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Dissociation of Rabbit γ-Globulin into Half-Molecules after Reduction of One Labile Disulfide Bond*

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Rabbit γ-globulin has previously been found to dissociate into half-molecules at low *p*H after reduction. The present investigation indicates that one-half to two-thirds of the molecules can be dissociated after reduction of one very labile disulfide bond. Dissociation of the remainder of the γ-globulin requires the reduction of more than one disulfide bond, indicating heterogeneity with respect to the number of interchain disulfide bonds, or to the relative lability of a single bond joining the half-molecules. The evidence is consistent with the conclusion that the bond joins two "A" chains. The amino acid composition of isolated half-molecules is consistent with other evidence indicating that each consists of an "A" and a "B" chain.

Recent investigations indicate that 6.5 S γ-globulins of several species consist of four polypeptide chains, two of approximate molecular weight 55,000 and two of molecular weight 20,000 (Edelman, 1959; Edelman and Poulik, 1961; Fleischman *et al.*, 1962, 1963; Pain, 1963; Marler and Tanford, 1963). The two types of polypeptide chain have been referred to as heavy ("H") and light ("L") chains (Edelman and Benacerraf, 1962), or as "A" and "B" chains (Porter, 1962), respectively. The combining sites of the molecule appear to be associated with "A" chains (Fleischman *et al.*, 1963; Utsumi and Karush, 1963); the participation of "B" chains, in a manner that is not as yet clearly defined, is also indicated (Edelman *et al.*, 1961, 1963; Franek and Nezlin, 1963; Roholt *et al.*, 1963; Metzger and Singer, 1963).

It was recently reported (Palmer *et al.*, 1963) that rabbit γ-globulin is cleaved into two subunits, approximately equal in size, by reduction with 0.1 M 2-mercaptoethylamine hydrochloride and subsequent acidification to *p*H 2.5 in 0.1 M NaCl. The conclusion that each subunit contains an A and a B chain was supported by the average molecular weight of the subunits; the agreement between the weight and *z*-average molecular weights, indicating homogeneity; the fact that the subunits migrate in the ultracentrifuge as a single symmetrical peak; and the low yield of B chains, obtained by gel-filtration on Sephadex in 1 M propionic acid, from a preparation which dissociated almost completely into half-molecules at low *p*H. Also, the present report will indicate that the half- and whole molecules are very similar in amino acid composition.

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After neutralization of a reduced, acidified preparation, the major product had a sedimentation coefficient almost identical with that of untreated (or reduced) γ-globulin, indicating that recombination of half-molecules occurs at neutral *p*H (Palmer *et al.*, 1963). Most of the specific combining capacity of an anti-hapten antibody is retained during these procedures, and the protein remains soluble. Subunits derived from specifically purified antiovalbumin are capable of combining with subunits of normal γ-globulin to form hybrid 6.2 S molecules with active combining sites (Nisonoff and Palmer, 1964).

Data presented here indicate that the dissociation of a large proportion of γ-globulin molecules at low *p*H occurs after the reduction of one disulfide bond per molecule. The bond is extremely labile to reduction by 2-mercaptoethylamine HCl. The results suggest that the disulfide bond links two A chains.

Materials and Methods

Three preparations of normal rabbit γ-globulin were used; each was obtained from the serum of an individual rabbit. The γ-globulin was prepared by three precipitations with decreasing concentrations of sodium sulfate (Kekwick, 1940) followed by passage through a column of diethylaminoethyl-cellulose (Levy and Sober, 1960) in 0.0175 M sodium phosphate buffer, *p*H 6.9. The $s_{20,w}$ values of the preparations were 6.2 or 6.3 S. Other serum proteins were not detectable by immunoelectrophoresis with sheep anti-rabbit serum.

S-Carboxymethyl-L-cysteine was obtained from the Nutritional Biochemicals Corp. 2-Mercaptoethylamine hydrochloride and sodium *p*-mercuribenzoate were obtained from the California Corp. for Biochemical Research.

Free —SH groups were estimated (Boyer, 1954) by addition of less than a 2-fold excess of sodium *p*-mercuribenzoate, followed by back-titrations, in duplicate, with a freshly prepared solution of recrystallized L-cysteine in the presence of 0.5% recrystallized sodium lauryl sulfate. The sodium *p*-mercuribenzoate solution was first standardized by titration of a cysteine solution. The concentration obtained in this way agreed closely with that estimated by using Boyer's extinction coefficient. These methods have been described in detail elsewhere (Mandy and Nisonoff, 1963). The average of all values of the differences between duplicate determinations was 0.1 —SH group per molecule.

