**—Sulfhydryl groups were estimated essentially as described by Boyer (17). Samples were treated with excess CMB and back-titrated with a freshly prepared solution of L-cysteine. In most instances the amount of CMB used was 80 to 110% in excess of the —SH groups present; the latter value was estimated on the basis of preliminary experiments. When the content of free —SH was less than 1 group per molecule of protein, the amount of CMB added before back-titration was equivalent to 2 —SH groups per molecule.

The CMB solution was standardized by titration with L-cysteine. The concentration thus obtained agreed in each case within 2% with the value estimated by using Boyer’s extinction coefficient for CMB. All titrations were carried out both in the presence and absence of 0.5% sodium lauryl sulfate, which had been recrystallized twice from hot ethanol. The effect of the detergent was small in every case and the data presented below are those obtained with detergent present.

**Other Methods—Sedimentation Analysis**—was carried out in the Spinco model E ultracentrifuge at 59,780 r.p.m. Temperature was controlled to within ±0.2° of the desired value. For correction of s values to s20, w, the partial specific volume was taken as that of untreated γ-globulin, 0.745 (18).

Viscosities were measured with an 8-ml Ostwald-Fenske viscometer, immersed in a constant temperature water bath at 20 ± 0.05°. Amounts of protein in γ-globulin preparations were estimated by use of the extinction coefficient, 1.50 optical density units per mg per ml.

**RESULTS**

Reduction with Increasing Concentrations of 3-Mercaptopropeptide—Hydrochloride, followed by Proteolysis with Pepsin—Portions of rabbit antiovalbumin γ-globulin (55 mg each) were treated with varying concentrations of MEA (0.01 M to 0.1 M) in 0.1 M sodium acetate buffer, pH 5.0; the protein concentration was 35 mg per ml. Reductions were carried out under nitrogen for 75 minutes at 37°. To remove the reducing agent, the pH of the solution was lowered to 4.5 by addition of 1 M acetic acid and it was passed through an 8-mm × 16-cm or, for the sample reduced with 0.1 M MEA, an 8-mm × 21-cm column of Amberlite IR-120 cation exchange resin in the sodium cycle; this was done in the cold room. The column was first equilibrated with 0.1 M sodium acetate buffer, pH 4.5. The size of the column required to remove the MEA was determined by preliminary experiments and a large safety factor was added. Immediately after collecting the protein, it was neutralized and sufficient CMB (2.22 mm) was added to give a final molar concentration in excess of the —SH groups present. The latter value was estimated on the basis of preliminary experiments. A portion of the sample treated with CMB was used for the determination, by back titration with cysteine, of the number of —SH groups liberated. The remainder was dialyzed against 4 liters of cold 0.1 M sodium acetate, then treated with an amount of pepsin equal to 2% of the weight of γ-globulin for 6 hours at 37°, pH 4.5. A control sample that had not been reduced was similarly incubated under nitrogen, passed through a column of IR-120 resin, treated with CMB (equivalent to 2 —SH groups per molecule), and then with pepsin. The content of —SH groups was determined on a portion of this material after passage through the column.

The results of these experiments are shown in Table I. After treatment of the unreduced sample with pepsin, the s20, w value of the major product was 4.6 S. This comprised about 70% of the area in the schlieren pattern. The remainder consisted of heterogeneous slower moving material. Samples that were first reduced with increasing concentrations of MEA yielded diminishing amounts of the 4.6-S component, and increasing proportions of slower moving material, upon treatment with pepsin. It was possible to determine the sedimentation coefficient of the slower component in the last two experiments of Table I. The values were 3.2 and 3.3 S, respectively.

Reduction with 0.05 M MEA was sufficient to permit essentially complete breakdown to 3.2 S on subsequent digestion. The reduction resulted in the liberation of 5.3 new —SH groups per molecule.

Since the formation of 3.5-S product can be brought about by treatment with pepsin first, followed by reduction (1), it appears that the same process was taking place in these experiments, with the sequence of events reversed. Complete reduction of the disulfide bond which links the univalent fragments appears to require the cleavage of 2.7 disulfide bonds, of a total of approximately 23 disulfide bonds in the molecule (19). (When the disulfide bond is broken subsequent to peptic digestion it is the first to be reduced in the 5.8 fragment (3)).

The results in Table I are expressed in terms of the amount of the faster component because smaller peptides were not adequately resolved from the slower peak.

