

Peptic Fragmentation of Rabbit γ G-Immunoglobulin*

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ABSTRACT: Rabbit γ G-immunoglobulin has been subjected to peptic digestion at pH 4.5 and the resulting fragments separated into four major populations by gel filtration through Sephadex G-75. The largest fragment, Pep-I, was shown to be antigenically identical to papain fragment I (II) but different from it with respect to molecular weight (92,000/2 for Pep-I versus 40,700) and carbohydrate content. Since the carbohydrate moiety of Pep-I was very similar to the glycopeptide resulting from papain digestion, it could be inferred that its point of attachment to the heavy chain is near the C terminus of papain fragment I (II). The other three peptic fragments constitute portions of papain fragment III. One of them, Pep-III', has a molecular weight of 27,000 compared to 50,000 for papain fragment III, and possesses some but not all of the antigenic determinants of the latter. Pep-III' also differs from the papain fragment in its lack of carbohydrate and the absence of interchain disulfides. The third fragment,

Pep-IV, was recognized only by the ninhydrin reaction. It carries a second carbohydrate moiety which contains all of the fucose of γ G-immunoglobulin and 70% each of the hexose and hexosamine. This carbohydrate appears to be identical with that contained in papain fragment III. Pep-IV is evidently immunogenically inactive since it showed no reaction with antiserum against the heavy chain. The final group of fragments, Pep-V, was characterized by its small size, probably not exceeding 5000 in molecular weight, and by its antigenic activity. At least 50% of the antibody specific for the heavy chain was capable of reaction with this fragment.

A tentative disposition of the peptic fragments within the γ G-globulin molecule has been proposed. A model has also been formulated involving two asymmetric interheavy-chain disulfide bonds to account for the apparently divergent evidence regarding the number of such bonds.

The proteolytic breakdown of immunoglobulins into macromolecular fragments has provided an important avenue for the study of the relation between the functional properties of these proteins and the chemical basis of these characteristics. The original demonstration by Porter (1959) that papain digestion of γ G-globulin gave rise to two kinds of fragments led to the association of immunologic activity with one kind of fragment (I, II) while the other kind of fragment (III) was related to many of the species-specific and invariant functional properties of the original protein, such as complement fixation, placental transmission, skin fixation, and Gm allotyping (in humans). It was later shown (Nisonoff *et al.*, 1960b) that peptic digestion at pH 4.5–5 followed by mild reduction also produced a fragment with antibody activity similar to papain fragment I (II) (Mandy *et al.*, 1961) and antigenically identical with it (Goodman and Gross, 1963). However, a fragment similar to papain fragment III did not survive the pepsin treatment. The further breakdown of fragment III by pepsin has been

investigated by Goodman (1963), who demonstrated that such treatment produces several smaller, antigenically active fragments, some of which are dialyzable (see Marrack *et al.*, 1961).

In this report we present the results of a detailed study of four groups of peptic fragments derived from the electrophoretically slow-moving fraction of rabbit γ G-immunoglobulin. These groups of fragments have been separated and compared to those obtained by papain treatment with respect to molecular weight, antigenic specificity, carbohydrate content, and N-terminal residues. Two of these four groups are constituted of dialyzable fragments and within one of them are molecules which have been shown to possess some of the antigenic specificity of the heavy chain. In addition the existence of two carbohydrate moieties has been demonstrated and their localization on the heavy chain has been clarified.

Materials and Methods

Preparation of Rabbit γ G-Immunoglobulin. Normal rabbit γ G-globulin was prepared from pooled rabbit serum by precipitation with 33%-saturated ammonium sulfate at room temperature followed by reprecipitation three times. This product was further purified by chromatography on DEAE-cellulose (Sigma Chemical Co.). A sample of 250 mg was loaded on a 2.4 × 30-cm column and eluted with 0.02 M sodium phosphate, pH 7.2, at room temperature. The first major peak was

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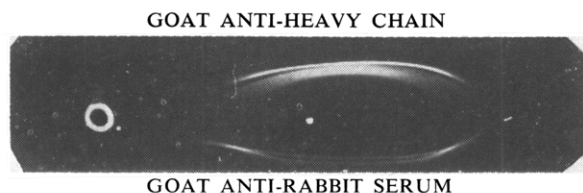