Alternatively, —SH groups released upon reduction were estimated by treatment with excess iodoacetate, as described below, and determination of *S*-carboxymethylcysteine in the Spinco amino acid analyzer. Samples were prepared for analysis by hydrolyzing with constant-boiling HCl for 22 hours at 110° in an evacuated sealed tube. The procedure recommended by Crestfield *et al.* (1963) for complete removal of oxygen was used for evacuation. To obtain *S*-carboxymethylcysteine peaks sufficiently large for accurate measurement, relatively large samples (approximately 8 mg) were applied to the long column of the analyzer. The peaks for nearly all the other amino acids were thus too large for measurement. An aliquot equal to exactly one-eighth of the volume placed on the long column was passed through the short column of the analyzer for determination of lysine, arginine, and histidine.

The amount of protein applied to the long column was determined in two ways. First, the amount to be hydrolyzed was estimated by use of the extinction coefficient, 1.50 OD units per mg/ml at 280 mμ, and quantitative transfers were made from the sealed tubes. Second, the amount of protein applied to the short column was determined from the recoveries of lysine and arginine by using the values of Smith *et al.*, (1955) for the content of these amino acids per unit weight of γ-globulin (0.443 μmole lysine and 0.287 μmole arginine per mg protein). The mean value thus obtained was multiplied by 8 to give the amount of protein applied to the long column. This procedure provided an independent check on the amount of protein analyzed. The values based on lysine and arginine always agreed within 2%. The maximum difference between the value based on optical density and the mean value obtained from the lysine and arginine content was 6%. The data given in the tables are based on values of protein content estimated from the mean of the lysine and arginine values. The area per μmole of *S*-carboxymethylcysteine was taken as 0.88 times the average for the amino acids, other than proline, cysteine, serine, and threonine, recovered from the long column in runs made with standard samples (Spackman, 1960); the same batch of ninhydrin solution was used for the unknown and the standard.

Recoveries of *S*-carboxymethylcysteine from known mixtures are shown in Table I. The mixtures containing protein were hydrolyzed in the presence of the added *S*-carboxymethylcysteine prior to application to the column. A separate symmetrical peak corresponding to *S*-carboxymethylcysteine was obtained in each case. The results in Table I indicate that 8 mg of hydrolyzed protein does not overload the long column of the analyzer and prevent the resolution of *S*-carboxymethylcysteine.

The minimum amount of *S*-carboxymethylcysteine in reduced alkylated protein samples analyzed in the experiments described below was 0.035 μmole. In the case of an unreduced alkylated sample 0.013 μmole was present. Analyses for *S*-carboxymethylcysteine were carried out with duplicate samples, hydrolyzed separately, with one exception (3rd sample, Table IV). A single analysis was carried out with the latter preparation. The average of differences between the results of duplicate determinations was 0.04 *S*-carboxymethylcysteine residue per molecule of protein.

TABLE I

RECOVERY OF *S*-CARBOXYMETHYLCYSTEINE IN THE AMINO ACID ANALYZER[a]

Carboxymethylcysteine Applied to Long Column (μmole)	Other Components Present[b]	Pretreatment	Carboxymethylcysteine Recovered (μmole)
0.020	A	None	0.024
0.200	A	None	0.216
0.200	A	None	0.218
0.020	B	Hydrolysis[c]	0.021
0.120	B	Hydrolysis	0.115

[a] The area per μmole of *S*-carboxymethylcysteine was estimated as indicated under Methods. [b] A = 1 μmole of each of 18 amino acids; B = 8 mg of rabbit γ-globulin. [c] Hydrolyzed for 22 hours at 110° in constant-boiling HCl.

Reductions were carried out with 2-mercaptoethylamine HCl for 75 minutes at 37° in 0.1 M sodium acetate buffer, *p*H 5.0. Excess 2-mercaptoethylamine HCl was removed by passage through a column of IR-120 resin at *p*H 5.0 in the cold room. The size of the column necessary for removal of the reducing agent was estimated by preliminary experiments and a large safety factor was added. Immediately after passage through the column, excess sodium *p*-mercuribenzoate was added to inactivate free —SH groups and the *p*H was raised to 8.0 by addition of sodium hydroxide solution. When samples were to be treated with iodoacetate the reagent was added to the mixture containing reducing agent. The conditions are given with the description of the experiments.

Weights of protein were estimated with the extinction coefficient, $E^{1\%}_{1cm} = 15.0$ at 280 mμ. The absorbancy was not significantly affected by reduction of four disulfide bonds. Subsequent addition of a 20% excess of sodium *p*-mercuribenzoate, after removal of 2-mercaptoethylamine HCl, increased the value, measured at *p*H 4.5, by 3%. This was determined by adding the sodium *p*-mercuribenzoate, in a small volume, to a spectrophotometer cuvet containing the solution of reduced protein. The same volume of water was added to a reference cuvet containing the same protein solution.

Sedimentation velocities were measured in a Spinco Model E ultracentrifuge at 59,780 rpm and 20°. A double 2° sector, 12 mm, filled-epoxy centerpiece was used. The outer solution of the last dialysis of the protein was placed in the second compartment. When the *p*H of the sample to be tested was adjusted prior to ultracentrifugation (usually to *p*H 2.4), the outer solution was similarly adjusted.