Reoxidation of —SH Groups on the Ion Exchange Column—To investigate the possibility that reoxidation might be occurring on the ion exchange column, 115 mg of antiovalbumin γ-globulin in 3.8 ml were reduced with 0.06 M MEA as described above, and passed through an 8 × 175-mm column of IR-120 resin at pH 4.5 in the cold room. A portion was removed for estimation of free —SH. The remainder was immediately passed through another column, of the same diameter but twice as long as the
first column, at the same rate of flow. The —SH content was then determined. The two values obtained were 5.7 and 5.8 —SH groups, respectively, per molecule (molecular weight, 150,000). This indicates that reoxidation of —SH on the column is slow. It argues against, but does not preclude, the possibility that one or more disulfide bonds are reformed very rapidly. The only part of the conclusions on which this bears is that relating to the lability of the critical disulfide bond, relative to that of other disulfide bonds in the molecule.

**Effects of Reduction on Sedimentation Rate and Specific Viscosity, and on Precipitability before and after Treatment with Pepsin**—As indicated above, treatment for 75 minutes with 0.06 M MEA was sufficient to reduce the disulfide bond linking the univalent fragments of the γ-globulin molecule; subsequent treatment with pepsin yielded a 3.2-S, rather than a 4.6-S product. The effects of reduction of this bond on the specific viscosity, sedimentation rate, and immunologic activity were determined next. Rabbit antiovalbumin γ-globulin (519 mg) was treated with 0.06 M MEA under nitrogen for 75 minutes at pH 5.0 and 37°C. The pH was lowered to 4.5 and the sample was passed through a 14- × 230-mm column of IR-120 resin in the cold room to remove the MEA. The pH of the eluate was adjusted to 8.0, and excess CMB was added immediately. The following determinations were carried out with this sample: (a) number of free —SH groups liberated on reduction; (b) sedimentation velocity; (c) relative viscosity; (d) precipitation with varying concentrations of antigen; (e) nature of the product formed on pepsin digestion (sedimentation velocity, precipitability, or capacity to inhibit the precipitin reaction of untreated antibody with antigen). Experiments b, c, d, and e were carried out after dialyzing the sample against 4 liters of cold 0.1 M sodium acetate.

As a control, 500 mg of the same antiovalbumin γ-globulin preparation (not reduced) were similarly incubated at 37°C, pH 5.0, and passed through a column of IR-120 resin at pH 4.5 in the cold room. The solution was neutralized, treated with CMB and experiments identical with Experiments a, b, c, d, and e in the preceding paragraph were carried out.

As indicated in Table II, reduction with 0.06 M MEA resulted in the release of 5 new —SH groups per molecule. There was no appreciable change in the sedimentation coefficient or specific viscosity; the latter was measured at protein concentrations of 10 and 20 mg per ml. The amount of precipitate formed upon addition of an optimal quantity of antigen was 92% of that formed by the unreduced protein.

On treatment of the reduced sample with pepsin, followed by dialysis, the major component had a sedimentation coefficient of 3.3 S as compared with 4.6 S for the pepsin-treated, unreduced sample. Also, the reduced, pepsin-treated protein had lost the capacity to form specific precipitates and was a potent inhibitor of the homologous precipitin reaction. The presence of 4 mg of such fragments caused a 93% reduction in the amount of precipitate formed by 2.5 mg of untreated antibody γ-globulin with slightly less than an optimal concentration of ovalbumin (from 580 µg of precipitate to 43 µg). This provided further evidence that the disulfide bond linking the univalent fragments had been cleaved by treatment with MEA; a univalent rather than a bivalent product was evidently formed on treatment with pepsin.

The unreduced sample still formed specific precipitates after pepsin digestion (Table II).

**Reoxidation of Reduced Disulfide Bonds**—To study reoxidation of the reduced, critical disulfide bond, a sample of antiovalbumin γ-globulin was treated as above for 75 minutes at 37°C with MEA at a concentration of 0.06 M. After passage, as before, through a column of IR-120 resin, the protein was neutralized to pH 8.0. An aliquot was treated immediately with excess CMB; the remainder was placed in a long test tube immersed in a constant temperature bath at 25 ± 0.05°C. At intervals up to 2 hours, samples were removed, excess CMB was added, and the concentration of free —SH groups determined by back-titration with cysteine. A portion of each CMB-treated sample was dialyzed against 4 liters of cold 0.1 M sodium acetate and treated with pepsin for 6 hours under the conditions described above. Sedimentation analysis was carried out after neutralizing and dialyzing once more against the same solution. The results are given in Table III.