FIGURE 1: Immunelectrophoresis of slow-moving γ G-immunoglobulin. Upper well contained goat anti-heavy-chain serum and lower well goat antiserum to rabbit serum.

separated from a slower component, which appeared as a shoulder, and constituted 30% of the total protein sample. It was entirely a slow-moving γ G-globulin as shown by immunelectrophoresis (Figure 1). Only one component (6.5 S) was seen when the sample was examined in the analytical ultracentrifuge. When antiserum to the heavy chain of γ G-globulin was used for the development of the immunelectrophoretic pattern, the precipitin band was clearly a doublet (Figure 1). This observation is similar to that previously described for the heavy chains of antihapten antibody (Karush and Utsumi, 1965) and serves to demonstrate the antigenic heterogeneity of γ G-globulin with respect to its heavy chains. The two antigenic components observed with rabbit γ G-globulin probably are analogous to the γ Ga- and γ Gb-globulins described in the horse (Rockey *et al.*, 1964) and to the γ G subclasses in the human (Dray, 1960) and in the mouse (Fahey *et al.*, 1964). No other antigenic component was found in our preparations of rabbit γ G-globulin.

Preparation of Anti-Lac Antibody. Rabbit antibody to *p*-azophenyl- β -lactoside (anti-Lac)¹ was purified from pooled antiserum as described previously (Utsumi and Karush, 1964). The preparation also contained only a 6.5 S γ G-globulin.

Antisera. Goat anti-fragment I (papain) was generously provided by Dr. Mel Cohn, and sheep anti-rabbit γ -globulin (RGG) and antirabbit serum were obtained from Baltimore Biological Laboratory. Goat anti-Pep-I (4.5 S protein obtained from peptic digest of γ G), goat anti-heavy chain and goat anti-light chain antisera were prepared in this laboratory as previously described (Utsumi and Karush, 1964). Rabbit allotypic antisera were kindly supplied by Dr. S. Dray of the National Institutes of Health.

Papain Fragments of γ G-Globulin. γ G-Globulin was digested by papain (13 μ /mg, Worthington Biochemical Corp.) at a ratio of substrate to enzyme of 100:1 (w/w) in the presence of 0.01 M cysteine, 0.002 M EDTA, 0.2 M sodium acetate buffer, pH 5.5–5.7, for 20 hours at 37° (Putnam *et al.*, 1962). The fractionation on a CM-cellulose column was carried out according to Porter (1959). The yield in the second peak was gen-

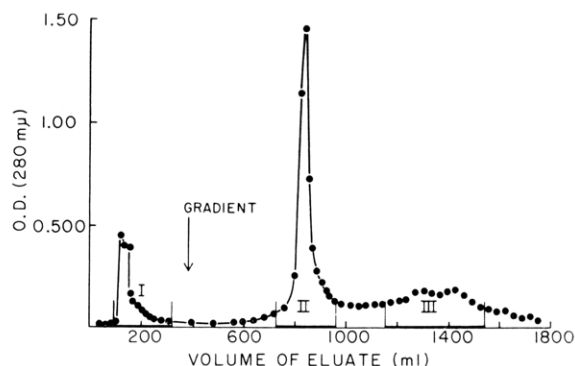


FIGURE 2: Chromatography on CM-cellulose of papain-digested anti-Lac in acetate buffer, pH 5.5. Acetate gradient from 0.01 to 0.90 M.

erally much higher than that in the first peak when chromatographically purified γ G-globulin was used. This was also the case for anti-Lac antibody (Figure 2). Since fragment II was rarely obtained free from contamination by fragment III or fragment III-like substance, further purification of this fraction was carried out by chromatography on a DEAE-cellulose column as described.

DEAE-Cellulose Chromatography of Fragment II. Fragment II obtained from CM-cellulose chromatography of a papain digest of γ G-globulin was dialyzed against 0.01 M sodium phosphate, pH 7.9. The sample (75 mg) was placed on a DEAE-cellulose column (1.2 \times 50 cm) and eluted with 0.01 M phosphate, pH 7.9, to give a yield of almost 90% in the first fraction (Fr I of Figure 4b). A linear concentration gradient of sodium chloride, up to 0.4 M in 800 ml with constant phosphate concentration and pH (Figure 4b), was then applied. Another few per cent of the sample was recovered in the second fraction (Fr II) and both Fr I and Fr II were found to contain only fragment II. A third fraction (Fr III), also comprising only a few per cent, constituted the impurity present in the original material.

