is that an A chain from a given antibody molecule would be able to interact more favorably with one or another of the variety of B chains available. This selection would be governed by the conformational relationship of the interacting portions of the two chains and would be presumed to occur during biosynthesis. The studies of Edelman *et al.* (1961) on guinea pig antibodies provide cogent evidence for such a selective process. Following subsequent dissociation the homologous B chain would be expected to form more stable complexes with the A chain than the other kinds of B chains. However, because of the limited variety of B chains and because of their similarity, interaction of the A chain with some or all of the B chains provided by a different population of γ-globulin would be expected. Thus the limited choice in the structural variants of the B chain could result in a partial specificity in the interaction of the A and B chains.

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Evidence for Linkage of Univalent Fragments or Half-Molecules of Rabbit γ-Globulin by the Same Disulfide Bond*

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Previous work has shown that rabbit γ-globulin dissociates into half-molecules at low *p*H after mild reduction. Also, peptic digestion of γ-globulin removes an inactive fragment, corresponding to a large part of fragment III of a papain digest, leaving a 4.6 S bivalent residue. The latter is cleaved into two univalent fragments, approximately equal in size, by reduction of one labile disulfide bond. Univalent products are similarly obtained by reduction, followed by peptic digestion. The present results indicate that the single, exceptionally labile disulfide bond which links half-molecules in a large part, if not all, of the γ-globulin population is the same bond as that which joins univalent fragments after peptic digestion. The results are also consistent with the hypothesis that half-molecules, consisting of an A and a B chain, are linked in the native molecule by a disulfide bond that joins two A chains. The data provide supporting evidence as to the nature of the structural relationship between the fragments liberated by pepsin and the polypeptide chains of the molecule.

The work of Edelman, Porter, and their collaborators (Edelman and Poulik, 1961; Edelman and Benacerraf, 1962; Fleischman *et al.*, 1962, 1963; Porter, 1962) indicates that the 6.5 S γ-globulin of various species consists of two types of polypeptide chain, designated as "H" (heavy) and "L" (light) (Edelman and Benacerraf, 1962) or, equivalently, as "A" and "B" chains (Fleischman *et al.*, 1962).[1] Each molecule appears to consist of two A chains, each of molecular weight

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[1] The symbols A and B will be used here since they were applied to rabbit γ-globulin.

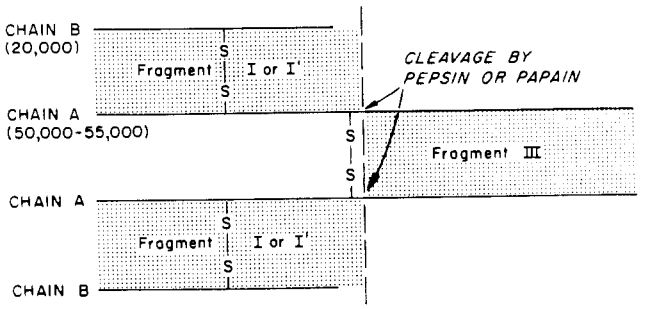

FIG. 1.—Possible arrangement of polypeptide chains and fragments of rabbit 6.5 S γ-globulin (after Fleischman *et al.*, 1963). Their proposed structure has been modified to indicate the presence of 1, rather than 3 disulfide bonds joining the A chains. Treatment with pepsin leaves a bivalent residue, in which univalent fragments are joined by a disulfide bond. However, after digestion with papain, this bond may actually be located in fragment III rather than in the region of I and I′ as shown (see Discussion).

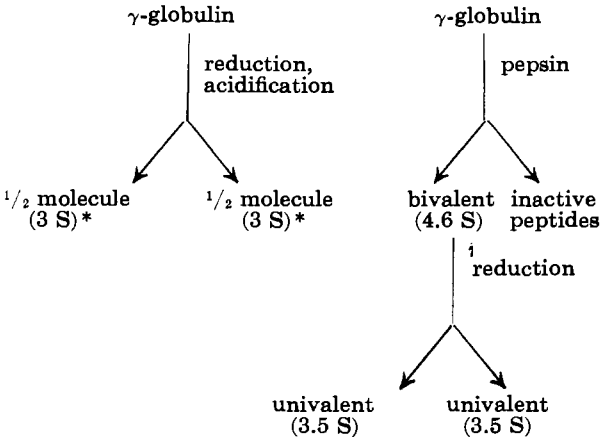

FIG. 2.—Effects of reduction and acidification, or of peptic digestion followed by reduction, of rabbit 6.5 S γ-globulin. * In 0.025 M NaCl, *p*H 2.4.