The fact that the critical disulfide bond had been cleaved was indicated by the result of hydrolysis with pepsin of the sample treated with CMB immediately after removal of the reducing agent. No detectable amount of S-S protein was formed. Samples treated with CMB after standing at pH 8.0 for periods of time up to 120 minutes showed a progressive loss of free —SH groups (from 5.1 to 0.6, Table III). Also, there was a change in the nature of the products observed when the samples were treated with pepsin. (They were dialyzed before and after proteolysis.) Increasing amounts of a 4.4-S component were formed. The products of pepsin digestion of the sample that had been allowed to stand for 120 minutes before addition of CMB were very similar to those of the unreduced, pepsin-treated

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**Table II**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>S.@-W</th>
<th>Relative viscosityb</th>
<th>—SH groups per protein molecule</th>
<th>Amount of precipitate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not reduced</td>
<td>6.1 S*</td>
<td>1.079</td>
<td>1.159</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Reduced</td>
<td>6.2 S*</td>
<td>1.079</td>
<td>1.156</td>
<td>5.8 µg</td>
</tr>
<tr>
<td>Not reduced, treated with pepsin</td>
<td>4.6 S*</td>
<td>1.079</td>
<td>1.156</td>
<td>5.8 µg</td>
</tr>
<tr>
<td>Reduced, treated with pepsin</td>
<td>3.3 S*</td>
<td>1.079</td>
<td>1.156</td>
<td>5.8 µg</td>
</tr>
</tbody>
</table>

| Material did not yield precipitates with antigen. Four milligrams caused a reduction of 93% in the amount of precipitate formed (580 µg) by 2.5 mg of untreated antibody globulin and slightly less than an optimal concentration of antigen. |

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*MEA (0.06 M) was used for each reduction.

† Relative to the salt solution against which the protein had been dialyzed (0.1 M sodium acetate). The values 10 and 20 mg per ml are protein concentrations.

‡ Significant amounts of other components were not observed.

§ Obtained with 2.5 mg of protein and an optimal antigen concentration.

||* Represented 70% of the total area in the schlieren pattern; the remainder was slower moving material.

∥ Obtained with 3 mg of protein and an optimal antigen concentration.

* Material did not yield precipitates with antigen. Four milligrams caused a reduction of 93% in the amount of precipitate formed (580 µg) by 2.5 mg of untreated antibody globulin and slightly less than an optimal concentration of antigen.
TABLE III
Reformation of disulfide bond linking univalent fragments after reduction of antiovalbumin γ-globulin

<table>
<thead>
<tr>
<th>Period of reoxidation</th>
<th>No. of -SH groups per protein molecule</th>
<th>Recovery of protein after peptic digestion</th>
<th>Protein formed on peptic digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>5.1</td>
<td>71%</td>
<td>3.1 S and slower (96%)</td>
</tr>
<tr>
<td>15 min</td>
<td>4.0</td>
<td>70%</td>
<td>3.2 S and slower (96%)</td>
</tr>
<tr>
<td>30 min</td>
<td>2.8</td>
<td>69%</td>
<td>3.0 S (85%) faster material</td>
</tr>
<tr>
<td>60 min</td>
<td>1.8</td>
<td>70%</td>
<td>4.4 S (58%)*</td>
</tr>
<tr>
<td>120 min</td>
<td>0.6</td>
<td>71%</td>
<td>4.4 S (66%)*</td>
</tr>
<tr>
<td>Unreduced</td>
<td>0.5</td>
<td>80%</td>
<td>4.3 S (71%)*</td>
</tr>
</tbody>
</table>

* Reoxidation at 25°, pH 8.0; protein concentration, 25 mg per ml. Details of procedure are in the text. Excess CMB was added to stop the reoxidation at the times specified.

b Molecular weight of the protein was taken as 150,000.

c Values refer to dialyzed protein; calculated on the basis of amounts treated with pepsin.

d Determined by measurements of areas from enlargements of schlieren patterns obtained after 80 minutes at 59,780 r.p.m.

e The remainder consisted of slower moving components.

γ-globulin. It thus appears that the disulfide bond linking the univalent fragments was largely reformed during the 2-hour period.

