*DISSOCIATION OF RABBIT GAMMA GLOBULIN INTO SUBUNITS BY REDUCTION AND ACIDIFICATION**

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The work of Edelman and collaborators[1-3] and of other investigators[4-6] has demonstrated that 6.5 S gamma globulin molecules of various species consist of several polypeptide chains. Recent experiments of Fleischman, Pain, and Porter[7, 8] and of Marler and Tanford[9] indicate the presence in rabbit gamma globulin of two chains of molecular weight of 55,000–60,000 each, and two of molecular weight 20,000–25,000. Fleischman *et al.* denote the two types of polypeptide chains as "A" and "B," respectively, corresponding to the "H" and "L" chains of human gamma globulin, described by Edelman and associates. On the basis of experiments with horse antibody, Porter has concluded that the "A chains" contain the active combining sites of the antibody molecule.[8] Utsumi and Karush,[10] and Jaquet, Bloom, and Cebra,[11] have obtained active subunits of rabbit antibody by reduction and treatment with detergent.

It was recently demonstrated that the disulfide bond which links the univalent fragments of rabbit antibody after treatment with pepsin can readily be reduced prior to enzymatic digestion.[12] Complete reduction of this bond was observed when a total of 5–6 —SH groups had been liberated, and was characterized by the formation of univalent, 3.5 S fragments upon subsequent treatment with pepsin. (Peptic digestion of the unreduced antibody molecule yields a bivalent, 5 S fragment which is split into 3.5 S fragments by reduction of one labile disulfide bond.[13, 14]) We have now found that acidification of a solution of gamma globulin in 0.1 *M* NaCl, after reduction of 2–3 disulfide bonds, results in a decrease in molecular weight to approximately half that of the original molecule. The product forms one symmetrical peak in the ultracentrifuge. The results suggest that two subunits, approximately equal in size, are liberated. When the pH is then raised to neutrality, varying proportions of two major components, sedimenting with velocities of approximately 4 S and 6 S, are observed. The latter value is almost identical with that of untreated gamma globulin, which suggests that spontaneous recombination occurs at neutral pH. There is no appreciable formation of insoluble material, and a large fraction of the specific antibody activity of an antihapten antibody was retained.

Materials.—Methods used to obtain rabbit antisera against chicken ovalbumin or the *p*-azobenzoate haptenic group, and for the preparation of gamma globulin fractions, have been described elsewhere.[15-18] Immunoelectrophoresis of each gamma globulin preparation with sheep anti-rabbit serum showed only a single band, characteristic of gamma globulin. Chicken ovalbumin, twice crystallized, was obtained from the Pentex Company. 2-Mercaptoethylamine hydrochloride (MEA) was purchased from the California Corporation for Biochemical Research. *p*-Nitrobenzoic acid labeled with C^{14} in the carboxyl group (1.2 mc/mmole) was obtained from the New England Nuclear Corporation, and recrystallized. The radiochemical purity, estimated by the isotope dilution method, was found to be greater than 97%.

Methods.—Measurements of the capacity of anti-*p*-azobenzoate preparations to bind *p*-nitrobenzoate-C^{14} were made by the method of equilibrium dialysis at 5.0°C, in saline-borate buffer, pH 8.0, ionic strength 0.16.[19] Samples to be compared were dialyzed against a common outer solution.

Measurements of radioactivity per unit volume of the inner and outer solutions were made after dialysis to equilibrium. Samples were evaporated to dryness in transparent cellulose acetate planchets, placed in cylindrical glass vials filled with 15 ml of redistilled toluene containing 1.5 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and 60 mg of 2,5-diphenyloxazole (PPO), and counted at −20°C in a Packard Tri-Carb liquid scintillation counter. Protein was added to the outer solution prior to evaporation to equalize the amounts present in the samples.

Molecular weights were determined by the methods of sedimentation diffusion or sedimentation equilibrium in a short column. Diffusion constants were determined in a synthetic boundary cell in the ultracentrifuge at 9,341 rpm, by the method of Ehrenberg,[20] and calculated from graphs of $1/y^2_{max}$ against time, where y_{max} is the height of the peak in the schlieren pattern. The sedimentation equilibrium experiment was carried out at 20°C and 12,590 rpm; a 1.7 mm column in a filled epoxy double sector cell was used. Sedimentation velocities were determined at 59,780 rpm and 20° ± 0.2°C in 12 mm, 2°, single or double sector Kel-F cells. In correcting values to $s_{20,w}$ the partial specific volume was taken as that of untreated gamma globulin, 0.745.[21, 22] Except as indicated, the protein concentration was 5–8 mg/ml.