50,000–55,000, and two B chains of approximate weight 20,000 (Edelman and Poulik, 1961; Pain, 1963; Small *et al.*, 1963; Marler *et al.*, 1964). Information as to the structural relationships among the polypeptide chains and the fragments formed by digestion of γ-globulin with papain (Porter, 1959) has been obtained by comparison of their antigenic determinants (Porter, 1962; Olins and Edelman, 1962; Fleischman *et al.*, 1963). The position of interchain disulfide bonds was investigated by measurement of the *S*-carboxymethylcysteine content of individual chains separated from γ-globulin after reduction and alkylation with iodoacetate (Fleischman *et al.*, 1963). On the basis of such studies, Fleischman *et al.* (1963) proposed the structural model shown in Figure 1, which has been modified here to show the presence of one, rather than three, disulfide bond between the A chains (Palmer and Nisonoff, 1964). One purpose of the present investigation was to provide additional evidence as to the validity of this postulated structure.

Reduction with 0.1 M mercaptoethylamine at *p*H 5, followed by acidification to *p*H 2.5 in dilute salt solution (Palmer *et al.*, 1963) results in symmetrical cleavage of the rabbit 6.5 S γ-globulin molecule (Fig. 2) into two subunits, each evidently consisting of an A and a B chain. Supporting evidence includes the average molecular weight (75,000–80,000); the agreement between the weight- and *z*-average molecular weights; the symmetry of the peak observed in the ultracentrifuge at low *p*H; the very close agreement between the amino acid compositions of separated half- and whole molecules; and the fact that separation can be effected after a mild reduction which results in the release of only small amounts of B chains by 1 M propionic acid (Palmer *et al.*, 1963).

Additional evidence is the fact that about two-thirds of the rabbit γ-globulin population can be dissociated into half-molecules at low *p*H after reduction of a single, highly labile disulfide bond (Palmer and Nisonoff, 1964).[2] An hypothesis consistent with the structure in Figure 1 is that this bond joins the two A chains.

Another approach to the localization of the labile bond, with respect to the fragments and chains, is based on the effects of proteolysis and reduction (Nisonoff *et al.*, 1960a). The reaction of rabbit γ-globulin with pepsin results in removal and partial degradation of a fragment corresponding roughly to fragment III of a papain digest (see Fig. 2). The residual portion of the molecule has a sedimentation coefficient of approximately 4.6 S and a molecular weight of 106,000, and is still bivalent. Subsequent reduction of a labile disulfide bond splits this fragment into two parts, approximately equal in size (Nisonoff *et al.*, 1960a,b, 1961). The resulting fragments are univalent and are similar to papain fragments I and I′ (Fig. 1) in sedimentation coefficient (3.5 S), amino acid composition (Mandy *et al.*, 1961), and antigenic properties (Goodman and Gross, 1963). Comparison with recent data on the molecular weights of the univalent papain fragments indicates that those made with pepsin are somewhat larger (Pain, 1963; Marler *et al.*, 1964; Nisonoff *et al.*, 1960b). Similar results are achieved by reducing the molecule prior to proteolysis (Palmer and Nisonoff, 1963). Reduction of 2–3 disulfide bonds has no effect on the sedimentation coefficient, specific viscosity, or specific antibody activity of the molecule, but subsequent treatment with pepsin yields 3.5 S univalent fragments, rather than the 4.6 S bivalent product. Fragmentation in two stages can also be carried out by treatment with papain, covalently linked to an insoluble synthetic polypeptide, followed by reduction after removal of the papain (Cebra *et al.*, 1961). (The brief treatment with papain, necessary for the conversion to 3.5 S products on subsequent reduction, does not result in a decrease in sedimentation coefficient).