Reduction and Alkylation of γ G-Globulin. The protein was generally reduced at a concentration of 1–2% by treatment with 0.14 M β -mercaptoethanol in Tris-HCl buffer and 0.005 M EDTA, pH 8.0, for 1 hour. For alkylation iodoacetic acid or iodoacetamide was used, at 10% molar excess of the mercaptoethanol, in the presence of an appropriate concentration of Tris (about 0.5 M) and 0.005 M EDTA in the pH range of 8.3–8.0 for 30 minutes. The entire reaction was carried out in an atmosphere of nitrogen in a closed vessel with two side arms from which the alkylating agent and Tris were introduced into the reduction mixture in the main chamber. The reaction mixture was then dialyzed to remove the excess reagents.

Preparation of Subunits of γ G-Globulin. The heavy and light chains from γ G-globulin and anti-Lac antibody were prepared by gel filtration of the reduced and

¹ Abbreviations used in this work: anti-Lac, rabbit antibody to *p*-azophenyl- β -lactoside; RGG, rabbit γ -globulin; SDeS, sodium decyl sulfate; MRA, mildly reduced and alkylated.

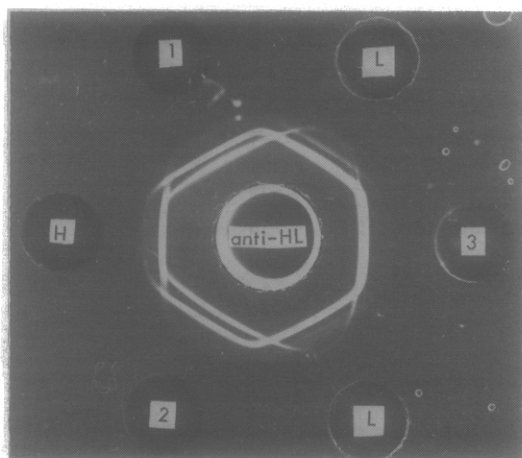


FIGURE 3: Gel-diffusion pattern of pepsin-digested γ G-globulin. Anti-HL, a mixture of goat antirabbit heavy-chain and goat antirabbit light-chain antisera; H, heavy chain; L, light chain; 1, supernatant of pepsin-digested γ G-globulin; 2, precipitate from digestion mixture; 3, digest fractionated with sodium sulfate.

alkylated proteins on Sephadex G-200 in the presence of 0.04 M sodium decyl sulfate (SDeS) in 0.01 M sodium phosphate buffer, pH 7.5, and 0.005 M EDTA as described previously (Utsumi and Karush, 1964).

Analytical Methods. Protein concentrations were determined by the measurement of optical density at 280 and 250 $m\mu$ in a Zeiss spectrophotometer. Extinction coefficients used were 14 ($E_{280}^{1\%}$) for intact rabbit γ G-globulin in neutral buffer, 14 for fragment I, and 10 for fragment III (Porter, 1959), 14.5 for the heavy chain in SDeS at neutral pH, and 13.2 for the light chain in SDeS (Utsumi and Karush, 1964).

The determination of the N-terminal amino acids was done by Sanger's dinitrophenylation method according to Porter (1957). Hydrolysis of DNP-proteins was carried out in a vacuum-sealed test tube for 12 hours at 110° and the hydrolysates were analyzed by paper chromatography with *tert*-amyl alcohol phthalate, pH 6. Unidentified DNP-amino acids were eluted in 0.1 N HCl and rechromatographed with 1.5 M sodium phosphate, pH 6. Only ether-soluble DNP-amino acids were examined.

Hexose content was determined according to Momose and Inaba (1959) and Momose *et al.* (1960). The protein sample was first heated at 100° for 16 hours in 0.5 N HCl and, after removal of HCl by flash evaporation, passed through a column of Dowex 50 (H^+) (Fleischman *et al.*, 1963). The recovery of a standard sugar from the hydrolysis was 92% and from the Dowex column 100%.

Hexosamine was estimated by a modification of the Elson-Morgan method as described by Belcher *et al.* (1954). Hydrolysis was carried out in 4 N HCl at 100° for 6 hours according to Fleischman *et al.* (1963), and the reaction mixture was treated with Dowex 50 (H^+) as described by Boas (1953). A recovery correction was not

made for the hexosamine not released from the protein by hydrolysis. A recovery of 81% of a control (glucosamine) was found with the Dowex column when eluted with 2 N HCl.