To study the reoxidation further, another experiment was carried out in which a 300-mg sample of antiovalbumin γ-globulin, reduced with 0.06 M MEA, was allowed to reoxidize after removing the reducing agent on an ion exchange column. The methods were as described above. A portion of the reduced sample, which was treated with CMB before reoxidation, was found to have 5.5 free -SH groups per molecule and yielded a 3.3-S product on treatment with pepsin. The remainder of the sample was allowed to reoxidize at pH 8 and 25° for 6 hours, and was then dialyzed against 4 liters of cold 0.1 M sodium acetate. By addition of excess CMB and back-titration it was found that there was 0.3 free -SH group per molecule. A portion of this CMB-treated sample was dialyzed again, treated with pepsin for 6 hours at pH 4.5, neutralized, and dialyzed once more.

Photographs of the schlieren patterns of these samples are shown in Fig. 1. Diagrams A and B indicate the close similarity of the unreduced and reduced γ-globulins, respectively. Photographs taken after shorter periods of time similarly showed no appreciable differences, except for the presence of a slight amount of aggregated material in the reduced sample. As expected, there was a marked difference between Diagrams C and D, which correspond to the pepsin-treated, reduced, and unreduced samples, respectively. Diagram E shows that the schlieren pattern of reoxidized, pepsin-treated protein is essentially the same as that for the unreduced, pepsin-treated material (Diagram D).

Specific Precipitability, after Treatment with Pepsin, of Reoxidized Samples—As already indicated, the reduced, pepsin-treated antiovalbumin γ-globulin failed to form specific precipitates, and inhibited the precipitation of untreated antibody with antigen. This was confirmed with the reduced, pepsin-treated sample described in the preceding paragraph. This material did not precipitate with a wide range of concentrations of antigen. Four milligrams caused a reduction from 580 μg to 37 μg in the amount of precipitate formed by 2.5 mg of unreduced antiovalbumin γ-globulin, reacting with slightly less than an optimal concentration of antigen.

In contrast, the portion of the material which was permitted to reoxidize before peptic digestion and dialysis formed specific precipitates. With an optimal amount of antigen, 2.8 mg yielded 558 μg of precipitate, as compared with 590 μg from 2.8 mg of unreduced, pepsin-treated antiovalbumin γ-globulin.

Comparison of Rates of Hydrolysis by Pepsin of Unreduced and Reduced γ-Globulin—The reduced and unreduced samples of antiovalbumin γ-globulin previously used for viscosity measure-

FIG. 1. Photographs of schlieren patterns taken in the Spinco model E ultracentrifuge after 80 minutes at 59,780 r.p.m. Sedimentation is from left to right. Samples in 0.1 M sodium acetate, pH 7 to 8. A, unreduced rabbit antiovalbumin γ-globulin; B, reduced with 0.06 M MEA and treated with excess CMB; C, reduced, treated with CMB, then with pepsin; D, unreduced, treated with pepsin; E, reduced, reoxidized, then treated with pepsin. Details are in the text. The numerals are s20,w values.
ments (Table II) were also employed for a comparison of rates of proteolysis. Each had been treated with CMB in excess of the —SH groups present, then dialyzed against 0.1 M sodium acetate. Acetic acid was added to lower the pH to 4.4 for treatment with pepsin. The protein concentration of each sample was approximately 13.5 mg per ml and the amount of pepsin used was 2% of the weight of protein. Proteolysis was carried out at 37°. Samples were removed and neutralized, to inactivate the enzyme, after 0, 15, 30, 60, and 150 minutes, then dialyzed against 0.1 M sodium acetate and examined in the ultracentrifuge. Recoveries of protein were 75 to 84%.

Within 15 minutes the reduced sample was almost completely broken down to 3.1 S, whereas approximately 60% of the unreduced sample remained undegraded. After 1 hour of peptic digestion the extent of breakdown of the unreduced sample to 4.6 S was about 90%, and it was essentially complete in 150 minutes. The rate of breakdown of the reduced γ-globulin appears from these results to be more than 4 times as rapid as that of the unreduced γ-globulin.

**DISCUSSION**

It has previously been shown that the breakdown of rabbit antibody into univalent fragments can be accomplished by successive treatments with pepsin and a reducing agent. The first stage leaves a bivalent residue. The second step requires the cleavage of one disulfide bond which is relatively labile, as shown by the fact that it is the most readily reduced of approximately 14 disulfide bonds in the major product of peptic digestion (3).

