# *LOCALIZATION OF NONCOVALENT INTERACTIONS BETWEEN THE HEAVY CHAINS OF RABBIT γG-GLOBULIN**

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After reduction of an interchain disulfide bond, rabbit γG-globulin dissociates into half molecules, each comprising one light and one heavy chain, in aqueous salt solution at pH 2.5.[1] The dissociation is reversed at neutral pH;[1, 2] since sulfhydryl groups are masked after the preliminary reduction, reassociation evidently occurs through noncovalent interactions. Half molecules of rabbit antiovalbumin recombine at random, within experimental error, with half molecules derived from nonspecific γG-globulin.[2] Also, essentially random recombination occurs when half molecules of rabbit γG-globulins, differing in allotypic specificities at both the *a* and *b* loci, are mixed and allowed to reassociate.[3] That the two heavy chains are in contact within the molecule was deduced from several lines of indirect evidence by Fleischman *et al.*,[4] and confirmed by the demonstration that the disulfide bond joining half molecules connects the two heavy chains.[5]

Reduced, alkylated fragment Fc of a papain digest (the crystallizable fragment; molecular weight, 50,000) dissociates into two half fragments in 4 *M* guanidine hydrochloride.[6] Since fragment Fc comprises portions of two heavy chains,[4] it was anticipated that it might dissociate in dilute salt solution at pH 2.4, conditions which yield half molecules. This was found to occur and, as is true of half molecules, reassociation at neutral pH was observed.[7]

These results suggested that the noncovalent interactions joining the heavy chains might be largely localized within fragment Fc. They are consistent with the finding that, subsequent to peptic digestion, the univalent (Fab′) fragments separate at a pH near neutrality following reduction of a disulfide bond.[8, 9] This suggests that strong noncovalent interactions do not link the Fab′ fragments, and that such interactions may therefore be confined to the region of the heavy chain within fragment Fc. However, the data are not conclusive since pepsin might conceivably disrupt noncovalent bonds between the Fab′ fragments in the native molecule. The fact that fragments Fab′ are linked by a disulfide bond shows that they are in close proximity. (The symbol Fab′ refers to each of the two fragments, containing an active site, liberated by pepsin in the presence of reducing agent. It has a molecular weight of 45,000–50,000 and comprises a light chain and most or all of the heavy chain not present in fragment Fc.[4] The univalent fragments of a papain digest are designated Fab.)

As a further test of the hypothesis that noncovalent interactions are confined to the fragment Fc region of the molecule, we have allowed half fragments Fc, formed at low pH, to reassociate with half molecules of γG-globulin. If noncovalent interactions between half molecules occurred in the Fab′ as well as the Fc region of the molecule, the energy of interaction between two half molecules would exceed that of a half molecule interacting with a half fragment Fc, and preferential recombination of half molecules would be expected. Our results, however, indicate that the recombination is essentially random and thus support the view that noncovalent interactions between heavy chains are confined to the region of fragment Fc.

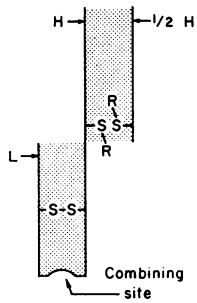


FIG. 1.—Structure of the product obtained by recombination of a half molecule with a half fragment Fc. It is slightly modified from that of Nelson[10] who prepared a similar molecular fragment by limited digestion of γG-globulin with papain. The *H* and *L* (heavy and light) chains comprise a half molecule. The symbol $^1/_2$ *H* designates the C-terminal half of a heavy chain (half fragment Fc).

The product of the recombination of a half molecule and a half fragment Fc is evidently the same as that obtained by Nelson[10] and by Goodman[11] during limited digestion of γG-globulin with papain, in which only one of the two heavy chains of the molecule is cleaved. It has the interesting property of comprising one Fab (univalent) fragment and a complete fragment Fc. A molecular model is shown, for the purpose of illustration, as Figure 1.

*Materials and Methods.*—Nonspecific γG-globulin was prepared from the pooled sera of several rabbits by two precipitations with sodium sulfate[12] and passage through DEAE-cellulose in 0.0175 *M* phosphate buffer, pH 7.0.[13] Other serum proteins were not detectable in the product by immunoelectrophoresis with a multispecific sheep antirabbit serum. A portion was reduced with 0.01 *M* or, in another experiment, 0.015 *M* 2-mercaptoethanol for 75 min at pH 8.2 and room temperature, then alkylated by overnight treatment in the cold with iodoacetamide (0.1 *M*).

