The univalent fragments of rabbit antibody released by simultaneous or successive treatments with pepsin and a reducing agent are similar in a number of respects (1-3) to those formed by the action of papain (4). The similarities include the average molecular weight, sedimentation coefficient (3.5 S), amino acid composition, capacity per unit weight to inhibit specific precipitation, and chromatographic behavior. It was proposed on this basis that the mechanism of action of pepsin and a reducing agent was similar to that of papain, which is used in conjunction with a reducing agent as activator. There appear to be one or more regions in the molecule that are particularly susceptible to the action of either proteolytic enzyme. Subsequent to this attack, which results in the removal of a large inactive fragment (Porter's Fraction III (4)), the univalent fragments remain linked only by a single, labile disulfide bond. Cebra et al. (5) have shown recently that a two-stage reaction can be carried out with papain, covalently linked to an insoluble polypeptide, and a reducing agent.

The reaction with pepsin alone yields a 5-S bivalent fragment with a molecular weight of 109,000, which is decreased to 56,000 upon reduction (1, 2). The fact that the reduced fragments migrate as a single 3.5-S peak in the ultracentrifuge suggests that the 5-S molecule is split approximately in half on reduction. The 3.5-S fragments can readily be reoxidized in good yield to 5-S material. When specifically purified antibodies are used, the capacity to form precipitates is largely restored upon reoxidation (3). Hybrid antibodies can be formed by the reoxidation of a mixture of antibody fragments of different specificity (6, 7). The recombination of univalent fragments appears to be random (7).

The transformation of 5-S to 3.5-S protein is brought about by the reduction of one labile disulfide bond (8). This bond can be reduced by treatment with 0.008 M 2-mercaptoethylamine hydrochloride for 1 hour at 37°C and pH 5. Under these conditions, it is the only disulfide bond broken in the 5-S molecule, of approximately 14 bonds that appear to be present on the basis of amino acid analysis (3). It was of interest to study the nature of the aggregation that occurred after additional disulfide bonds were broken. If other sulfhydryl groups became available for intermolecular reaction, one would expect that larger aggregates would be formed upon reoxidation. The results of such an investigation are reported here. Some of the properties of the reduced antibody molecule and the effects of various parameters on the oxidation of 3.5-S to 5-S protein were also studied.

In addition, the chromatographic behavior of the fragments of a papain digest of rabbit antibody has been investigated further. In a recent report (9), it was demonstrated that the fractions designated I and II (4) do not correspond to two parts of individual molecules, but that the univalent fragments of an individual molecule are ordinarily eluted in either Fraction I or II. The fact that only two univalent fractions were obtained in the original procedure was attributed to the chromatographic method used. A similar conclusion has been reached by Amirian and Leikhim (10) and by Stelos, Radzimski, and Pressman (11) on the basis of results indicating that some antibodies yield unequal amounts of Fractions I and II.

Our experiments showed (9) that there is a direct parallel between the relative ease of elution of a γ-globulin molecule and that of its univalent fragments, and thus indicated that the differences in net charge among γ-globulin molecules are related to differences among their univalent fragments. Evidence was adduced which suggested that the two univalent portions of an individual molecule may be very similar. (The similarities of Fractions I and II no longer can be taken as evidence on this point, since they are derived from different molecules.)

In the present investigation, the chromatographic properties of univalent fragments derived from different γ-globulin molecules have been further characterized by using labeled γ-globulin as starting material. By mixing fractions of labeled globulin with unfractionated, unlabeled globulin it was possible to compare directly the chromatographic behavior of fragments from the two populations of molecules.

**EXPERIMENTAL PROCEDURE**

**Antiserum.—**Rabbit antiovalbumin was prepared by inoculating rabbits intravenously three times weekly with 10 to 15 mg of twice crystallized hen ovalbumin. After 6 to 8 weeks of immunization, a rabbit was allowed to rest for 1 week, 20 to 30
ml of blood were taken from an ear vein, and the animal was again inoculated. Bleeding and inoculation were repeated once a week. A number of samples of antisera of high titer, obtained from several rabbits, was pooled.