Fucose content was determined by the cysteine-sulfuric acid method described by Dische and Shettles (1948) using L-fucose as a standard. The optical densities at 396 and 430 $m\mu$ varied linearly with the amount of the protein sample, and both were found to extrapolate to zero.

The ninhydrin analysis of protein and peptide was carried out in 0.11 M citrate buffer, pH 5, according to Kabat (1961). When the sample contained 1 N acetic acid, 0.1 ml of 1 N NaOH was added to 0.2 ml of the sample to bring the pH to the proper value.

Measurement of Molecular Weight. Molecular weights were determined by the method of sedimentation equilibrium in a short column (1.7 mm) as described by Van Holde and Baldwin (1958) and analyzed for homogeneity according to these authors. A Spinco Model E ultracentrifuge with a filled-Epon, double-sector cell was used. The initial refractive index was determined in a filled-Epon, double-sector, synthetic-boundary cell at 5227 rpm.

Peptic Digestion of γ G-Globulin. The protein was hydrolyzed by pepsin to obtain the 5 S fragment described by Nisonoff *et al.* (1960b). The sample, at a concentration of 1–2%, was incubated with pepsin (twice crystallized, Worthington Biochemical Corp.) at a protein to enzyme ratio between 100:1 and 100:2 in 0.2 M acetate buffer, pH 4.5, at 37°. The incubation time was varied between 4 hours and 96 hours and no change in the pH was noted during the incubation. The turbidity which appeared on mixing pepsin with γ G-globulin persisted during the digestion period.

Results

Antigenic Properties of Peptic Digest of γ G-Globulin. The precipitate obtained after 20 hours of digestion was separated from the supernatant fluid by centrifugation before neutralization of the digestion mixture and dissolved in 0.5 M phosphate buffer, pH 8.6, or dissolved by adjusting the pH of the suspension to 11 and subsequent neutralization. The amount of protein in the precipitate was 2–4% of the total protein. This was examined with anti-heavy chain and anti-light chain sera by the immunodiffusion technique and compared with the supernatant fraction. The supernatant phase and precipitate showed identical pairs of distinct precipitin lines in the Ouchterlony plate (Figure 3). This observation was independent of the amount of the precipitate formed during digestion. Therefore, subsequent fractionation of the digest was done after dissolution of the precipitate by adjusting the pH to 8.5.

Of the two precipitin lines formed by the peptic digest, the outside line, nearest the antigen well, fused with the precipitin line of the light chain, while the inside line cross-reacted with the heavy chain, the latter forming a spur. From the stoichiometric recovery of the 5 S protein and the antigenic relationship between the

subunits and the fragments of papain-digested rabbit γ G-globulin (Utsumi and Karush, 1964), it appears that peptic fragmentation also splits γ G-globulin into two major, antigenically distinct fragments corresponding to papain fragment I (II) and fragment III. Since papain fragment III, however, showed no antigenic deficiency compared with the heavy chain (Utsumi and Karush, 1964), the spur formation observed here suggested the presence of a fragment smaller than papain fragment III.

The precipitation by sodium sulfate of the peptic digest of γ G-globulin at 18% (w/v) was carried out as described by Mandy *et al.* (1961). The dissolved protein was precipitated a second time, dissolved in a few ml of buffer, and dialyzed against three 4-liter portions of phosphate buffer, pH 8.0, for 3 days in the cold. After removal of a small amount of precipitate the recovery was about 50%. The material thus obtained also showed both components in the immunodiffusion test (Figure 3) as observed in the original whole digest, although the amount of the component cross-reacting with the heavy chain was somewhat lower.