Fragment Fc[14] was prepared by digestion of γG-globulin with 1% by weight of papain at 37° in 0.1 *M* phosphate buffer, pH 7.5, containing 0.05 *M* L-cysteine and 0.002 *M* disodium ethylenediamine tetraacetate. After 6 hr, sodium iodoacetate was added to a concentration of 0.1 *M*, the mixture was allowed to stand overnight, then dialyzed against cold water. The presence of 0.05 *M* L-cysteine during digestion has previously been shown to permit subsequent dissociation of fragment Fc into halves at pH 2.4.[7] Crystals of fragment Fc that formed were separated by centrifugation and recrystallized from saline-borate buffer.[7]

The reduced γG-globulin and fragment Fc were labeled with $I^{131}$ and $I^{125}$, respectively, by the method of McFarlane[15] which utilizes ICl; one to three atoms of iodine were incorporated per molecule of protein in the four preparations used. Iodination of fragment Fc was carried out at 40–45° because of its limited solubility at lower temperatures. The iodinated proteins were dialyzed against four 4-liter portions of cold NaCl-borate buffer, pH 8, containing 0.02 *M* KI, and finally against the buffer alone. More than 99% of the radioactivity in each preparation was precipitable by trichloracetic acid, added to a concentration of 5%.

The radioisotopes, $I^{131}$ and $I^{125}$, were measured individually with a well-type gamma scintillation counter and pulse height analyzer. Appropriate, small corrections were made for the contribution of each isotope to the counts recorded at the setting used for the other.

Extinction coefficients, $E^{1\%}_{1\,cm}$, at 280 mμ, utilized for fragment Fc and γG-globulin were 12.2 and 14.5, respectively.

Specific precipitations of labeled proteins were carried out with an excess of sheep anti-Fab or anti-Fc antiserum; each was made specific by exhaustive absorption with the other protein (fragment Fc or Fab, respectively). To ensure complete precipitation of the labeled protein, a second precipitin reaction was carried out in the presence of unlabeled γG-globulin, added as carrier, and a portion of the supernatant of the first precipitation.

*Results.*—The iodinated, reduced γG-globulin and fragment Fc were dialyzed separately against 0.04 *M* NaCl, acidified to pH 2.4 with 1 *N* HCl, and examined in the ultracentrifuge. The sedimentation coefficient, $S_{20,w}$, of the major peak of the reduced γG-globulin was 3.3*S* (Fig. 2*A*), indicating that dissociation into half molecules had occurred.[17] Ten per cent of the protein migrated with a greater