As will be indicated below, many of the samples examined in the ultracentrifuge consisted of two components, corresponding to whole and half-molecules of γ-globulin. The solvent was 0.025 M NaCl or 0.03 M NaCl, adjusted to *p*H 2.4 with HCl. To determine the magnitude of the Johnston-Ogston effect (Johnston and Ogston, 1946) on the areas in schlieren patterns, artificial mixtures were prepared in 0.025 M NaCl,

pH 2.4, of an unreduced preparation of γ-globulin ($s_{20,w}$ = 3.9 S), and the same γ-globulin was treated with 0.1 M 2-mercaptoethylamine HCl and sodium *p*-mercuribenzoate as described *vide supra* ($s_{20,w}$ = 2.8 S); the latter sample contained less than 3% of undissociated protein. Total protein concentrations in the mixtures were 8 mg/ml. The mixtures contained 19, 39, 59, and 78% of the slower component and measurements of areas gave values of 30, 49, 68, and 84%, respectively. A graph of these results was used to correct experimental values based on area measurements (Tables II and III). Areas were measured on enlargements of photographs taken after 128 minutes at 59,780 rpm (Table II), or after 80 minutes at 59,780 rpm (Table III).

TABLE II

REDUCTIONS OF RABBIT γ-GLOBULIN WITH INCREASING CONCENTRATIONS OF 2-MERCAPTOETHYLAMINE HCl, FOLLOWED BY ACIDIFICATION[a]

2-Mercapto-ethylamine HCl Concentration (M)	—SH Liberated[b] (groups/molecule)	Slow Component[c] (%)	—SH Liberated (groups/molecule dissociated)
0.010	0.8	35	2.3
0.015	1.2	46	2.6
0.020	1.6	66	2.4
0.030	3.5	80	4.4
0.050	4.2	88	4.8
0.10	8.4	96	8.8

[a] The γ-globulin was pretreated with iodoacetamide (see text). Sedimentation runs were carried out in 0.025 M NaCl, pH 2.4. [b] Determined by addition of excess sodium *p*-mercuribenzoate and back-titration with L-cysteine. The molecular weight of the γ-globulin was taken as 150,000. [c] The sedimentation coefficient, $s_{20,w}$, was 2.9 ± 0.1 S. Area measurements were corrected for the Johnston-Ogston effect as described in the text.

TABLE III

REDUCTION OF RABBIT γ-GLOBULIN WITH INCREASING CONCENTRATIONS OF 2-MERCAPTOETHYLAMINE HCl, FOLLOWED BY ACIDIFICATION[a]

2-Mercapto-ethylamine HCl Concentration (M)	—SH Liberated[b] (groups/molecule)	Slow[c] Component (%)	—SH Liberated (groups/molecule dissociated)
0.005	0.7	29	2.4
0.010	1.2	48	2.5
0.030	3.5	70	5.0
0.050	4.3	91	4.7
0.10	8.0	94	8.5

[a] The γ-globulin was not pretreated with iodoacetamide. Sedimentation runs were carried out in 0.03 M NaCl, pH 2.4. [b] Estimated by the sodium *p*-mercuribenzoate titration method. The molecular weight of the γ-globulin was taken as 150,000. [c] The $s_{20,w}$ value was 2.9 S in each case. Area measurements were corrected for the Johnston-Ogston effect, as described in the text.

RESULTS

To relate the extent of dissociation into half-molecules at low pH to the number of disulfide bonds reduced, two series of reductions were carried out, each with increasing concentrations of 2-mercaptoethylamine HCl. In the first series the γ-globulin was pretreated with iodoacetamide; in the second it was not. Pretreatment with iodoacetamide was done to reduce the value of the free —SH content, which had to be subtracted as a blank from values obtained with reduced samples. It was expected that the decrease in the —SH content of the control sample should somewhat improve the accuracy of subsequent measurements by the titration method.

A 400-mg portion of γ-globulin was allowed to react with 0.1 M iodoacetamide at pH 5.0 for 12 hours in the refrigerator. This lowered the —SH titratable by sodium *p*-mercuribenzoate from 0.8 to 0.5 group per molecule. Thus, 0.5 —SH group which was not available for reaction with iodoacetamide at pH 5.0 did react with sodium *p*-mercuribenzoate. This is not attributable to the use of detergent with the sodium *p*-mercuribenzoate since nearly the same number of —SH groups in the untreated molecule were titratable by sodium *p*-mercuribenzoate in the presence or absence of detergent (0.8 and 0.7 —SH, respectively).