Since the univalent fragments consist of a B chain and part of an A chain (Porter, 1962; Olins and Edelman, 1962; Fleischman *et al.*, 1963), a consistent model is obtained on the assumption that the disulfide bond joining half-molecules is the same bond which serves as the link between the two univalent fragments after treatment with pepsin. The present experiments were designed to provide direct evidence as to the validity of this hypothesis.

The method consisted in reduction with varying concentrations of 2-mercaptoethylamine (MEA);[3] concentrations used covered a range resulting in partial to complete dissociation into half-molecules after acidification. Part of each reduced preparation was tested for the extent of dissociability into half-molecules at low *p*H; another portion was treated with pepsin. If the same disulfide bond is involved in the two proc-

[2] Reduction of the remaining one-third of the γ-globulin population required the cleavage of more than one disulfide bond per molecule. This finding is considered under Discussion.

[3] The abbreviation used is: MEA, 2-mercaptoethylamine hydrochloride.

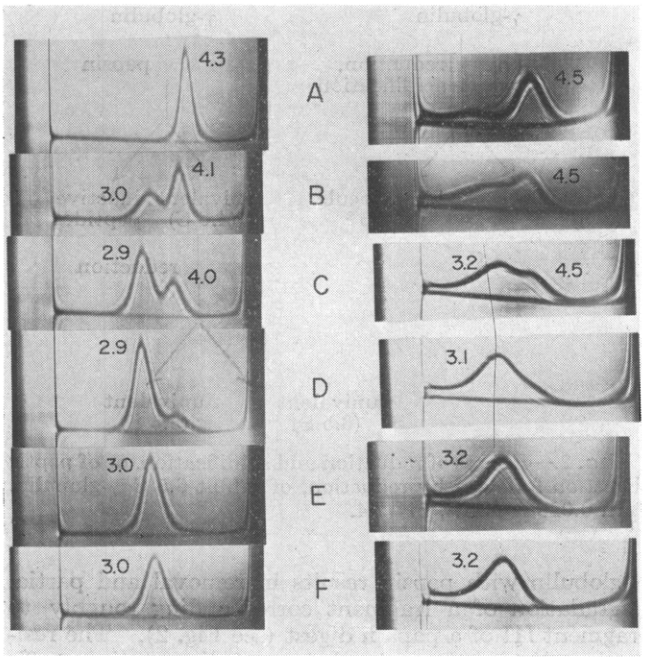

FIG. 3.—Sedimentation patterns of reduced preparations of γ-globulin after acidification to *p*H 2.4 in 0.025 M NaCl (photographs in left column), or treatment with pepsin (photographs on the right). Photographs were taken after 112 minutes (left column) or 96 minutes (right column) at 59,780 rpm (20°). A, unreduced γ-globulin (control); B, C, D, E, and F, γ-globulin prereduced with 0.005, 0.01, 0.02, 0.05, or 0.1 M MEA, respectively as described in the text. The solvent for the peptic digestion is described under Methods. Sedimentation is from left to right. The numerals are $s_{20,w}$ values.

esses, the increased formation of half-molecules at low *p*H, with increasing extent of reduction, should be inversely related to the amount of 4.6 S product in the portions treated with pepsin.

METHODS

γ-Globulin was prepared from the pooled serum of several rabbits by three precipitations with sodium sulfate (Kekwick, 1940) followed by passage through diethylaminoethyl-cellulose (Levy and Sober, 1960) in 0.0175 M phosphate buffer, *p*H 7.0. The product had a sedimentation coefficient ($s_{20,w}$) of 6.1 S and exhibited only a single component when tested by immunoelectrophoresis with a sheep antirabbit serum containing antibodies against numerous components of rabbit serum, including γ- and β-globulins.

Reduction with MEA was carried out for 75 minutes at *p*H 5.0 and 37°. At the end of this time the mixture was passed through a column of IR-120 resin at *p*H 5.0 in the cold room to remove the reducing agent. *p*-Mercuribenzoate in excess of the sulfhydryl groups liberated, as estimated from preliminary experiments, was then added to the eluate, and the number of —SH groups released during reduction was estimated by back-titration of an aliquot with L-cysteine (Boyer, 1954). The methods used have been described in detail elsewhere (Mandy and Nisonoff, 1963).