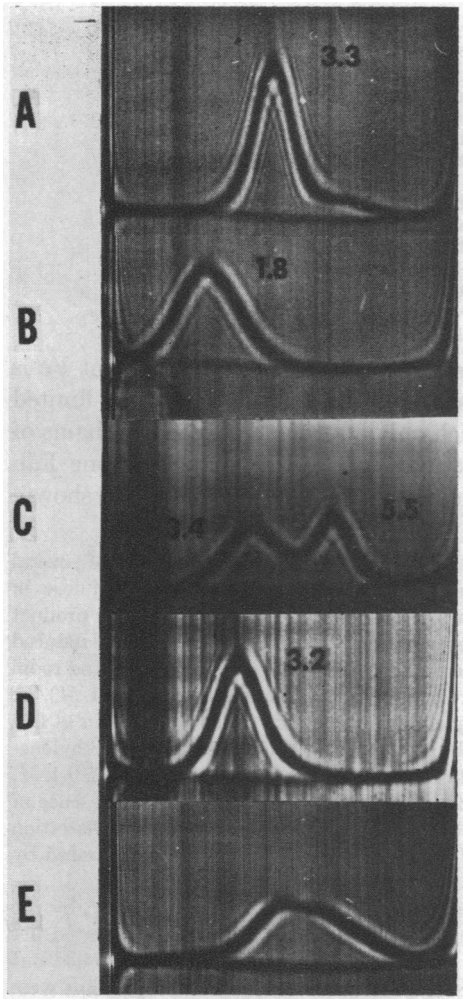


FIG. 2.—Schlieren patterns photographed in the model E ultracentrifuge. (*A*) Reduced γG-globulin-$I^{131}$, pH 2.4; (*B*) fragment Fc-$I^{125}$, pH 2.4; (*C*) γG-globulin of (*A*), after neutralization and adjustment to pH 4; (*D*) fragment Fc of (*B*) after neutralization and adjustment to pH 4; (*E*) mixture of fragment Fc and reduced γG-globulin, prepared in a 1:1 molar ratio at low pH, neutralized, then brought to pH 4. Solvents were 0.05 *M* NaCl adjusted to pH 2.4 with HCl, or 0.1 *M* acetate buffer, pH 4. Photographs were taken after 96 min (*A,B,E*) or 80 min (*C,D*) at 59,780 rpm. Protein concentrations, 6–7 mg/ml.

velocity, corresponding roughly to that of undissociated γG-globulin. The $S_{20,w}$ value of reduced fragment Fc (Fig. 2*B*) at pH 2.4 was 1.8*S*, a value characteristic of half fragments.[7] (Undissociated fragment Fc migrates with a velocity of 2.4*S* to 2.5*S* under these conditions.[7])

Portions of the preparations of half molecules and half fragments were mixed at pH 2.4 in a 1:1 molar ratio (90 and 30 mg, respectively). The mixture was brought to neutrality by successive dialyses in the cold against 0.16 *M* NaCl and 0.1 *M* sodium acetate. At the same time, portions of the dissociated half molecules and of half fragments were brought to neutrality separately in a similar manner for use as controls. Each of the three preparations was then dialyzed against 0.1 *M* acetate buffer, pH 4, and examined in the ultracentrifuge. This relatively low pH was used to minimize aggregation of fragment Fc. The schlieren pattern of the mixture of half molecules and half fragments that had been prepared at pH 2.4, neutralized, and adjusted to pH 4 indicated the presence of two or more components with sedimentation coefficients in the range 3–6*S* (Fig. 2*E*).

Forty-eight per cent of the area in the schlieren pattern of the preparation of recombined half molecules (Fig. 2*C*) comprised protein having a sedimentation coefficient of 5.5*S*, a value close to that of unreduced γG-globulin in this buffer (5.7*S*); that of the remaining, slower-moving protein, presumably consisting of unrecombined half molecules, was 3.4*S*. Owing to the Johnston-Ogston effect, the actual extent of recombination must have been greater than 48 per cent. If the corrections for this effect, appropriate to pH 2.4,[17] are applied, the calculated value is 58 per cent. This correction factor for the Johnston-Ogston effect is probably fairly accurate since the ratio of the sedimentation coefficients of half and whole molecules at pH 2.4[17] is close to that observed at pH 4 (Fig. 2*C*). The sedimentation coefficient of the reconstituted fragment Fc (3.2*S*) was slightly lower than that of un-

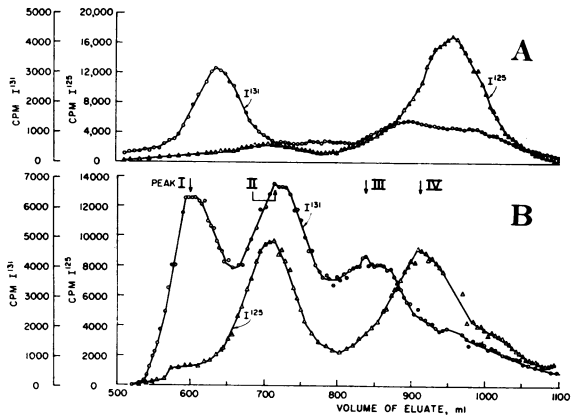


FIG. 3.—(*A*) Gel filtration on a 4 × 125-cm column of Bio-Gel P-200 in pH 4 acetate buffer, of recombined half molecules ($I^{131}$) and recombined half fragments Fc ($I^{125}$). The mixture was prepared in a 1:1 molar ratio after separate reassociation at neutral pH; total weight of protein, 22 mg. (*B*) Gel filtration, as above, of 20 mg of a recombined mixture of half molecules of γG-globulin ($I^{131}$) and half fragments Fc ($I^{125}$), prepared in a 1:1 molar ratio at pH 2.4 and then neutralized.

reduced Fc under the same conditions (3.5*S*). This suggests that some unfolding may have occurred at low pH.