Portions of the pretreated samples (50 mg) were then reduced with increasing concentrations of 2-mercaptoethylamine HCl for 75 minutes at pH 5.0 and 37°. The protein concentration during reduction was 25 mg/ml. The reduced sample was passed through a column of IR-120 resin at pH 5.0 in the cold room to remove 2-mercaptoethylamine HCl. Excess sodium *p*-mercuribenzoate was added and duplicate aliquots were back-titrated with L-cysteine at pH 4.5 in the presence of detergent to estimate the free —SH liberated by the reduction. The remainder of the sodium *p*-mercuribenzoate–treated protein was dialyzed in the cold against a 4-liter portion of 0.1 M sodium acetate, and then for 2 days against two 4-liter portions of 0.025 M sodium chloride. The pH was adjusted to 2.4, and the solution was examined in the ultracentrifuge to determine the extent of dissociation into half-molecules. The dissociation was carried out in 0.025 M NaCl, rather than 0.1 M NaCl (Palmer *et al.*, 1963), because the lower salt concentration has been found to result in more nearly complete separation of half-molecules after extensive reduction.

Figure 1 shows sedimentation patterns at pH 2.4 of unreduced γ-globulin and of γ-globulin reduced with increasing concentrations of 2-mercaptoethylamine HCl and treated with excess sodium *p*-mercuribenzoate after removal of the reducing agent. The 3 S component present in reduced samples consists of half-molecules of γ-globulin. The evidence for this is, first, that it corresponds to the 3.4 S protein (molecular weight, 75,000–80,000) observed in 0.1 M NaCl, pH 2.5, after reduction (Palmer *et al.*, 1963). There is a gradual transition in the S value as conditions are changed from 0.1 M NaCl, pH 2.5, to 0.025 M, pH 2.4. (Evidence indicating that the 3.4 S component consists of half-molecules was summarized in the introduction.) Second, we have determined the apparent molecular weight of the 3 S protein by sedimentation and diffusion measurements at concentrations of 8, 5, and 2 mg/ml in 0.025 M NaCl, pH 2.4. The values, extrapolated to zero concentration, give a molecular weight of 71,000 (unpublished results).

It is apparent (Fig. 1) that there was a progressive increase in the extent of dissociation, at low pH and ionic strength, after reduction with increasing concentrations of 2-mercaptoethylamine HCl. Dissociation was nearly complete after reduction with 0.1 M 2-mercaptoethylamine HCl. Calculations based on these data and on titrations of —SH groups are given in Table II. The results obtained after reduction with 0.03 M or 0.05 M 2-mercaptoethylamine HCl would suggest that an average of about 2.5–3 disulfide bonds per molecule must be reduced for essentially

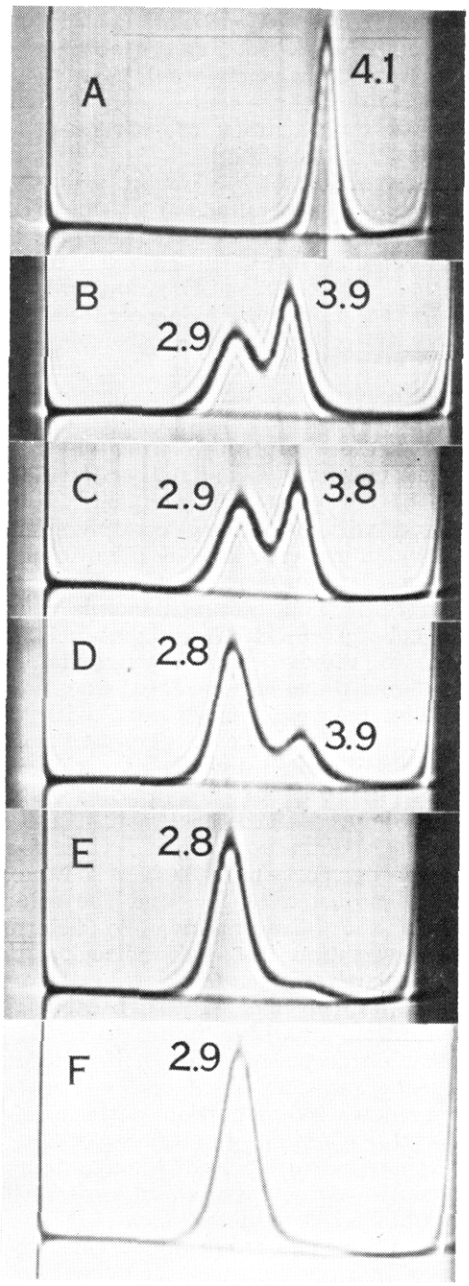

FIG. 1.—Photographs of schlieren patterns taken after 128 minutes at 59,780 rpm and 20° in the Spinco Model E ultracentrifuge. Sedimentation is from left to right. A, unreduced rabbit γ-globulin. B, C, D, E, and F, γ-globulin reduced with 0.01, 0.015, 0.02, 0.03, and 0.1 M 2-mercaptoethylamine HCl, respectively. In each case the solvent is 0.025 M NaCl, *p*H 2.4. The numerals are $s_{20,w}$ values. Protein concentration, 8 mg/ml except for A (6 mg/ml).

complete separation of half-molecules. Liberation of 4.2 —SH groups per molecule originally present resulted in 88% dissociation. This corresponds to the release of 4.8 —SH groups for each molecule cleaved (4.2/0.88). However, in terms of the number of bonds actually linking these subunits, this represents a maximal value since other disulfide bonds may be reduced at the same time.