Sulfhydryl groups were estimated essentially as described by Boyer.[23] Details are given in reference 15. After reduction and removal of the reducing agent, samples were treated with an excess of *p*-chloromercuribenzoic acid (CMB) and back-titrated in the presence of 0.5% sodium lauryl sulfate with a freshly prepared solution of recrystallized L-cysteine.

Gamma globulin preparations were reduced with 2-mercaptoethylamine hydrochloride (MEA) at pH 5 and 37°C for 75 min, then passed through a column of IR-120 cation-exchange resin at pH 5.0 in the cold room to remove the MEA. Details of the methods have been described elsewhere.[15] The eluate was neutralized, and excess CMB was added immediately. In some experiments CMB was added just before neutralization.

Results.—Reduction of rabbit anti-ovalbumin gamma globulin, followed by acidification: Figure 1 shows sedimentation patterns obtained after reduction of gamma globulin (preparation A) with decreasing concentrations of MEA, and subsequent adjustment of the pH to 2.5 with 1 *M* HCl. Samples were dialyzed against 0.1 *M* sodium chloride at neutral pH before acidification. With 0.5 or 0.1 *M* MEA, the product, at pH 2.5, migrated almost entirely as a single peak ($s_{20,w}$ = 3.4 S in each case—Figs. 1*A* and 1*B*). Small amounts of a faster-moving component were present. At the same pH and salt concentration, the $s_{20,w}$ value of the unreduced gamma globulin was 5.0 S (Fig. 1*E*). When 0.05 *M* or 0.01 *M* MEA was used for the reduction, incomplete transformation to the product having a lower sedimentation co-

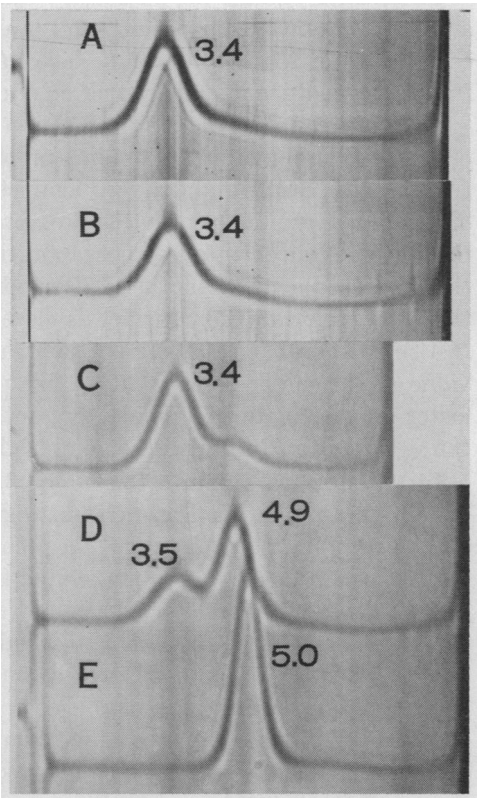

FIG. 1.—Photographs of schlieren patterns taken in a Spinco Model E ultracentrifuge after 80 min at 59,780 rpm. Sedimentation is from left to right. Samples in 0.1 *M* NaCl plus HCl, pH 2.5. (*A*) Gamma globulin reduced with 0.5 *M* MEA, 9.3 —SH groups per molecule; (*B*) 0.1 *M* MEA, 9.1—SH groups; (*C*) 0.05 *M* MEA, 5.2 —SH groups; (*D*) 0.01 *M* MEA, 1.8 —SH groups; (*E*) unreduced gamma globulin, 0.7 —SH groups. Details are in the text. The numerals in the figure are $s_{20,w}$ values.

efficient was observed (Figs. 1*C* and 1*D*); this was especially pronounced for the sample treated with 0.01 *M* MEA. The sedimentation velocity of the faster component in each case was very similar to that of the unreduced control (Fig. 1*E*). The number of free —SH groups per molecule of protein, for each preparation, is indicated in the legend of Figure 1. The value, 0.7 —SH per molecule, was obtained for the unreduced control, which was similarly incubated at pH 5.0 and passed through a column of IR-120 resin. This value, when subtracted from each of the other values, gives the number of —SH groups liberated by reduction. The data indicate that 2–3 disulfide bonds must be reduced for complete transformation to the slower-moving form. The value, 9.3 —SH groups per molecule, obtained after reduction with 0.5 *M* MEA, was 2–3 —SH groups lower than that observed in similar experiments with other preparations.