Portions of the preparations of half molecules (16.6 mg) and half fragments (5.5 mg) that had been neutralized separately were mixed at neutral pH. Gel filtration was then carried out on the same column as that used for the recombined mixture of half molecules and half fragments (mixed at low pH and then neutralized).

Figure 3*A* shows the elution profile of the mixture prepared after separate neutralization of the two proteins. A large proportion of the $I^{131}$ label, associated with γG-globulin, was eluted as the first major peak, comprising approximately half of the total $I^{131}$ activity. The remainder of the $I^{131}$ was eluted later (mainly between 800 and 1100 ml). These components evidently correspond to the 5.5*S* and 3.4*S* components observed at pH 4 in the ultracentrifuge (Fig. 2*C*). Nearly all of the $I^{125}$ label, associated with the reconstituted fragment Fc, was eluted as a single, fairly symmetrical peak.

Figure 3*B* represents the elution pattern of radioactivity in the recombined mixture of half fragments and half molecules, prepared at low pH and then neutralized. In contrast with Figure 3*A*, a large proportion of the $I^{125}$ (associated with fragment Fc) was eluted from the column much earlier. In addition, an $I^{131}$ peak, not observed in Figure 3*A*, corresponds in its position to that of the first $I^{125}$ peak. The elution volume of the second $I^{125}$ peak corresponds to that of the major $I^{125}$ component in Figure 3*A* (reconsituted fragment Fc). These results suggest the possibility that the first $I^{125}$ peak (peak II) consists of a recombinant of a half molecule of γG-globulin-$I^{131}$ with a half fragment Fc.

The results of a number of additional experiments carried out with the radioactive protein in peak II of Figure 3*B* support this conclusion. (*a*) The molar ratio of fragment Fc to γG-globulin calculated from the $I^{125}$ and $I^{131}$ activities in peak II is close to unity; the value for a pool of protein from nine tubes at the peak was 1.13. (The molecular weights assumed for this calculation were 150,000 and 50,000, respectively, for γG-globulin and fragment Fc.) (*b*) The position of elution of peak II, intermediate between that of the two major peaks in Figure 3*A*, indicates a molecular weight between 50,000 and 150,000, a result consistent with recombination of a half molecule with a half fragment. (*c*) An antiserum specific for fragment Fab precipitated 97 per cent of the $I^{125}$ label in an aliquot taken from a pool of

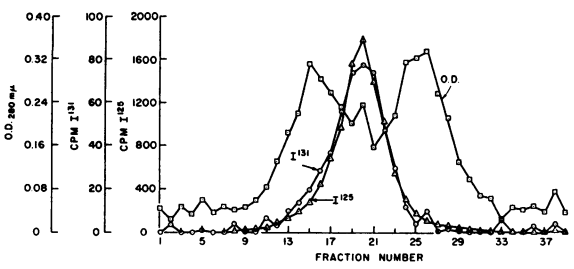


FIG. 4.—Sucrose density gradient centrifugation, for 21 hr at 39,000 rpm, of 0.6 mg of labeled protein taken from a pool of the nine tubes at the maximum of peak II in Fig. 3*B* (see text). Also present were 1 mg of unlabeled γG-globulin and 1 mg of unlabeled fragment Fab of γG-globulin. Radioactivity measurements were made on samples of eight drops each. Optical densities were read after dilution of these samples to 0.5 ml.

nine tubes comprising the maximum of peak II; 98 per cent of the $I^{131}$ was precipitated at the same time. In contrast, only 6 per cent of the $I^{125}$ label in an aliquot of a pool of six tubes at the maximum of peak IV (Fig. 3*B*) was precipitable by the same antiserum. An antiserum specific for fragment Fc precipitated over 95 per cent of the $I^{125}$ (Fc) label of each pool. Unlabeled γG-globulin was added as carrier for the second precipitation in each case (see *Methods*). These results are consistent with association of a half fragment Fc with a half molecule upon neutralization of the mixture prepared at low pH. (*d*) Sedimentation experiments, to be discussed next, provided strong additional evidence for such an association.