A $\gamma$-globulin fraction of the pooled antisera was prepared (12) by three precipitations with sodium sulfate at final concentrations of 0.18, 0.14, and 0.125 g per ml. The product migrated as a single peak in the ultracentrifuge ($s_{20, w} = 6.4$) and exhibited a single band in the $\gamma$-globulin region on paper electrophoresis. On testing with a series of concentrations of ovalbumin, it was found that 13% of the protein was precipitable by an optimal amount of the antigen (36 $\mu$g of antigen for 1.5 mg of $\gamma$-globulin).

The method used for estimating the amount of antigen in precipitates in the equivalence zone is described in (3).

Other Materials—Pepsin, papain, and hen ovalbumin were obtained as twice crystallized preparations from the Worthington Biochemical Corporation. 2-Mercaptoethanol hydrochloride and the sodium salt of $p$-chloromercuribenzoic acid were obtained from the California Corporation for Biochemical Research. Carboxymethyl cellulose (0.7 meq per g) was purchased from the Brown Company, Berlin, New Hampshire.

Treatment of Antisera—The preparation of the 5-S hydrolysis product of rabbit antibody was carried out essentially as described previously (3). Antiovalbumin $\gamma$-globulin, 1.4 g, was mixed with 28 mg of crystallized pepsin in 0.1 M sodium acetate buffer, pH 4.5, and allowed to react for 8 hours at 37°. The mixture was adjusted to pH 8 to inactivate the pepsin, and a solution of sodium sulfate, 0.25 g per ml, was added to a final concentration of 0.18 g per ml. The precipitate was separated by centrifugation, washed once with a small amount of sodium sulfate solution (0.18 g per ml), dissolved in a sodium chloride-borate buffer of pH 8 and ionic strength 0.16, and dialyzed against cold 0.1 M sodium acetate. The product migrated as a single peak ($s_{20, w} = 4.7$) in the ultracentrifuge. Twelve per cent of the protein was precipitable by an optimal concentration of antigen.

A $\gamma$-globulin fraction was also prepared from normal rabbit serum, treated with pepsin, and precipitated with sodium sulfate as described above. The $s_{20, w}$ values of the $\gamma$-globulin and the pepsin-treated protein were 6.8 and 4.8 S, respectively. In both cases, single symmetrical peaks were observed in the ultracentrifuge. Another preparation of normal $\gamma$-globulin (6.5 S) was also used.

Titration of SH Groups—Sulfhydryl groups were estimated by titration with the sodium salt of $p$-chloromercuribenzoic acid essentially as described by Boyer (13). Samples were treated with a 50 to 100% excess of 1.85 $\times$ $10^{-4}$ M CMBl and back-titrated with a freshly prepared solution of reagent grade L-cysteine which had been recrystallized from hot water and dried under vacuum. The CMBl solution was first standardized by titration with a solution of the L-cysteine. The concentration of CMBl thus obtained agreed within 5% with that estimated by using Boyer's extinction coefficient. Titrations were carried out in 0.3 M sodium acetate buffer, pH 4.6, in both the presence and absence of 0.5% sodium lauryl sulfate, which had been crystallized twice from hot ethanol.

Radioiodination of $\gamma$-Globulin—Normal rabbit $\gamma$-globulin (100 mg), which moved as a single 6.5-S peak in the ultracentrifuge, was iodinated with 131I monoiodide by the procedure of McFarlane (14). The unreacted 131I was removed by passing the mixture through a column of an anion exchange resin, Amberlite IR-45, followed by dialysis against 4 liters of 0.02 M potassium iodide in saline-borrate buffer, pH 8.0. The protein solution was then dialyzed against 4 liters of 0.02 M sodium acetate. The labeling efficiency was 70%, and the average amount of iodine coupled to protein was 0.8 atom per molecule. The molecular weight of the $\gamma$-globulin was taken as 160,000. More than 90% of the radioiodine in the product was precipitated by trichloroacetic acid, added to a final concentration of 5%.

Other Methods—Amounts of protein in the 5-S preparation or in the 3.5-S product obtained from it by reduction were estimated by using the extinction coefficient, 1.48 optical density units per mg per ml.

Sedimentation analysis was carried out in the Spinco model E ultracentrifuge at 59,780 r.p.m. Temperature was controlled to within ±0.1° of the desired value.