At neutral pH the $s_{20,w}$ value of the sample treated with 0.5 *M* MEA (6.5 S) was similar to that of the unreduced gamma globulin.

These data suggest the possibility that the reduction and acidification result in a decrease in molecular weight, and that the samples reduced with the lower concentrations of MEA (0.01 *M* or 0.05 *M*) exhibit 2 components in the ultracentrifuge because of inadequate reduction of disulfide bonds.

Molecular weight of the 3.4 S component: A 500 mg sample of another preparation of antiovalbumin gamma globulin (preparation B) was reduced with 0.1 *M* MEA, treated with CMB, and dialyzed against cold 0.1 *M* NaCl at neutral pH. A total of 8.9 —SH groups per molecule of protein was liberated in this procedure. A portion was concentrated by pervaporation to a protein concentration of 30 mg/ml, then dialyzed against another 4 liter portion of the same salt solution. At pH 7, the $s_{20,w}$ value was 6.2 S, which is close to that of the untreated gamma globulin (6.1 S). The pH of a 30 mg portion was lowered to 2.5 with 1 *M* HCl. The $s_{20,w}$ value of the major component at this pH was 3.4 S. An appreciable amount of faster-moving material was also present. At the same pH and salt concentration the $s_{20,w}$ value of the unreduced gamma globulin was 4.6 S.

In an effort to separate the major 3.4 S protein from the faster-moving components, 30 mg in 1 ml was chromatographed on a 110 cm^3 column of Sephadex G-200, equilibrated with 0.1 *M* NaCl containing sufficient HCl to lower the pH to 2.5. Two thirds of the protein, eluted first from the column, was discarded. The last third was used for determinations of molecular weight, following dialysis against cold 0.1 *M* NaCl, pH 2.5. The $s_{20,w}$ value was the same as that of the major component observed prior to passage through Sephadex (3.4 S). The sedimentation velocity was measured in a double-sector cell, with a portion of the outer solution of the dialysis present in the other compartment of the cell. The gradient curve obtained in this experiment was analyzed by subtracting from the leading side of the principal (3.4 S) boundary the mirror image of the trailing side. This resolved the "shoulder" into a small peak, with area corresponding to 10% of the whole, and sedimentation coefficient of approximately 5 S.

The molecular weight was determined at pH 2.5 by the sedimentation equilibrium method with a protein concentration of 3.1 mg/ml, and by sedimentation and diffusion measurements at concentrations of 7.0 and 2.9 mg/ml. The results are in Table 1. The molecular weight is also given for unreduced gamma globulin at pH 2.5. The sedimentation equilibrium data were analyzed by the methods

suggested by Van Holde and Baldwin.[24] The slight curvature in the Z/r *vs.* $\int_a^r Z dr$ graph (Fig. 2), as well as the difference between M_w and M_z (Table 1), is probably a consequence of the small amount of 5 S material remaining in the sample. If the data are treated as resulting from a mixture of 90% of a monomer with molecular weight 75,000 and 10% dimer of weight 150,000, the values of M_w and M_z predicted are 82,100 and 88,100, in good agreement with the observed values. Since the sedimentation equilibrium method is extremely sensitive to small amounts of high molecular weight aggregates, it is important that the reduced material be passed through Sephadex G-200 before experiments of this kind are attempted. Preliminary experiments, in which this separation was not performed, gave erratic and high results.

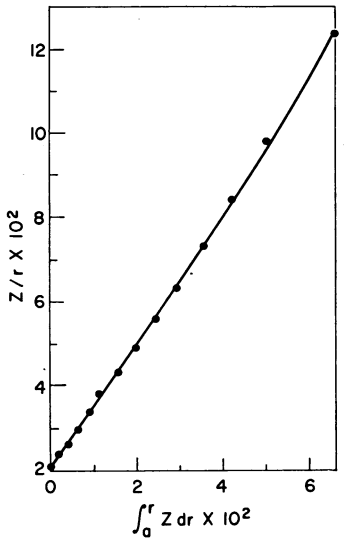

FIG. 2.—Sedimentation equilibrium of reduced gamma globulin in 0.1 *M* NaCl, pH 2.5. The data are graphed according to equation 35 of reference 24. Z is schlieren image deflection (cm). r is the distance from the center of rotation (cm), and a is the meniscus position.