The results of density gradient centrifugation in sucrose of 0.6 mg of the radioactive protein, taken from a pool of nine tubes comprising the maximum of peak II in Figure 3*B* and then concentrated, are shown in Figure 4. γG-globulin and fragment Fab (mol wt 45,000), 1 mg of each, were added as markers, which are represented by the two major optical density peaks in Figure 4. The two labels, $I^{125}$ and $I^{131}$, were evidently associated in the same molecular species, since their sedimentation velocities were identical. This experiment also supports the results of gel filtration in indicating that the molecular weight of the hybrid species is intermediate between that of fragment Fab and γG-globulin. Thus, the results of several experiments demonstrate that a large proportion of the half fragments combined with half molecules upon neutralization of a mixture prepared at low pH.

It seemed probable that peak I in Figure 3*B* ($I^{131}$) comprises reconstituted whole molecules, since its position corresponds closely to that of the reconstituted γG-globulin in Figure 3*A*. Confirmation was obtained by density gradient centrifugation. The $I^{131}$-labeled protein taken from a pool of four tubes at the maximum of peak I sedimented at a velocity essentially identical with that of unlabeled γG-globulin added in large excess as a marker.

In another experiment, radioactive protein from the top of peak III was centrifuged in a density gradient in the presence of unlabeled Fab and γG-globulin. The rate of sedimentation of the $I^{131}$-label was slightly greater than that of fragment Fab and less than that of γG-globulin, a result consistent with the premise that the protein in this peak consists of half molecules that had failed to recombine.

A second recombination of half fragments with half molecules was carried out in an exactly analogous manner, with the exception that fragment Fc and γG-globulin were mixed, at low pH, in a 2:1 rather than a 1:1 molar ratio. Also, in this experiment, the γG-globulin was reduced with 0.015 *M* 2-mercaptoethanol, instead of 0.01 *M*, and dissociation into half molecules at low pH was essentially complete.

Results of gel filtration of the recombined mixture are shown in Figure 5. Again,

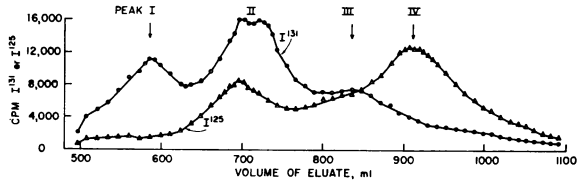


FIG. 5.—Gel filtration of 22 mg of a recombined mixture of half fragments Fc ($I^{125}$) and half molecules of γG-globulin ($I^{131}$), prepared in a 2:1 molar ratio at pH 2.4 and then neutralized. Conditions as in Fig. 3.

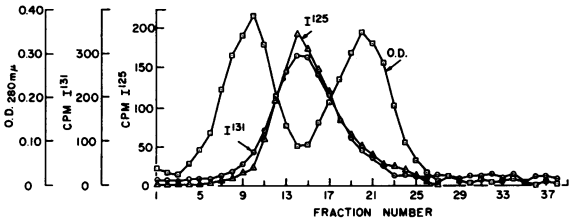


FIG. 6.—Sucrose density gradient centrifugation at pH 4, for 30 hr at 39,000 rpm, of 0.017 mg of labeled protein taken from a pool of the nine tubes comprising the maximum of peak II in Fig. 5. Unlabeled γG-globulin (1.1 mg) and fragment Fc (1.2 mg) were added as markers.

part of the $I^{125}$-labeled fragment Fc was eluted early (peak II) in a position corresponding to an $I^{131}$ peak. The calculated molar ratio of fragment Fc to γG-globulin in a pool made up of an aliquot of the nine tubes comprising the maximum of the peak was 1.11. The fraction of $I^{125}$ label in peak II (assuming that the leading edge represents part of a symmetrical peak) was 28 per cent of the total eluted from the column. For complete and random recombination of half fragments with half molecules the calculated value is $33^1/_3$ per cent.

Density gradient centrifugation (Fig. 6) also indicated that the labels were associated in the same molecules. The sedimentation rate was intermediate between that of the unlabeled γG-globulin and fragment Fc, added as markers. Since unlabeled fragment Fc, rather than Fab as in Figure 4, was used as a marker, centrifugation was carried out at pH 4 to minimize aggregation.

*Discussion.*—Our previous work has demonstrated that reduced γG-globulin and fragment Fc dissociate at low pH into half molecules or half fragments, respectively, and that the dissociation is reversed at neutral pH.[1, 2, 7] The present data indicate that a half molecule is capable of association with a half fragment Fc to form a molecular species with a weight intermediate between those of the two parent molecules. The product apparently corresponds to that obtained, in relatively low yield, during a very limited digestion of γG-globulin by papain, in which only one of the two heavy chains of the molecule is cleaved.[10, 11] An alternative method is therefore provided for the preparation of this molecule which, if derived from antibody, should be univalent and contain a complete fragment Fc.