RESULTS

Magnitude of Johnston-Ogston Effect—Many of the experiments reported below involve the reoxidation of 3.5-S to 5-S protein. The relative amounts of each component in mixtures were estimated by measurement of areas in enlargements of photographs of schlieren patterns obtained in the ultracentrifuge. Owing to the Johnston-Ogston effect (13), some error in these estimations was anticipated. The apparent amount of 5-S protein was expected to be smaller than that actually present. To obtain information as to the magnitude of the effect, synthetic mixtures of 3.5-S and 5-S protein were prepared and tested in the ultracentrifuge. The reduced 3.5-S fragments had been treated with iodoacetamide, added to a final concentration of 0.03 M, to prevent reoxidation. The mixtures contained 20, 40, and 60% by weight of 5-S protein in 0.1 M sodium acetate adjusted to pH 7.0; the total protein concentration was 10 mg per ml. The percentages of 5-S protein estimated from the schlieren patterns (shown in Fig. 1) were 21, 38, and 59%, respectively. It is concluded that within this range of concentrations, errors due to the Johnston-Ogston effect are small.

Reactions of 5-S Antiovalbumin with Increasing Concentrations of 2-Mercaptoethanol Hydrochloride—Portions of 50 to 100 mg each of 5-S protein derived from antiovalbumin $\gamma$-globulin were treated with 0.0025, 0.01, or 0.5 M MEA for 75 minutes at 37° in 0.1 M sodium acetate buffer, pH 5.0. The pH was then lowered to 4.5 with 1 M acetic acid, and the solution in each case was passed through a column, 8 $\times$ 280 mm, of IR-120 cation exchange resin in the sodium cycle to remove MEA. This was done in the cold room. The size of the column to be used for removal of MEA was determined on the basis of preliminary experiments; a large safety factor was added. An amount of standardized CMBl solution 50 to 100% excess of SH groups present, as estimated on the basis of preliminary experiments, was then added to a portion of each eluate. Aliquots of the protein-CMB mixture were removed for back-titrations with cysteine in the presence and absence of 0.5% sodium lauryl sulfate, and also for immediate examination in the ultracentrifuge. The samples treated with 0.01 M or 0.5 M MEA (followed by CMBl) were adjusted to pH 8.0 with 2 N sodium hydroxide, dialyzed overnight against 4 liters of cold 0.1 M sodium acetate, and tested again in the ultracentrifuge. This was done to insure that recombination to 5-S had not taken place in the presence of CMBl. In each case it was found that there was no appreciable formation...
Reduction of Disulfide Bonds in Univalent Antibody

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Reduction of 5-S protein derived from antiovalbumin γ-globulin with increasing concentrations of 2-mercaptoethylamine hydrochloride (MEA)

<table>
<thead>
<tr>
<th>Concentration of MEA used</th>
<th>Molar of SH groups liberated per mole of 5-S protein</th>
<th>Blocking activity expressed as amount of precipitate (as per cent of control), with:</th>
<th>s, w, b</th>
<th>1.0 mg of fragments</th>
<th>2.0 mg of fragments</th>
<th>3.0 mg of fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0025</td>
<td>1.2</td>
<td>0.9</td>
<td>Partial breakdown</td>
<td>16</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>0.01</td>
<td>2.5</td>
<td>2.6</td>
<td>3.4</td>
<td>18</td>
<td>3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>0.5</td>
<td>12.0</td>
<td>11.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a In the untreated 5-S protein, the numbers of SH groups titratable in the presence and absence of detergent (0.5% sodium lauryl sulfate) were, respectively, 0.4 and 0.3 moles per mole of protein. These values were subtracted from the corresponding values obtained on titration of the reduced protein with or without detergent present.

b Recombination was prevented by addition of excess CMB.

c In the control experiment, 1.5 mg of antiovalbumin γ-globulin were used together with an optimal concentration of antigen; 197 μg of precipitate formed. No appreciable blocking was observed in a heterologous system (bovine γ-globulin reacting with rabbit antiovalbumin γ-globulin; see the text).