The data obtained after reduction with 0.01 M, 0.015 M, and 0.02 M 2-mercaptoethylamine HCl indicate that a large proportion of the molecules is dissociable at low *p*H after reduction of a single disulfide bond. Thus, after treatment with 0.01 M 2-mercaptoethylamine HCl, 35% of the molecules were dissociated at *p*H 2.4 and 0.8 —SH group was liberated for each molecule originally present (Fig. 1, Table II). This corresponds to 2.3 —SH groups per molecule dissociated (0.8/0.35), or 1.2 disulfide bonds. With 0.015 M and 0.02 M 2-mercaptoethylamine HCl the percentages dissociated at *p*H 2.4 were 46 and 66%, respectively. The calculated values of disulfide bonds reduced per molecule dissociated are 1.3 and 1.2. Again, these values are maximal, for the reason mentioned above. The results indicate that more than half the γ-globulin can be dissociated after reduction of one disulfide bond for each molecule cleaved.

Data obtained by the titration method with γ-globulin from another rabbit are shown in Table III. In this case the γ-globulin had not been pretreated with iodoacetamide and it contained 1.0 titratable —SH group per molecule; this number was subtracted from values obtained after each reduction.

With 0.005 and 0.01 M 2-mercaptoethylamine HCl, 1.2 and 1.25 disulfide bonds were cleaved for each molecule dissociated. After reduction with 0.01 M 2-mercaptoethylamine HCl, 48% of the molecules were dissociated at low *p*H. Thus at least half the molecules were dissociable after reduction of one disulfide bond. As was observed with the other preparation of γ-globulin (Table II), reduction of three disulfide bonds per molecule resulted in nearly complete dissociation at low *p*H. The data in Table III differ from those in Table II in that reduction with 0.01 M 2-mercaptoethylamine HCl caused a greater extent of dissociation at *p*H 2.4 (48% vs. 35%). Also a somewhat smaller percentage of the γ-globulin used in the second series of experiments was dissociable after reduction of one disulfide bond per molecule; this is indicated by a comparison of the number of —SH groups released, corresponding to 70% dissociation at low *p*H.

Determinations of S-Carboxymethylcysteine Content of Half-Molecules of γ-Globulin.—To obtain an independent estimate of the number of disulfide bonds linking the half-molecules, the *S*-carboxymethylcysteine content of a partially reduced, alkylated sample and the percentage of protein dissociable at low *p*H were determined and compared. Half-molecules were then separated from the partially dissociated protein and analyzed for *S*-carboxymethylcysteine content. A 400-mg portion of γ-globulin was pretreated with 0.1 M sodium iodoacetate for 12 hours at *p*H 5 and 5°. After extensive dialysis a sample was removed, hydrolyzed, and *S*-carboxymethylcysteine was estimated in the Spinco analyzer. Another portion (200 mg) of the pretreated material was reduced with 0.015 M 2-mercaptoethylamine HCl for 75 minutes at 37° in 0.1 M acetate buffer, *p*H 5.0. Iodoacetate was added to a concentration of 0.1 M and the mixture was allowed to stand in the cold room overnight. The *p*H decreased to 4.9. This solution was dialyzed against two 4-liter portions of 0.025 M NaCl, and the *p*H was adjusted to 2.4. In the ultracentrifuge two peaks were observed (Fig. 2, upper diagram). The fraction of protein in the slower peak, corrected for the Johnston-Ogston effect, was 56%. Values of the *S*-carboxymethylcysteine content, determined as described under Methods, are given in Table IV. The difference between the values for the reduced-alkylated and unreduced-alkylated γ-globulin is 1.2 groups per molecule. This corresponds to a release of 2.1 —SH groups per molecule dissociated (1.2/0.56).

In an effort to isolate the half-molecules 120 mg of the reduced alkylated preparation in 5 ml was put through a 1.8 × 120-cm column of Sephadex G-200, equilibrated with 0.025 M NaCl, *p*H 2.4. The elution pattern is shown in Figure 3. Eighty-six per cent of