Evidence for the association includes the elution of the two differently labeled proteins in the same peak during gel filtration (Figs. 3*B* and 5); the identical rates of sedimentation in a density gradient of the two labels taken from this peak (Figs. 4 and 6) and the precipitability of the $I^{125}$ label, originally associated with fragment Fc, by an antiserum specific for fragment Fab. Measurements of the radioactivity of the two labels in the mixed recombinant provided evidence that recombination occurs in approximately a 1:1 molar ratio. The results of gel filtration and density gradient ultracentrifugation indicate that the molecular weight of the recombinant lies between 50,000 and 150,000. Its sedimentation velocity, in density gradient ultracentrifugation (Figs. 4 and 6), was about midway between that of fragment Fab or fragment Fc and γG-globulin.

Whether the association is entirely random was a question of particular interest, since random recombination would provide evidence that the noncovalent interactions between half molecules are largely or completely confined to the region of the heavy chain comprising fragment Fc (see the introduction). The data are not adequate to permit the conclusion that recombination is entirely random. Incomplete recombination, especially of half molecules (Fig. 2), complicates attempts to quantify the results. Nevertheless, it is clear that there is no strong bias favoring the preferential recombination of two half molecules. Measurements of $I^{125}$, used to label fragment Fc, after gel filtration of the recombined mixture are perhaps the most reliable indicator of the extent of hybridization since half fragments tended to reassociate more completely in these experiments than half molecules. In the experiments of Figure 3*B*, nearly all of the $I^{125}$ label was found in two major peaks (II and IV) corresponding to the mixed recombinant and to recombined half fragments Fc, respectively. If recombination were complete and random, 50 per cent of the $I^{125}$ label would have been present in mixed molecules, since a 1:1 molar ratio of $\gamma$G-globulin and fragment Fc was employed. The observed value, estimated from the area of peak II in Figure 3 and the total area corresponding to the $I^{125}$ label, was 38 per cent. As indicated, incomplete recombination would influence this result; in addition, only 90 per cent of the $\gamma$G-globulin dissociated in this experiment into half molecules at pH 2.4 (Fig. 2). The results show that there is no strong bias toward preferential recombination of two half molecules and suggest that it is random.

In the second recombination experiment the molar ratio of $\gamma$G-globulin to fragment Fc, in the mixture prepared at pH 2.4, was 1:2. For random, complete association, $33^1/_3$ per cent of the $I^{125}$ label should have appeared in the peak representing the recombinant; approximately one fourth of the $I^{125}$ was actually present. In this experiment, as well as in the first, the molar ratio of the two labeled proteins in the recombinant was approximately 1:1. The results of density gradient centrifugation (Fig. 6) were similar to those of the first experiment.

As indicated in the introductory section, the separation of two fragments Fab′, after reduction of pepsin-digested $\gamma$G-globulin under nondissociating conditions, suggests but does not prove that the two fragments are linked only by the disulfide bond and not by noncovalent interactions in the native molecule. The present experiments provide strong support for this view.

Several lines of evidence,[14, 8, 16] discussed by Noelken *et al.*,[16] have indicated that the three major fragments of a papain digest are compact subunits which are joined in the $\gamma$G-globulin molecules by loosely folded polypeptide strands. However, the existence of an interchain disulfide bond in the $(Fab')_2$ product of peptic digestion shows that the Fab′ fragments are in close physical contact in at least one region. The present results, and the lability of this disulfide bond to reduction even prior to peptic digestion,[17, 18] indicate that the bond is not stabilized by significant noncovalent interactions. The data are consistent with the view[19] that this disulfide bond may act as a "hinge point" about which the univalent fragments can rotate. The flexibility of the molecule has been established by electron microscopy.[19–21]

*Summary.*—Half molecules of rabbit $\gamma$G-globulin, prepared by acidification of the reduced protein, recombine with half fragments Fc to form molecules of approximate molecular weight 100,000. comprising one Fab (univalent) and one Fc frag-

ment. There is little or no bias toward preferential recombination of two half molecules. This indicates that the noncovalent interactions between heavy chains are localized in the fragment Fc region of the molecule.

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