A portion of the remaining *p*-mercuribenzoate-treated protein was then dialyzed against two 4-liter portions of cold 0.025 M NaCl. The *p*H was lowered to 2.4 with 1 N HCl and the mixture was examined in the ultracentrifuge to determine the extent of dissociation into half-molecules (Palmer *et al.*, 1963; Palmer and Nisonoff, 1964). Relative amounts of half- and whole molecules were determined by measurement of

TABLE I

COMPARISON OF EFFECTS OF LOW *p*H AND PEPTIC DIGESTION ON REDUCED RABBIT γ-GLOBULIN

Sample (*cf.* Fig. 3)	Concn of MEA (moles/liter)	—SH Released[a] (groups per molecule)	Dissociation at Low *p*H: Half-Molecules Formed[b] (% total area)	Dissociation at Low *p*H: Half-Molecules Formed Corrected[c] (% total protein)	Peptic Digestion 4.5 S Component Formed (% total area)
A	0	0	0	0	62
B	0.005	0.8	39	29	43
C	0.01	1.4	67	58	[d]
D	0.02	3.1	80	73	[d]
E	0.05	5.4	>95	>95	<10
F	0.10	7.1	>95	>95	<10

[a] In addition to 0.7 titratable —SH groups per molecule of unreduced γ-globulin. [b] 3 S component, in 0.025 M NaCl, *p*H 2.4 (Fig. 3). [c] Corrected for the Johnston-Ogston effect (see Methods). [d] Area could not be measured accurately.

areas of schlieren patterns. Because of the Johnston-Ogston effect (Johnston and Ogston, 1946), areas obtained with known mixtures of half- and whole molecules were used as a basis for converting relative areas into relative concentrations. For mixtures containing 19, 39, 59, and 78% of half-molecules, area measurements gave values of 30, 49, 68, and 84%, respectively (Palmer and Nisonoff, 1964). A graph of these results was used for corrections.

Another portion of the reduced protein that had been treated with *p*-mercuribenzoate was dialyzed against 4 liters of cold 0.1 M sodium acetate. The *p*H was lowered to 4.3 with 2 M acetic acid, and the protein was allowed to react with pepsin at this *p*H for 8 hours at 37°. The weight of pepsin used was 2% of the weight of γ-globulin present. The reaction was stopped by adjusting the *p*H to 8.0, and ultracentrifugation of the pepsin-treated mixture was carried out without further dialysis.

Sedimentation velocities were measured in double-2°-sector 12-mm cells in the Spinco Model E ultracentrifuge operated at 59,780 rpm. The solvent was placed in the second compartment. The temperature was maintained at 20° ± 0.1°. In correcting S values to $s_{20,w}$ the partial specific volume of the protein was taken as 0.745 (Kabat, 1939). To obtain the correction factor for the solvent used in the peptic digestion, a portion of 0.1 M sodium acetate was adjusted to *p*H 4.3 with acetic acid and then neutralized (as was done with the protein solution). The relative viscosity and density of the resulting solution were measured. The relative viscosity and density of the solvent used for the other portion of the protein (0.025 M NaCl, *p*H 2.4) were estimated from values in the literature; this correction factor was small. Concentrations of γ-globulin were estimated by use of the extinction coefficient, $E^{1\%}_{1\,cm} = 15$ at 280 mμ.

RESULTS

Data obtained following reductions with increasing concentrations of MEA are given in Table I and Figure 3. As the concentration of reducing agent was increased from 0.005 to 0.1 M, the number of —SH groups per molecule released increased from 0.8 to 7.1. After removal of the reducing agent and inactiva-

tion of free —SH groups, the extent of dissociation into half-molecules at low *p*H was estimated from the sedimentation pattern in 0.025 M NaCl, *p*H 2.4 (Fig. 3, photographs on the left), as described under Methods. As in previous investigations, the use of increasing concentrations of MEA resulted in a progressive increase in the amount of the 3 S component, characteristic of half-molecules (Palmer *et al.*, 1963; Palmer and Nisonoff, 1964), that formed at low *p*H. (The $s_{20,w}$ value is about 10% lower in 0.025 M NaCl, *p*H 2.4, than in 0.1 M NaCl, *p*H 2.5 [Palmer *et al.*, 1963; Palmer and Nisonoff, 1964; Nisonoff and Palmer, 1964].)