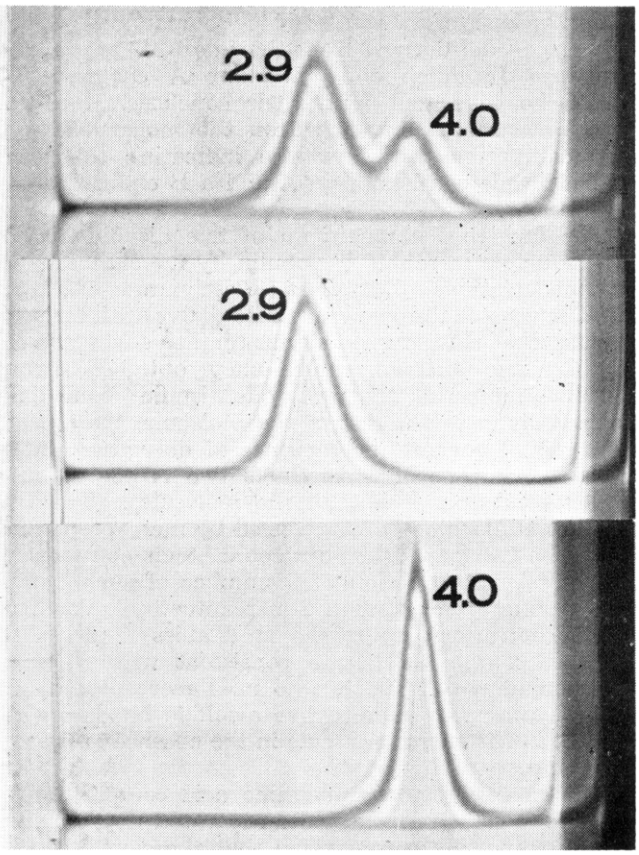

FIG. 2.—Photographs of schlieren patterns taken in the ultracentrifuge. Conditions as in Fig. 1; protein concentration, 8 mg/ml. Solvent, 0.025 M NaCl, *p*H 2.4. Upper diagram, γ-globulin reduced with 0.015 M 2-mercaptoethylamine HCl and treated with excess iodoacetate. Center diagram: fraction of the above protein obtained from the second major peak eluted from Sephadex G-200 at low *p*H (see Fig. 3). Lower diagram: fraction obtained from first major peak (Fig. 3). The numerals are $s_{20,w}$ values.

TABLE IV
CARBOXYMETHYLCYSTEINE CONTENT OF ALKYLATED PROTEINS[a]

Sample	*S*-Carboxymethylcysteine Content (moles/150,000 g)	Net *S*-Carboxymethylcysteine Content[b] (moles/150,000 g)
Unreduced γ-globulin	0.3	
Reduced γ-globulin[c]	1.5	1.2
Separated whole molecules[d]	0.7	0.4
Separated half-molecules[e]	2.1	1.8

[a] Procedures are described in the text. Each sample was treated with excess iodoacetate. [b] Corrected for the *S*-carboxymethylcysteine content of unreduced alkylated γ-globulin. [c] Reduced with 0.015 M 2-mercaptoethylamine HCl. [d] First major peak eluted from Sephadex G-200 (Figs. 2 and 3). [e] Second major peak eluted from Sephadex G-200 (Figs. 2 and 3).

the protein, estimated from optical density measurements at 280 mμ, was eluted in the two major peaks. This value was obtained by assuming symmetry of the major peaks and resolving the leading and trailing shoulders into separate peaks. Samples were removed for analysis from the regions between the pairs of arrows shown in Figure 3. The nature of the protein in the smaller peaks has not yet been investigated. It is improbable that it consists of free A

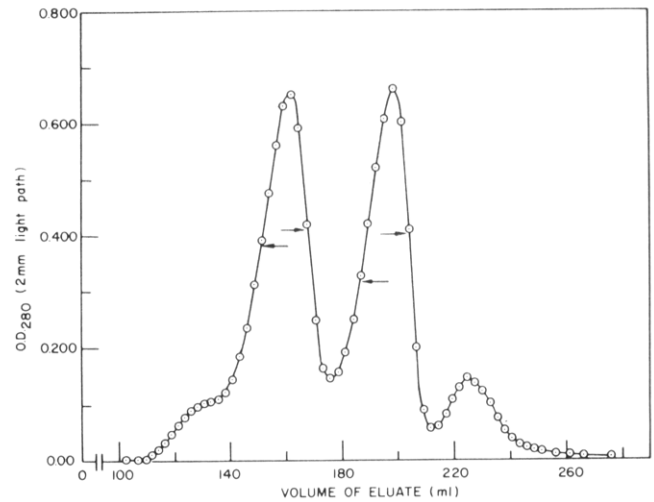

FIG. 3.—Elution from Sephadex G-200 of 120 mg of γ-globulin, reduced with 0.015 M 2-mercaptoethylamine HCl and alkylated. Solvent, 0.025 M NaCl, *p*H 2.4. Column size, 1.8 × 120 cm. The protein eluted between the arrows in each peak, including the points indicated, was pooled separately and examined by ultracentrifugation and amino acid analysis.

or B chains, since under much more stringent conditions, i.e., reduction with 0.1 M 2-mercaptoethylamine HCl and gel filtration in 1 M propionic acid, only 6% of the protein is recovered as B chains (Palmer *et al.*, 1963).

The sedimentation patterns, obtained in 0.025 M NaCl, *p*H 2.4, are shown in Figure 2, center and lower diagrams. The sedimentation velocities correspond very closely to those of half- and whole molecules, respectively, at *p*H 2.4. This, together with the symmetry of the ultracentrifugal patterns and the resolution on Sephadex, indicates that separation was effected by this procedure.