The values obtained by measurements of sedimentation velocity and diffusion constant (Table 1) are in good agreement with the results of sedimentation equilibrium. The molecular weight obtained for unreduced gamma globulin at pH 2.5 falls within the range of values reported in the literature for rabbit gamma globulin at neutral pH.[21, 22, 25]

Reduction alone, without acidification, did not significantly affect the sedimentation coefficient; the $s_{20,w}$ value was 6.2 S in saline-borate buffer, pH 8.0, ionic strength 0.16. (It has previously been shown that treatment with 0.1 *M* MEA does not appreciably affect the sedimentation coefficient or specific viscosity.[12])

The results indicate that the 3.4 S component represents half molecules of gamma globulin. This is supported by the values obtained for the molecular weight and by the fact that it migrates as a single symmetrical peak in the ultracentrifuge. Additional support for this hypothesis is given by data (discussed below) indicating that the smaller subunits can apparently be formed without breaking the disulfide bonds linking "B chains" to the rest of the molecule.

Effect of varying pH on the reduced gamma globulin molecule: Portions of the

TABLE 1

MOLECULAR WEIGHTS AT pH 2.5*

Gamma globulin preparation	Method	Protein concentration mg/ml	$s_{20,w}$ × 10^{13}	$D_{20,w}$ cm^2/sec × 10^7	Molecular weight
Reduced†	Sedimentation equilibrium	3.1			M_w = 81,800 M_z = 85,500
Reduced†	Sedimentation diffusion	2.9	3.65	4.31	80,000
Reduced†	"	7.0	3.40	3.88	82,000
Untreated	"	8.1	4.55	2.75	155,000

* Solvent, 0.1 *M* NaCl containing sufficient HCl to lower the pH to 2.5.
† Details of the procedures used are in the text. The reduced samples were passed through Sephadex G-200 prior to determinations of molecular weight.

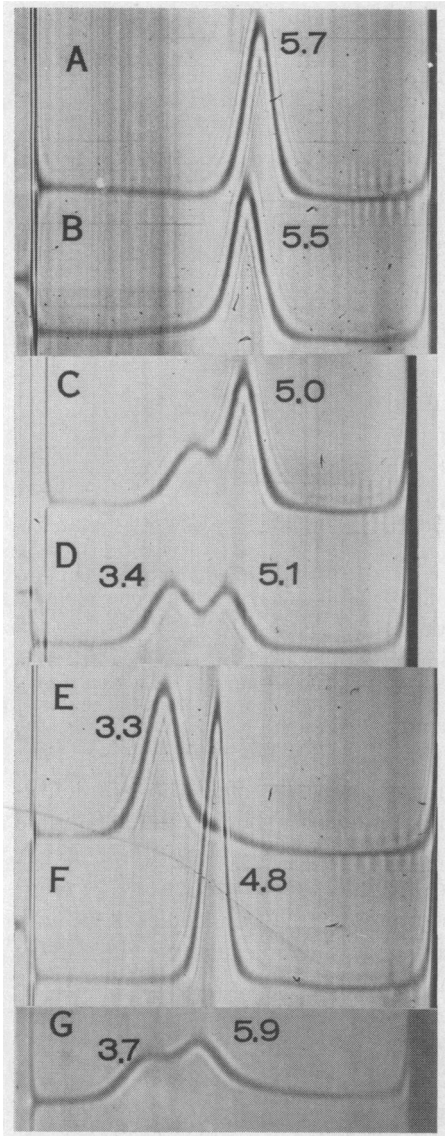

FIG. 3.—Photographs of schlieren patterns taken in a Spinco Model E ultracentrifuge after 80 min at 59,780 rpm (except *G*, 64 min). Sedimentation is from left to right. The numerals are $s_{20,w}$ values. *A*, unreduced gamma globulin, pH 3.3. *B*, *C*, *D*, and *E*: gamma globulin reduced with 0.1 *M* MEA and then adjusted to the following pH's: *B*, pH 3.3; *C*, pH 2.9; *D*, pH 2.7; *E*, pH 2.5. *F* is unreduced gamma globulin at pH 2.5. *G*, reduced sample adjusted to pH 7.0 after acidification to pH 2.5. Solvent, 0.1 *M* NaCl with sufficient HCl added to lower the pH to the desired value.