Reduction of 6-S protein derived from 5-S protein. The samples reduced with 0.01 M or 0.5 M MEA were also tested at this stage for capacity to inhibit the homologous precipitin reaction and a heterologous reaction (bovine γ-globulin reacting with its rabbit antiserum). The quantities used in the homologous system are given in Table 1. In the heterologous system, 3 mg of the reduced protein were tested with an amount of bovine γ-globulin and antibody sufficient to yield 263 μg of precipitate in the absence of inhibitor. In all tests of inhibition, untreated antibody was added to mixtures last.

Those portions of the protein preparations reduced with 0.01 M or 0.5 M MEA that had not been treated with CMB were allowed to reoxidize at pH 8. This was done by stirring in an open beaker for 2 hours at room temperature and dialyzing overnight against 4 liters of cold 0.1 M sodium acetate, pH 7. The extent of recombination to 5-S was determined in the ultracentrifuge. The number of free -SH groups per mole was also measured on a portion of this reoxidized material. The reoxidation procedure was omitted in the case of reduction with 0.0025 M MEA, where only partial breakdown occurred. The results of these experiments are summarized in Tables I and II.

With 0.01 M MEA there was essentially complete breakdown to 3.4 S. This was accompanied by the formation of 2.5 or 2.6 new -SH groups per mole, as measured in the presence or absence of detergent, respectively (Table I). This corresponds to 45 to 60% of one disulfide bond and thus agrees quite well with the fraction of 5-S protein reduced.

TABLE II

<table>
<thead>
<tr>
<th>Nature of reoxidation products formed after reduction of varying numbers of disulfide bonds in 5-S protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of MEA used for reduction</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>0.01</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.01 (CMB added)</td>
</tr>
<tr>
<td>0.5 (CMB added)</td>
</tr>
</tbody>
</table>

a From Table I. Detergent was present during the titration.

b No appreciable concentration of components moving more rapidly than those indicated was observed.

c Detergent was present during the titration.

d The S value of the slow component could not be measured accurately, but its rate of movement was approximately the same as that observed before reoxidation.

* CMB was added to a final concentration of 8.0 × 10^-4 or 1.7 × 10^-4 M after passage of the reduced protein through a column of IR-120 resin, as described in the text.
When the 5-S protein was treated with 0.5 mM MEA, or more than 50 times the minimal concentration required to reduce one disulfide bond, 12 new -SH groups per mole of protein were liberated (Table I). The $s_{20, w}$ value was 3.6 S. Thus, the reduction of five disulfide bonds, in addition to the critical bond linking the univalent fragments, does not result in the formation of smaller fragments.

Data on the capacities to block specific precipitation of the proteins formed by reduction with 0.01 M and 0.5 M MEA are also given in Table I. At the three concentrations of each preparation tested, there were no significant differences in the capacity to inhibit precipitation in the homologous system (ovalbumin-antiovalbumin). No appreciable inhibition was observed when 3.0 mg of either preparation were tested in a heterologous system as described above. It is evident that the reduction of approximately six disulfide bonds does not fix the specific combining sites of the 3.5-S molecules. Evidence indicating that the initial reduction from 5-S to 3.5-S protein involves very little destruction of combining sites has been reported previously (1). It should be noted that the five additional disulfide bonds are divided in an unknown proportion between the two univalent subunits.

Upon reoxidation of the protein that had been reduced with either 0.01 M or 0.5 M MEA, only two components were observed in the ultracentrifuge. The extent of recombination was about the same in each case (70% or 75%, Table II). The $s_{20, w}$ values of the faster components were 4.7 and 4.8 S after reoxidation of the samples that had been reduced with 0.01 M or 0.5 M MEA, respectively. The sedimentation values of the slower components could not be measured accurately, but the distance traversed after 80 minutes at full speed corresponded closely to that of 3.5 S protein. No higher polymere were formed on reoxidation, even in the sample in which 12 -SH groups had been liberated. This indicates that the -SH groups formed, other than the two formed from the critical disulfide bond linking the 3.5-S fragments, are not available for the formation of bonds between fragments.

The number of -SH groups remaining was determined after each reoxidation (Table II). These values were small in each case and comparable to that of the unreduced 5-S protein. Whether all of the -SH groups had recombined to form disulfide bonds is uncertain.