The net values for *S*-carboxymethylcysteine content of the half- and whole molecules, obtained by subtracting the value for unreduced alkylated γ-globulin, were 1.8 and 0.4 moles of —SH groups, respectively, per 150,000 g (Table IV). The former value corresponds to 0.9 —SH group per half-molecule. The *S*-carboxymethylcysteine content of the partially reduced preparation and of the separated 2.9 S component are thus consistent in indicating that dissociation of a molecule can occur after reduction of one disulfide bond.

Amino Acid Analyses.—The amino acid composition of the 2.9 S and 4 S components, separated on Sephadex G-200, are given in Table V, together with results obtained with untreated γ-globulin. The data are expressed as mole-per cent of the amino acids determined (100 × μmoles of amino acid recovered/total μmoles recovered). It is evident that the half- and whole molecules eluted from Sephadex are very similar in composition to one another and to untreated γ-globulin. Since the results for B chains, expressed in the same way, are widely different, with respect to several amino acids, from those of γ-globulin (Crumpton and Wilkinson, 1963), any appreciable enrichment with B chains of either fraction eluted from Sephadex would have been detected. Since a single period of hydrolysis was used, the results for serine, threonine, valine, and isoleucine are probably somewhat low. Because of their unreliability, results for cysteine are not included.

The close agreement with respect to each of the amino acids in Table V contrasts with the large dif-

TABLE V
AMINO ACID COMPOSITION OF FRACTIONS OF REDUCED γ-GLOBULIN ELUTED FROM SEPHADEX G-200 AT LOW *p*H

Amino Acid	First Major Peak[a]	Second Major Peak[b]	Untreated γ-Globulin
	(mole %)[c]		
Lys	4.67	4.74	4.90
Hist	1.15	1.17	1.18
Arg	2.94	3.19	3.21
Asp	8.24	8.18	8.11
Thr	12.50	12.30	12.22
Ser	10.88	10.74	10.87
Glu	9.45	9.09	9.17
Pro	8.03	8.08	8.23
Gly	8.37	8.27	8.22
Ala	6.01	5.98	5.98
Val	9.50	9.71	9.46
Meth	0.98	1.00	1.02
Isoleu	3.30	3.35	3.40
Leu	6.53	6.59	6.63
Tyr	4.21	4.22	4.13
Phe	3.29	3.40	3.30
	100.05	100.01	100.03

[a] See Fig. 3. (whole molecules). [b] Half-molecules. [c] Number of μmoles in sample divided by the total recovery, in μmoles, of the amino acids listed in the table, × 100. Averages of duplicate determinations.

ference in the *S*-carboxymethylcysteine content of the half- and whole molecules (Table IV).

DISCUSSION

The results indicate that more than half the molecules of rabbit γ-globulin can be dissociated after reduction of one disulfide bond per molecule. This conclusion was reached by relating the dissociability at low *p*H to the number of sulfhydryl groups released, as estimated either by titration with sodium *p*-mercuribenzoate or by analysis for *S*-carboxymethylcysteine after alkylation with iodoacetate. Also, half-molecules isolated from γ-globulin that had been reduced under mild conditions and alkylated were found to contain 0.9 *S*-carboxymethylcysteine group.

In the experiments of Table II, 66% dissociation was observed after the reduction of 1.3 bonds for each molecule dissociated. The value 1.3 represents a maximum, with respect to the number of bonds joining the half-molecules, since other disulfide bonds might have been reduced at the same time. The bond is evidently extremely labile since it is reduced in a large proportion of the molecules before any of the other eighteen to twenty disulfide bonds that are present (Smith *et al.*, 1955; Crumpton and Wilkinson, 1963). In the experiments of Table III, carried out with γ-globulin from another rabbit, approximately half the population of γ-globulin molecules was dissociable at low *p*H after reduction of one disulfide bond; a minimum of 56% was dissociable after reduction of one disulfide bond in the experiment in which the protein was alkylated and the *S*-carboxymethylcysteine content was determined.

The fact that essentially complete dissociation requires the reduction of about three disulfide bonds per molecule indicates either that the half-molecules in the remaining one-third of the protein are held together by more than one disulfide bond, or that γ-globulin is heterogeneous with respect to the relative lability of this bond. The latter appears to be the simpler hypothesis in view of the large number of disulfide bonds in the molecule.

On the basis of the results of Fleischman *et al.* (1963), and the model derived from their data, it is probable that the disulfideb ond joins two A chains. This would be consistent with their conclusion that the A chains are in contact within the molecule. It is also compatible with evidence indicating that each half-molecule consists of an A and a B chain (Palmer *et al.*, 1963).