subunits which had not recombined.

reduced gamma globulin described in the preceding section were adjusted to various levels of pH after dialysis against 0.1 *M* NaCl. As already indicated, the sedimentation patterns of reduced and unreduced gamma globulins were similar at neutral pH. Figures 3*A* and 3*B* are photographs of the schlieren patterns of unreduced and reduced gamma globulin, respectively, at pH 3.3 (0.1 *M* NaCl + HCl). Again, little difference is noted in the patterns of S values (shown on the figure). Figure 3*F* indicates that lowering the pH from 3.3 to 2.5 decreased the $s_{20,w}$ value of the unreduced gamma globulin from 5.7 to 4.8 S. However, at this pH, the value for the reduced sample (3.3 S, Fig. 3*E*) is only 70% as great as that of the unreduced gamma globulin, as compared with 96% at pH 3.3. At intermediate pH's, 2.9 and 2.7, two distinct components are observed in the reduced sample (Figs. 3*C* and 3*D*). There is an increasing fraction in the form of the slower component as the pH is lowered; at pH 2.5 the transition is nearly complete (Fig. 3*E*). The small amount of faster-moving material visible in Figure 3*E* still remained at pH 2.0 (photograph not shown). These results indicate that the transition from whole to half molecules occurs between pH 3.3 and 2.5. Unreduced gamma globulin showed only a single component at pH's 2.9 and 2.7; $s_{20,w}$ = 5.0 and 4.9 S, respectively.

Recombination of subunits at neutral pH: The 3.4 S subunits formed by reduction and acidification appear to recombine spontaneously at neutral pH. Figure 3*G* shows the sedimentation pattern in 0.1 *M* NaCl after neutralization to pH 7. The $s_{20,w}$ value of the faster of the two major components, 5.9 S, agrees closely with the value for the same untreated gamma globulin (6.2 S); that of the slower component was approximately 3.7 S. This result suggests that the half molecules recombine to a large extent at neutral pH and that the slower component represents In a number of similar experiments 2 compo-

nents with similar S values were always obtained; however, the ratio of the amount of the faster to that of the slower component was usually greater than in Figure 3. In each experiment 10–20% of more rapidly migrating protein, presumably consisting of aggregates, was also present.

While the process is clearly reversible, the equilibrium cannot be rapidly attained, since two maxima are observed in the schlieren pattern at pH's 2.7 and 2.9. The theory of Gilbert[26] predicts that rapidly equilibrating monomer-dimer mixtures will exhibit only one maximum in the concentration gradient, moving at an intermediate velocity.

Effect of reduction and acidification on the activity of antihapten antibody: Tests of specific precipitability on recombined antibody were not reproducible. One recombined preparation of antiovalbumin gamma globulin formed a small amount of specific precipitate with antigen. Another specifically inhibited the homologous precipitin reaction (of untreated antibody with antigen). In view of the presence of some aggregates of high molecular weight and the possible presence of univalent molecules in the recombined preparations, interpretation of precipitin data is difficult. Since the method of equilibrium dialysis permits measurement of combining activity without the formation of specific precipitates, an antihapten antibody was investigated.

Forty-nine mg of anti-*p*-azobenzoate gamma globulin (3% precipitable by an ovalbumin-*p*-azobenzoate test antigen) was reduced with 0.1 *M* MEA as described above. Excess iodoacetate (0.2 *M*), rather than CMB, was used to react with free —SH groups, because CMB would be expected to combine specifically with the antibenzoate antibdy. After standing overnight in the cold, the mixture was dialyzed twice against 0.1 *M* NaCl, and the pH was lowered to 2.5. More than 85% of the protein migrated with $s_{20,w} = 3.3$ S, indicating a large amount of splitting into half molecules. After neutralization, two major components were observed with $s_{20,w}$ values of 3.6 and 5.7 S. Some aggregates were also present. Equilibrium dialysis was carried out with this preparation, with untreated antibenzoate gamma globulin, and with normal gamma globulin, each at a protein concentration of 16 mg/ml. The free concentration of hapten (C^{14}-*p*-nitrobenzoate) at equilibrium was 4.48×10^{-6} *M*. The concentration of hapten bound by the treated and untreated antibodies and by normal gamma globulin were 2.46×10^{-6}, 2.91×10^{-6}, and 0.35×10^{-6} *M*, respectively (averages of triplicate determinations). The results indicate that the specific binding capacity of the antibody is largely retained during reduction and acidification. (At a fixed free concentration of hapten, the concentration bound should be in direct proportion to the concentration of combining sites, if the average binding constant is not altered.)