Chromatography of Pepsin- and Papain-digested γ G-Globulin on DEAE-Cellulose Column. Gel filtration of a sample from 20-hour peptic digestion with Sephadex G-25 in neutral buffer yielded 80% of the protein in the void volume fraction and 20% in the following several peaks. The first peak again contained two antigenic components, as found with the whole digest. No other fraction showed precipitation with any antiserum. The protein in the first peak fraction was reduced and the fraction was subjected to chromatography on DEAE-cellulose in the same manner already described for the purification of papain fragment II. Since Nisonoff *et al.* (1960a) had found that the 5 S protein could be split into 3 S components when it was chromatographed on CM-cellulose, probably owing to the presence of reducing groups in the cellulose, the material from the first peak was pretreated with 0.014 M β -mercaptoethanol at pH 7.7 for 1 hour to avoid complications. The reduced protein migrated as a single peak of 3.2 S in the ultracentrifuge after removal of reducing agent by dialysis. The material showed little recombination after standing in the cold for several days. The chromatography of this material in a DEAE-cellulose column yielded three peaks as shown in Figure 4a. The total recovery of protein was greater than 95% and the fractions I, II, and III contained 35, 40, and 25%, respectively, of the recovered protein. The first (Fr I) and second (Fr II) fractions reacted only with the anti-light chain serum and the third fraction (Fr III) contained the fragment which cross-reacted with the heavy chain. Both the heavy chain and papain fragment III formed a spur relative to Fr III. These two antigenically distinct types of fragments of the peptic digest, Fr I and II and Fr III, are designated Pep-I and Pep-III', respectively. Chromatography of a similar peptic preparation which had been reduced by 0.14 M mercaptoethanol and alkylated with iodoacetamide showed an almost identical pattern.

Chromatographic patterns of papain fragment II

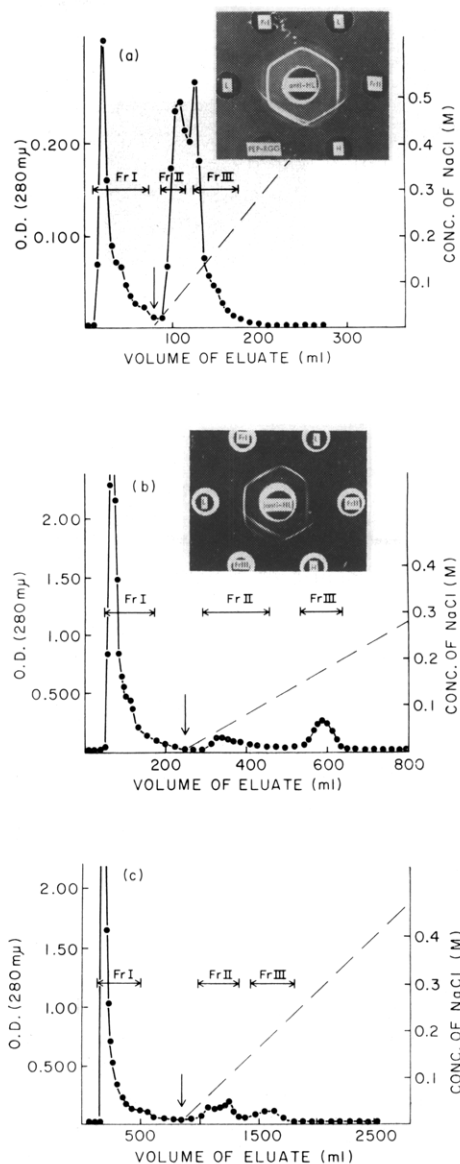


FIGURE 4: Chromatography of pepsin-digested γ G-globulin and papain fragments. (a) Chromatography of pepsin-digested γ G-globulin on a DEAE-cellulose column after treatment with Sephadex G-25 and reduction (see text). The protein sample (10 mg) was in 0.01 M sodium phosphate, pH 8.0, and the column was 1×24 cm. Concentration gradient of NaCl in same buffer started at arrow. Insert: Gel-diffusion pattern of pepsin-digested γ G-globulin (PEP-RGG) and Fr I and Fr II derived from it with anti-HL serum (see Figure 3). (b) Chromatography of papain fragment II (75 mg) on a DEAE-cellulose column (1.2×50 cm). Other conditions were the same as in (a). Insert: Gel-diffusion pattern of chromatographic fractions with anti-HL serum (see Figure 3). (c) Chromatography of papain-digested γ G-globulin (190 mg) after removal of the crystallized fragment III on a DEAE-cellulose column (2×36 cm). Other conditions were the same as in (a).