**Effect of Various Parameters on Recombination of 3.5-S Fragments**

To study the effects of experimental variables on the rate of recombination, portions of pepsin-treated normal rabbit γ-globulin (4.8 S) were first reduced by treatment with 0.01 M MEA in 0.1 M sodium acetate buffer, pH 5.0, for 75 minutes at 37°C. After the pH was lowered to 4.5, the MEA was removed by passage through an IR-120 column in the cold room. A portion of 3.5-S protein was then determined in the ultracentrifuge. When appreciable amounts of reoxidation occurred, it was possible to estimate the $s_{20, w}$ values of the faster component, and these were found to be 4.9 ± 0.2 S. Accurate measurements of the faster component were not practical when the amount of recombination was less than 40%, but the rate of movement was always comparable to that of 5-S protein.

The effects of experimental variables on the recombination are shown by the data in Tables III to VI. From the results in Table III it is apparent that, at pH 8, recombination is more rapid at 37°C or 25°C than at 5°C. Five hours is sufficient for maximal recombination (~70%) at 37°C, whereas after 24 hours at 5°C only 31% of the reduced protein had recombined. In our experience the maximal extent of recombination has been approximately 75%. Why the remaining 25% of 3.5-S protein fails to recombine is not known.

The recombination proceeds more rapidly at higher pH (pH 10 > pH 8 > pH 4.5 (Table IV)). In these experiments, maximal recombination was attained after 5 hours at either pH 8 or pH 10, whereas only 33% of the reduced protein had recombined at pH 4.5 in 24 hours. In 4 hour, the amount of recombination was greater at pH 10 than at pH 8.

The effect of protein concentration on recombination was also investigated. A temperature of 5°C was chosen, since the slow recombination at this temperature facilitates comparisons. The results are given in Table V. The rate of recombination is seen to increase with increasing protein concentration. Expressed as concentration of protein recombined, the extent of recombination was approximately 10 times as great in 1 hour at a concentration of 20 mg per ml as it was at 5 mg per ml. The results appear to approach an upper limit of about 30% at 5°C.

Since oxidations of sulphydryl compounds are frequently...

---

**Table III**

<table>
<thead>
<tr>
<th>Time allowed for recombination</th>
<th>3.5-S protein recombined to 5-S protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>24</td>
<td>31</td>
</tr>
</tbody>
</table>

**Table IV**

<table>
<thead>
<tr>
<th>Time allowed for recombination</th>
<th>3.5-S protein recombined to 5-S protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>%</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>3</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>11</td>
</tr>
<tr>
<td>pH 10.0</td>
<td>33</td>
</tr>
</tbody>
</table>
of EDTA. A control sample was also run
in which EDTA was not added at any stage. After the last
dialysis, reoxidation was stopped by the addition of CMB.
Only 15% of the protein was reoxidized when treated with
EDTA and dialyzed against EDTA. Removal of the EDTA by
tensive dialysis permitted reoxidation to an extent nearly as
great as that of the untreated control (71% versus 77%).

Chromatographic Properties of Univalent Fragments of Rabbit
Antibody Formed by Papain—The chromatographic behavior
of univalent fragments derived from different chromatographic
fractions of χ-globulin by digestion with papain was investi-
gated by using labeled χ-globulin as the starting material.
\( I^{38}, \chi \)-globulin, 30 mg, was separated into three fractions by step-
wise elution from a column of carboxymethyl cellulose
(11 \( \times \) 150 mm) with 0.06 M, 0.1 M, and 0.5 M sodium acetate
buffers, pH 5.4. The fractions contained 17, 9, and 3 mg of
protein, respectively. The protocol and elution patterns are
shown in Fig. 2.