The fact that a maximum of five disulfide bonds are reducible in rabbit γ-globulin by mercaptoethanol in concentrations up to 0.8 M (Fleischman *et al.*, 1962, 1963) suggested the possibility that all five are interchain bonds; that this conclusion was tentative was pointed out by the authors. Since they obtained strong evidence indicating that only one disulfide bond links each B chain to the rest of the molecule, the present data would suggest the presence of only three interchain bonds, one of which links two A chains, in a large proportion, if not all the molecules. The two additional disulfide bonds reduced by mercaptoethanol might be two particular intrachain bonds or represent the fractional reduction of a number of such bonds. The results obtained with 2-mercaptoethylamine HCl in concentrations up to 0.5 M (Tables II and III; [Palmer *et al.*, 1963]), are consistent with those of Fleischman *et al.* (1963), who used mercaptoethanol, in indicating that four to five disulfide bonds are reducible in the native molecule in the absence of urea or detergent.

The close agreement in amino acid composition of the half- and whole molecules separated on Sephadex G-200 supports the conclusion that the component of lower molecular weight consists of an A and a B chain.

In the native molecule 0.5 —SH group was found to be reactive with sodium *p*-mercuribenzoate but not with iodoacetamide or iodoacetate. The possibility was considered that it might become reactive with iodoacetate after reduction. However this would lead to a high value for the number of disulfide bonds linking the half-molecules and could not account for the observed dissociation after reduction of one disulfide bond. The fact that the same conclusion was reached on the basis of either analytical method also indicates that this was not a significant factor.

The possibility that more than one interchain bond was actually reduced, but that the additional sulfhydryl groups released failed to react with either sodium *p*-mercuribenzoate or iodoacetate, appears very unlikely. It is improbable that sulfhydryl groups released from interchain disulfide bonds would be completely inaccessible to either reagent. In addition, sulfhydryl groups that had been liberated, but not inactivated by reaction with sodium *p*-mercuribenzoate or iodoacetate, would have had the opportunity to reoxidize during the extensive dialysis at neutral *p*H prior to acidification, and thus prevent the observed dissociation at low *p*H. Other studies (S. R. Stein, J. L. Palmer, and A. Nisonoff, submitted for publication) have shown that reoxidation, resulting in stabilization at low *p*H, occurs after removal of the reducing agent.

Investigations are in progress to ascertain whether the disulfide bond linking the half-molecules is the same bond as that joining the two univalent fragments after treatment with pepsin (Nisonoff *et al.*, 1960, 1961).

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Active-Center Peptides of Liver-Alcohol Dehydrogenase. I. The Sequence Surrounding the Active Cysteinyl Residues*

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Previous work has demonstrated that the two zinc atoms and two of its twenty-eight sulfhydryl groups are essential to the catalytic activity of horse liver–alcohol dehydrogenase. The two essential —SH groups were carboxymethylated with [1-^{14}C]iodoacetate and the enzyme was digested with trypsin. Comparison of the resultant number of peptides with the number of trypsin-susceptible bonds suggests that horse liver–alcohol dehydrogenase consists of two similar or identical chains. The isolation of a single [^{14}C]carboxymethylcysteinyloctapeptide by gel filtration and ion-exchange chromatography indicates that the cysteinyl residues are part of an identical sequence in these two chains. The sequence of the peptide is Met-Val-Ala-Thr-Gly-Ileu-*S*-carboxymethylcysteine-Arg. This peptide does not bind the functional zinc atoms of the enzyme.

We have previously emphasized that the characteristics of metalloenzymes allow for experimental approaches which, in combination with other existent procedures, offer advantages in the elucidation of the active centers of such enzymes (Vallee, 1961). Our long-standing interest in the mechanism of action of the zinc-containing, DPN-dependent, horse liver–alcohol dehydrogenase has now led to an investigation of the composition and structure of its active center by means of site-specific and selective reagents. The loss of enzymatic activity due to the modification of side chains of amino acids has long served as a valuable means for the identification of residues which are indispensable in the mechanism of action. This mode of procedure has served further as a guide to the isolation of the peptide sequence surrounding such "active residues."

We have reported briefly (Li and Vallee, 1963) that iodoacetate preferentially carboxymethylates 2 of the 28 thiol groups of liver-alcohol dehydrogenase, resulting in simultaneous loss of activity. The present communication describes the isolation and characterization of the peptides containing these carboxymethylcysteinyl residues.

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MATERIALS AND METHODS

Crystalline alcohol dehydrogenase of horse liver was obtained from C. F. Boehringer und Soehne, Mannheim, W. Germany. Before use, the enzyme was dialyzed for 5 days against 0.1 M sodium phosphate buffer, *p*H 7.5, 4°, to remove low-molecular-weight impurities which absorb radiation at 280 mμ. The concentration of protein was determined by measurement of the absorbance at 280 mμ, based upon an absorptivity of 0.455 mg^{-1} cm^2 (Bonnichsen, 1950). Molar concentrations are based on a molecular weight of 83,300 (Ehrenberg and Dalziel, 1958). The turnover number of the enzyme preparations varied from 480 to 530 moles DPN/min per mole protein, and the —SH titer (Boyer, 1954) of such preparations varied between 26 and 28 moles —SH/mole protein.

Succinyltrypsin was prepared from twice-crystallized trypsin (Worthington Biochemical Corp.) ac-