Separation of "B chains" from reduced samples: To determine whether the disulfide bonds linking "B chains" to the remainder of the molecule are cleaved during reduction, we employed methods similar to those of Fleischman *et al.*,[7] except that Sephadex G-100 was used in place of G-75. Samples were dialyzed against two 4 liter portions of cold 1 *M* propionic acid, and the column was equilibrated with the same solvent. The ratio of column volume to the volume of sample exceeded 100 to 1. Samples of gamma globulin treated with 0.2 *M* mercaptoethanol and iodoacetate, as suggested by Porter,[8] yielded 2 peaks during chromatography. The second, which consists of "B chains,"[7] contained 20–25% of the protein, in close

agreement with the results of Fleischman *et al.* After treatment with 0.03 *M* MEA and CMB, by methods already described, 4.1 —SH groups per molecule were liberated. Only 6% of the protein was eluted from Sephadex G-100 as a second component; however, the same material gave 75% of a 3.3 S component in 0.1 *M* NaCl, pH 2.5. This indicates that the separation into half molecules does not require the release of free "B chains." A sample treated with 0.5 *M* MEA and CMB (11.2 —SH liberated) yielded 14% "B chains," and 85% of the 3.3 S component in 0.1 *M* NaCl, pH 2.5.

Discussion.—On the basis of analyses of carboxymethylcysteine content, after reduction of rabbit gamma globulin and alkylation with iodoacetate, Porter localized the interchain disulfide bonds and proposed that each "B chain" is held to an "A chain" by one disulfide bond and that the two "A chains" are linked by three disulfide bonds.[27]

Our results indicate that half molecules are formed from reduced gamma globulin at pH 2.5 and suggest that each consists of an "A chain" and a "B chain." This interpretation is compatible with the following facts: (*a*) the average molecular weight of the subunits formed at pH 2.5 closely approximates half the value for native gamma globulin. (*b*) The symmetry of the peak in the ultracentrifuge at pH 2.5 suggests that the subunits are similar in size. Other postulated combinations of "A" and "B chains" lead to molecular weights that are widely different from one another, or which disagree with the observed value. For example, a fairly symmetrical peak might result from the release of pairs of "B chains" and single "A chains," since the molecular weight of an "A chain" is about twice that of a "B chain," but the approximate average molecular weight would be only 50,000. (*c*) Only 2–3 disulfide bonds need be reduced for conversion to half molecules at low pH (0.1 *M* NaCl). This is consistent with Porter's model, if one postulates that the "B chains" remain attached to "A chains." (*d*) When an average of two disulfide bonds per molecule is cleaved, large amounts of half molecules are formed, but a low yield of free "B chains" is obtained by passage through Sephadex G-100 in 1 *M* propionic acid. This also indicates that the disulfide bonds linking the half molecules are more labile to reduction by MEA than those holding "B chains" to the remainder of the molecule.

When 0.5 *M* MEA is used for the reduction, a considerable proportion of "B chains" is separable in 1 *M* propionic acid, but remain attached to "A chains" in 0.1 *M* NaCl, pH 2.5, evidently through noncovalent bonds.

The transition to half molecules occurs between pH's 3.3 and 2.5 in 0.1 *M* salt (Fig. 3). The separation is probably attributable to electrostatic repulsion at low pH, which might disrupt hydrophobic bonds, or to the destruction of electrostatic bonds which require carboxylate ions. The apparent reformation of whole molecules at neutral pH (Fig. 3*G*) also supports the concept that noncovalent bonds are sufficiently strong to hold together the subunits. The large yield of 6 S protein that results after neutralization indicates that there is a strong tendency for these subunits to associate as pairs. The process is quite similar to the recently observed reversible dissociation of aldolase,[28, 29] the principal difference being the requirement in the case of rabbit gamma globulin for prior reduction of disulfide bonds.

Studies are in progress to attempt to define more precisely the conditions for dissociation and reformation, and to determine whether the half molecules are

univalent. The possibilities of reforming disulfide bonds after reassociation, by removal of CMB, and of hybridization of subunits from different antibody molecules are suggested.

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