One milligram each of the 0.06 M and 0.1 M fractions was mixed
separately with 50-mg portions of unlabeled, unfractionated
normal rabbit χ-globulin. Each mixture was treated with 1% of
by weight of crystallized papain for 2 hours at \( 37^\circ \) in phosphate
buffer at pH 7 and ionic strength 0.16, containing 0.01 M \( L \)-cys-
teine and 0.002 M EDTA. After dialysis against 4 liters of cold
0.1 M sodium acetate, the papain was inactivated by addition
of sodium iodosobenzoate to a final concentration of 0.005 M,
and the samples were dialyzed against 0.02 M sodium acetate.
In each case, 43 mg of protein were recovered, and there was com-
plete breakdown to \( 3.6 \) and \( 3.7 \) S, respectively. Crystals of
Fraction III (4) that formed on dialysis were removed for the
ultracentrifuge run but were resuspended and included in the
subsequent fractionation. Each mixture was diluted to give a
sodium acetate concentration of 0.01 M, and the pH was adjusted
to 5.4. Each preparation was then fractionated on a column of
carboxymethyl cellulose (19 \( \times \) 200 mm) surrounded by an
ice bath. The same buffer solutions and flow rate were used for
each sample. The amount of protein placed on the column was
41 mg for the mixture containing the 0.06 M fraction of labeled
globulin and 40 mg for the mixture prepared with the 0.1 M frac-
tion of labeled globulin. Fractions I, II, and III were then col-
clected as described by Porter (4). The protocol and results are
shown in Figs. 3 and 4. The respective quantities of Fractions
1, 11, and 111 collected were 11.7, 12.6, and 14.1 mg from the
0.06 M fraction and 12.1, 12.4, and 14.0 mg from the 0.1 M frac-
tion. Amounts of protein were estimated from the absorbancy
at 280 \( \text{nm} \) by using the extinction coefficients, 1.48 optical
density units per mg per ml for Fractions I and II and 1.2 units
per mg per ml for Fraction III.

In each of the above experiments, Fractions I and II contained
approximately the same amount of protein. However, in the
case of the solution containing 1 mg of the 0.06 M fraction of
labeled χ-globulin mixed with 50 mg of unlabeled χ-globulin,
nearly all of the radiolabel was found in Fraction I, whereas the
reverse was true of the 0.1 M fraction of labeled χ-globulin mixed
with excess normal χ-globulin. After correction for decay of
radioactivity, there was very little difference in the specific
activity of Fractions III of the two experiments. The relative
amounts of protein recovered as Fraction III agree fairly well
with the results of Porter (4).

Subfractionation of Fractions I and II—As indicated previously
(9), the relative quantities of protein obtained as Fractions I or
II change gradually as the pH used in the chromatographic pro-
cauterized by traces of metals, the effect of a chelating agent on
recombination was investigated. The reoxidation was carried
out in 0.1 M sodium acetate, at pH 8 and \( 25^\circ \), in the presence or
absence of 0.01 M EDTA. The protein concentration was 10 mg
per ml. The results, shown in Table VI, Experiments 1 and 2,
dicate marked inhibition of reoxidation in the presence of
EDTA. The extent of recombination in the presence of EDTA
was approximately 50 to 60% as great as in its absence.

The inhibitory effect of EDTA was also demonstrated (Table
VI, Experiments 3, 4, and 5) by treating the reduced protein
with 0.01 M EDTA and dialyzing portions against solutions
containing or lacking EDTA. A control sample was also run

Table V
Effect of protein concentration on recombination of 3.5-S
protein at pH 8 and 24 hr

<table>
<thead>
<tr>
<th>Time allowed for recombination</th>
<th>Recombination of 3.5-S to 3.5 protein at protein concentrations of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mg per ml</td>
</tr>
<tr>
<td>hrs</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>72</td>
<td>28</td>
</tr>
</tbody>
</table>

Table VI
Effect of EDTA on recombination of 3.5-S fragments after removal of reducing agent

The 3.5-S protein was prepared by reduction with 0.01 M MEA;
the latter was removed by passage through a column of IR-120
resin (see the text). The protein concentration was then adjusted
to 10 mg per ml.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Procedures*</th>
<th>Time allowed for recombination</th>
<th>Recombined to 3.5 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Recombination in open test tube at pH 8.0, 25º, 0.01 M EDTA present</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Recombination in open test tube at pH 8.0, 25º, without EDTA</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>0.01 M EDTA added immediately after removal of MEA; followed by dialysis versus 0.1 M sodium acetate, pH 8.0, containing 0.01 M EDTA</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>0.01 M EDTA added immediately after removal of MEA; followed by dialysis versus 0.1 M sodium acetate, pH 8.0, without EDTA</td>
<td>72</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>No EDTA added at any stage; dialyzed versus 0.1 M sodium acetate, pH 8.0</td>
<td>72</td>
<td>77</td>
</tr>
</tbody>
</table>

* Each solution was 0.1 M in sodium acetate. Dialysis was carried out at 4 ± 1º against three 2-liter portions of the outer solution specified.