The present results indicate, first, that the reverse procedure, i.e. reduction followed by enzymatic digestion, also yields univalent, 3.5-S fragments. However, it is necessary to prevent reoxidation of the liberated —SH groups before enzymatic digestion. Otherwise a product with a sedimentation coefficient of approximately 5 S is obtained, as in the peptic digestion of untreated antibody. The products obtained on proteolysis of native or reoxidized γ-globulin had the same sedimentation patterns and specific activities. It is evident that the critical disulfide bond is labile in the intact antibody as well as in the pepsin-treated molecule. Cleavage of the bond was substantially complete when fewer than 3 disulfide bonds had been reduced, of a total of approximately 25 in the molecule (19). It is not possible to determine from the data whether the additional —SH groups were derived from other particular disulfide bonds or from the partial reduction of a number of bonds. It seems improbable that more than one disulfide bond links the univalent fragments, since only one holds them together subsequent to the treatment with pepsin.

The rate of reformation of the disulfide bond joining the univalent fragments was somewhat greater than that previously observed for the reoxidation of separate 3.5-S fragments, released upon reactivation of pepsin-treated antibody (12). In the latter instance, recombination to give 5-S protein attains about one-fourth of its final value in 1 hour, whereas it appeared to have reached 80% of its final value in 1 hour in the present investigation, at the same pH and temperature. Within 5 hours the separate 3.5-S fragments combined to their maximal extent (12). Actually the difference in the rates of recombination in the two sets of experiments is surprisingly small. One might have expected that the proximity of the —SH groups in the reduced, undigested molecule would have greatly increased the rate as compared with that observed for the separated fragments. It seems probable that collision frequency is not the major factor in determining the rate of recombination within a reduced molecule.

No demonstrable effects were observed on preliminary reoxidation. The specific viscosity, sedimentation coefficient, and precipitability by antigen remained the same within experimental error, indicating that there was little change in the size, shape, or combining sites of the molecule associated with the reduction. The nature of the products formed on subsequent treatment with pepsin provided the analytical method for following the reduction and reoxidation of the disulfide bond linking the fragments.

The 7 S γ globulins of various species have been found to consist of several polypeptide chains held together by disulfide bonds (20–25). Porter (26) has proposed a model for rabbit γ-globulin based on the presence of two "A chains" of molecular weight 55,000 each, and two "B chains" (20,000 each). (The evidence for symmetry is summarized in the introduction.) He postulates that the disulfide bond linking the univalent fragments joins two "A chains." It would appear that this bond may be one of a very small number of covalent bonds holding together the polypeptide chains of the molecule.

**SUMMARY**

The disulfide bond which links the univalent fragments in pepsin-treated rabbit antibody can readily be reduced in the untreated molecule. This results in no appreciable change in specific immunological activity, sedimentation coefficient, or specific viscosity. However, on subsequent treatment with pepsin, 3.5-S univalent fragments are formed, whereas peptic digestion of the unreduced antibody molecule yields a bivalent product (~ 4.6 S).

Conversion of the antibody into a form which yields 3.5-S products is accomplished by reducing two to three disulfide bonds per molecule of antibody. Since there are approximately 23 disulfide bonds in the molecule, it is evident that the bond linking the univalent fragments is relatively labile in the intact antibody (as well as in the pepsin-treated molecule). Whether the additional —SH groups are released from one or two particular disulfide bonds, or are contributed by partial reduction of a number of bonds, was not determined.

Upon standing at neutral pH and 25° for 2 hours, after removal of the reducing agent, more than 90% of the liberated —SH groups were no longer available for reaction with CMB. This was accompanied by a change in the products of subsequent peptic digestion; after reoxidation the 4.6-S product is again formed. On reoxidation for varying periods of time, the amount of 4.6-S product paralleled the loss of titratable —SH groups. Precipitating antibody was obtained on treatment of reoxidized protein with pepsin.

Reoxidation was not very much faster than that previously observed for the recombination of separate 3.5-S fragments, despite the probable proximity of the —SH groups in the undigested molecule. This suggests that collision frequency is not the rate-determining factor.

The rate of breakdown of the reduced molecule to 3.5-S fragments on treatment with pepsin is somewhat more rapid than the proteolysis of the untreated γ-globulin.

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