After treatment of unreduced γ-globulin with pepsin, the major product had a sedimentation coefficient of 4.5 S (Fig. 3A, right). Prior reductions with increasing concentrations of MEA caused a progressive decrease in the amount of the 4.5 S component (Fig. 3), as previously observed (Palmer and Nisonoff, 1963).

It is apparent from Figure 3, first, that the extent of reduction required for complete dissociation into half-molecules (photographs on left) agrees closely with that required for complete reduction of the bond linking the univalent fragments (as indicated by absence of the 4.5 S component after treatment with pepsin; photographs on right). In Figure 3D a small leading shoulder is present in each pattern (left and right columns); these shoulders are not observed in Figure 3E. Thus 0.02 M MEA is not quite sufficient for complete transformation by either criterion, whereas reduction with 0.05 M MEA results in essentially complete dissociation into half-molecules at low *p*H, and also in the absence of a 4.5 S peak from the protein that was treated with pepsin.

Reduction with 0.005 M MEA resulted in the appearance of an appreciable proportion of half-molecules at low *p*H (Fig. 3B, left); also, there was a decrease in the relative size of the 4.5 S peak in the sample subjected to proteolysis, with a concomitant increase in the proportion of slower-moving components (Fig. 3B, right). Corrected for the Johnston-Ogston effect, the degree of dissociation into half-molecules at low *p*H was 29% (Table I). If the capacity to dissociate into half-molecules and the separation into univalent fragments were owing to reduction of the same bond, the fractional decrease in the amount of 4.5 S component after peptic digestion should have agreed, within experimental error, with the value, 29%. An accurate determination of this value was complicated by the heterogeneity of the slower components in the peptic digest; thus preparation of artificial mixtures of slower and faster components for evaluation of the magnitude of the Johnston-Ogston effect was not readily accomplished. However, it has been shown that, in the case of known mixtures of the 3.5 and 4.5 S fragments of γ-globulin, area corrections due to the Johnston-Ogston effect are less than 5% (Mandy and Nisonoff, 1963). In the latter experiments the proteins had been freed of smaller peptides, in contrast to the peptic digests in the present investigation. However, since the Johnston-Ogston effect diminishes as the ratio of sedimentation coefficients increases, the magnitude of the effect, with respect to the 4.5 S component, is probably small. This component comprises 62% of the total area in Figure 3A (right) and 43% in Figure 3B (right). These values were obtained by assuming that slower components did not contribute to the leading edge, and resolving the leading component into a symmetrical peak on this basis. The decrease in the amount of 4.5 S product of peptic digestion, owing to prior reduction with 0.005 M MEA, is approximately 30% (19/62), which agrees closely with the extent of dissociation of the same reduced sample into half-molecules at low *p*H.

Since this calculation is based on percentages of the total areas, it would be in error if small peptides which did not contribute to the area were formed in significantly different amounts in the two peptic digestions. That this was not the case is indicated by the fact that the total areas in Figures 1A and 1B (right) agreed within 6%. (The protein concentration, bar angle, and photographic-enlargement factor were the same in the two experiments).

The area of the faster peak in the peptic digest of Figure 3C could not be determined with accuracy, so that a quantitative comparison in this instance was not feasible. It should be noted that the 3.2 S component in the peptic digests reflects the presence of fragments I and I′ together with polypeptides derived from fragment III.

Discussion

Previous work has shown that mild reduction of rabbit 6.5 S γ-globulin results in dissociability into half-molecules at low *p*H. Also, reduction followed by peptic digestion yields 3.5 S, univalent fragments in place of the bivalent fragment ($s_{20,w}$ = 4.5–4.8), which is the major product of the peptic digestion of unreduced γ-globulin. The present data indicate that reduction of the same bond is responsible for both transformations.