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FIG. 2. Stepwise elution of 131I-labeled normal γ-globulin from carboxymethyl cellulose at pH 5.4. Open circles, net counts per minute per 0.1 ml of eluate. Closed squares, optical density at 280 μm. The small arrows indicate the start of a new eluting buffer.

FIG. 3. Elution from carboxymethyl cellulose of 41 mg of a papain digest of a mixture containing the 0.06 M fraction of labeled γ-globulin and a 50-fold excess of unlabeled, unfractionated γ-globulin. Closed squares, optical density at 280 μm. Open circles, net counts per minute per ml of eluate. The first fraction was collected by elution with 0.01 M sodium acetate, pH 5.4, followed by a gradient produced by the addition of 0.9 M sodium acetate, pH 5.4, to the mixing flask at a constant volume of 600 ml. The gradient was started after 100 ml of the starting buffer had passed through the column.

FIG. 4. Elution from carboxymethyl cellulose of 40 mg of a papain digest of a mixture containing the 0.1 M fraction of labeled γ-globulin and a 50-fold excess of unlabeled, unfractionated γ-globulin. Closed squares, optical density at 280 μm. Open circles, net counts per minute per ml of eluate. Fractions were collected as described in the legend of Fig. 3.

DISCUSSION

Our objective of these experiments was to determine the effect of reducing several disulfide bonds on the nature of the product that resulted on reoxidation. It has been observed previously that when a single disulfide bond is broken, only 5-S protein is formed after removal of the reducing agent (3, 6). The present experiments confirm this observation under a variety of experimental conditions. In addition it was found that there is no appreciable formation of larger aggregates upon reoxidation after additional disulfide bonds are broken (Table II). With a concentration of reducing agent 50 times as great as the minimum required for the liberation of 3.5-S fragments, a total of approximately six disulfide bonds was reduced. The product had a sedimentation coefficient of 3.6 S. After reoxidation, 75% of the protein migrated with a velocity of 4.8 S. No appreciable amount of faster moving material was formed. The reoxidation of two of these —SH groups to form a disulfide bond is responsible for the recombination of the two 3.5-S subunits. It is evident that the other —SH groups are not available for...
intermolecular interaction; otherwise higher polymers would have been produced. The simplest explanation would appear to be that the other —SH groups cannot interact in such a manner because of steric inaccessibility, or because of very rapid recombination with —SH groups within the same subunit after removal of the reducing agent. Whether or not all of the original disulfide bonds are re-formed was not ascertained; however, the free —SH groups disappeared on standing. The single disulfide bond responsible for the linkage of the two 3.5-S subunits is thus unique with respect to the type of interaction that it can undergo as well as to its great lability.

The fact that only six disulfide bonds, of 14 present, are reduced by 0.5 M reducing agent is in agreement with the studies of Markus, Grossberg, and Pressman (16). In the original molecule, additional disulfide bonds can be broken in the presence of detergent or urea with the loss of water solubility (17, 18). In the latter instance (18), a reduction in molecular size was reported, which indicates the presence in the molecule of more than one polypeptide chain. However, in the original molecule only one NH₂-terminal group, alanine, is available for reaction with dinitrophenolbenzene (19).

There appears to be no appreciable destruction of combining sites associated with the reduction of the six disulfide bonds. The capacity, per unit of weight, to inhibit the homologous precipitin reaction was approximately the same as that observed after reduction of one disulfide bond; previous work has shown that the reduction of the first disulfide bond has little effect on the activity of the combining sites (1). Also, the ratio of univalent to bivalent antibody required for essentially complete inhibition of specific precipitation in the present experiments is comparable to that observed previously (1). It should be noted that the distribution of the reduced disulfide bonds between the two univalent fragments of an individual molecule has not been determined, since we do not as yet know how to separate them.

The analytical method used to investigate the effects of various parameters on the rate of reoxidation, i.e. ultracentrifugal analysis, was too cumbersome for detailed kinetic studies. However, the data permit a number of conclusions. It was found, first, that only 5-S protein formed on reoxidation of 3.5-S fragments under the variety of experimental conditions used. This should make possible a more detailed investigation of the kinetics by a rapid technique such as light scattering, as in the study of the dimerization of serum albumin (20). Complications due to the presence of other components should not be serious.

The recombination proceeds more rapidly at pH 10 than at pH 8, and more rapidly at pH 8 than at pH 4.5 (Table IV). This is consistent with the possibility that reoxidation takes place through mercaptide formation. At 5°, the rate of recombination increases with increasing protein concentration, even if the data are expressed as fraction of the protein recombined (Table V). This suggests that the order of the reaction is greater than 1. At pH 8, the recombination proceeds much more rapidly at 25° than at 5° and is nearer completion after 1 hour at 37° than at 25° (Table III).

The decreased rate of reaction in the presence of a chelating agent (Table VI) suggests catalysis by trace amounts of metals, an effect frequently observed in the oxidation of sulfhydryl compounds.

The maximal amount of 5-S protein formed by recombination in these experiments was 77% of the total. This is as high a value as we have observed in a number of investigations. The reason for the failure of the remaining protein to recombine is not known. After recombination at 25°, no appreciable fraction of the —SH groups remain available for reaction with CMB, in either the presence or absence of detergent (Table II).

The chromatographic behavior of 3.5-S subunits derived from different fractions of labeled γ-globulin confirms the conclusion previously drawn (9) as to the nature of Fractions I and II obtained by the original procedure (4). It is evident that each fraction is derived from a different population of γ-globulin molecules and that there is a direct parallel between the ease of elution of the parent globulin molecule and that of its univalent subunits. The fact that only two fractions of univalent material were obtained in the original procedure is attributable to the rapid salt gradient used for collecting Fraction II. As indicated previously (9) and by the present results, the univalent protein can be subdivided further by stepwise alteration of the pH.

The use of radiolabeled γ-globulin in the present experiments permitted direct demonstration that the fractionation of the papain digest was being carried out in such a manner as to yield approximately equal amounts of Fractions I and II from a papain digest of whole γ-globulin. The sensitivity of the fractionation procedure to pH makes such a control desirable.

Since Fractions I and II do not represent two portions of individual molecules, their many resemblances, with respect to a number of properties, do not indicate similarity of the two univalent fragments of an individual molecule. Nevertheless, there is considerable evidence suggesting that these two fragments may be quite similar in their chemical structure (9), as originally proposed by Porter (4). The results of Franklin (21) and of Askonas and Fahey (22) indicate that the two univalent fragments of human and mouse γ-globulins are similar in their chromatographic properties.

In the case of guinea pig and human γ-globulins, Edelman et al. (23, 24) have shown that a polypeptide chain can be separated from the whole γ-globulin molecule or its immunologically active fragments by reduction in the presence of urea. Our own results (11 and unpublished data) indicate that the active fragments of the human γ-globulin molecule, as well as those of the rabbit, are linked by one or more disulfide bonds which are reducible without the addition of detergent or urea. It is evident that these two types of disulfide linkages are distinct and that such bonds are important structural features of γ-globulin molecules.

**SUMMARY**

Pepsin-treated rabbit antibody, which is bivalent and has a molecular weight of 106,000, has previously been shown to be split into 3.5-S univalent fragments by reduction of one labile disulfide bond. Reduction of five additional disulfide bonds is found to cause no further decrease in sedimentation coefficient or specific activity. The product obtained on reoxidation has the same sedimentation coefficient as that formed on recombination after the reduction of one disulfide bond; no higher polymers were observed. Thus, the additional sulfhydryl groups released are not available for interactions among the fragments. After standing at neutral pH, only a small fraction of the sulfhydryl groups in the reduced protein remain available for reaction with p-chloromercuribenzoate.

Effects on the reoxidation of pH, temperature, protein concentration, and the presence of a sequestering agent were investigated.
The use of labeled, fractionated \( \gamma \)-globulin permitted the confirmation of a direct relationship between the relative strength of adsorption to carboxymethyl cellulose of a \( \gamma \)-globulin molecule and that of its univalent fragments. The results provide a further indication that the univalent fragments of an individual molecule are similar to one another.

REFERENCES

Effect of Reduction of Several Disulfide Bonds on the Properties and Recombination of Univalent Fragments of Rabbit Antibody

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