After reductions with increasing concentrations of MEA the extent of dissociability into half-molecules at low *p*H was directly related to the decrease in the amount of 4.5 S product of peptic digestion; the two tests were carried out with separate portions of each reduced sample. By both criteria, the conversion was not quite complete after reduction with 0.02 M MEA (Fig. 3); the use of 0.05 M MEA resulted in essentially complete dissociation into half-molecules, and also in the absence of the 4.5 S product from a peptic digest. After reduction with 0.005 M MEA, the extent of dissociation into half-molecules and the percentage decrease in the amount of 4.5 S product formed by pepsin were the same (~30%) within experimental error. These data indicate that the disulfide bond joining half-molecules is the same bond which links two univalent fragments after peptic digestion. The results thus confirm the structural relationship of half-molecules, obtained after mild reduction and acidification, to the fragments formed by digestion with pepsin and reduction (Fig. 1).

The present data are in agreement with previous results indicating that a large proportion of the γ-globulin population can be dissociated into half-molecules after reduction of a single disulfide bond. Thus, after reaction with 0.005 M MEA, the number of —SH groups released per molecule dissociated (Table I) was 0.8/0.29, or 2.8, corresponding to the reduction of 1.4 disulfide bonds. After reduction with 0.01 M MEA the corresponding value was 1.4/0.58 or 2.4 —SH groups per molecule dissociated. In terms of the number of disulfide bonds linking half-molecules, these values are maximal, since some reduction of other disulfide bonds might occur at the same time.

On the same basis the results of reduction with 0.005 M MEA indicate that, after reduction of one disulfide bond per molecule, a 3.5 S product is formed by proteolysis with pepsin. The calculated number of —SH groups released is 0.8/0.30, or 2.7 groups for each molecule that yielded 3.5 S fragments upon subsequent treatment with pepsin. Area measurements on the 4.5 S peak are not sufficiently accurate in the samples

reduced with higher concentrations of MEA to permit similar calculations.

As in previous investigations (Palmer *et al.*, 1963; Palmer and Nisonoff, 1963), complete dissociation into half-molecules, or the absence of a 4.5 S component after peptic digestion, required the reduction of 2–3 disulfide bonds per molecule (Table I). It is uncertain whether, in the refractory one-third of the γ-globulin, more than one disulfide bond links the half-molecules; alternatively, a single bond may link the subunits in each molecule but this bond may be somewhat less labile, relative to other disulfide bonds, in part of the population. A third possibility is that the reaction with MEA is reversible and reaches equilibrium with an appreciable fraction of the molecules still unreduced.

The results, taken in conjunction with those of Fleischman *et al.* (1963), would indicate that all three interchain disulfide bonds are present in the 4.5 S fragment; i.e., that there are none in that part of fragment III which is removed by pepsin. This is of interest in connection with findings of Marler *et al.* (1964) and of R. H. Pain[4] that fragment III of a papain digest has a molecular weight of approximately 50,000 in concentrated urea or guanidine hydrochloride, but a value only half as great after reduction. This would suggest that the single disulfide bond joining the two A chains is in the region of fragment III. One possible explanation for the two sets of results is that papain may cleave the molecule in a manner such as to leave the disulfide bond in fragment III; i.e., that papain and pepsin cleave on opposite "sides" of the disulfide bond.

It is very probable that the disulfide bond joining half-molecules links two A chains. The presence of a single *S*-carboxymethylcysteine group in B chains, isolated after reduction and alkylation with iodoacetate, indicates that only one disulfide bond links each B chain to the rest of the molecule (Fleischman *et al.*, 1963). Also, half-molecules can be separated after reduction of one disulfide bond (Palmer and Nisonoff, 1964). It would then appear that this bond must join two A chains; cleavage of the single interchain bond through which a B chain is linked to the molecule could not yield half-molecules. This interpretation is also consistent with the presence of a B chain and part of an A chain in each univalent fragment liberated by papain (Porter, 1962) and with the present data indicating that the disulfide bond which joins univalent fragments after peptic digestion is also the bond which links half-molecules of γ-globulin.

[4] Personal communication.

The results confirm the unusual lability of this disulfide bond. The fact that it is reduced by MEA at concentrations which do not appreciably affect the other disulfide bonds in the molecule permitted the demonstration that only a single bond joins the half-molecules or fragments.

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