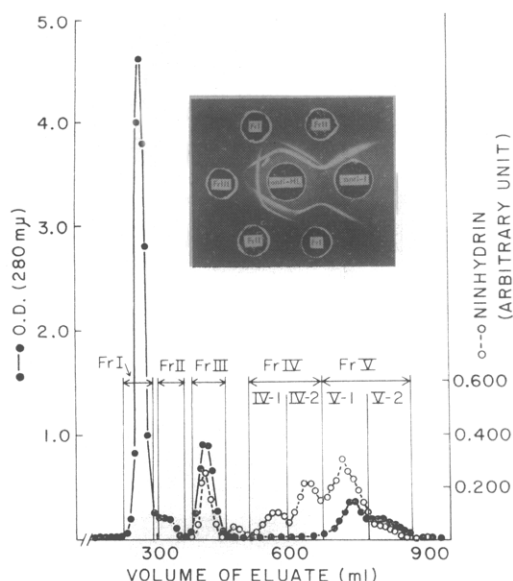


FIGURE 5: Gel filtration of peptic digest of γ G-globulin with Sephadex G-75. A sample (150 mg) of a 20-hour digest was passed through two 3- \times 54-cm columns in tandem at room temperature. Solvent was 0.2 M NaCl-0.01 M sodium phosphate buffer, pH 8.2, and 0.002 M EDTA. Insert: Gel-diffusion pattern of gel-filtration fractions with anti-HL serum (see Figure 3). Anti-I is a goat antiserum to papain fragment I absorbed with a light-chain preparation.

and papain-digested γ G-globulin are shown in Figure 4b,c. In Figure 4b, both Fr I and Fr II reacted only with anti-light chain serum, indicating pure fragment II, while Fr III exhibited antigenic deficiency with respect to the heavy chain. It was further found, as expected, that Fr III also was antigenically deficient when compared with papain fragment III. It is clear therefore that Fr III constituted a contaminant in the original preparation of papain fragment II. The recoveries of Fr I, Fr II, and Fr III were 86, 11, and 3%, respectively. The recovery of Fr III varied between 2 and 6% from one preparation to another.

Papain-digested γ G-globulin was dialyzed against 0.01 M sodium phosphate buffer, pH 7.9, in the cold and more than 80% of fragment III crystallized. The chromatography of the preparation after removal of the crystalline material also gave rise to three peaks with recoveries of 80, 16, and 4% for Fr I, Fr II, and Fr III, respectively, as shown in Figure 4c. Fr I largely comprised fragment I (II) but was contaminated with a small amount of fragment III. Fr II was a mixture of fragment III and fragment I (II). Fr III contained a component identical with Fr III obtained from chromatography of fragment II (Figure 4b), and likewise showed cross-reaction with the heavy chain and papain fragment III. This antigenically deficient fragment derived from papain digestion was designated Pap-III'.

Fractionation of Peptic Digest of γ G-Globulin on

Sephadex G-75. The most convenient method for the fractionation of the peptic digest of γ G-globulin was provided by gel filtration through Sephadex G-75. As shown in Figure 5, fragments of γ G-globulin derived from peptic digestion were separated according to their sizes. The first fraction, Fr I, including occasionally a shoulder in the faster-eluting position, contained only Pep-I with a recovery of $60 \pm 2\%$ of the total protein. The second fraction, Fr II, in the same elution position as pepsin, contained two types of antigens corresponding to papain fragments I and III and was mainly 3 S when examined in the ultracentrifuge. The recovery of this fraction was 2% in Figure 5 but varied considerably depending on the digestion period, as discussed later. The third fraction, Fr III, contained the Pep-III' fragment and migrated as a single homogeneous peak of 2.4 S in the ultracentrifuge. This fragment was crystallizable at 4° in dilute neutral buffer even at a protein concentration of 0.5%. The shape of the crystal was quadrilateral and closely resembled that of papain fragment III. A similar fragment has been reported by Goodman (1963) as a product of the peptic digestion of papain fragment III.

Following a 4-hour period of pepsin digestion, the distribution of the optical density among the fractions from Sephadex G-75 filtration was Fr I, 63; Fr II, 22.5; Fr III, 6; and Fr V, 8%. The increased amount of Fr II, compared with 2% for 20 hours' digestion, comprised mostly the fragment III-type antigen and exhibited antigenic identity with papain fragment III. In the ultracentrifuge this component migrated as 3.3 S. Fr I from the same digestion period (4 hours) was contaminated with a small amount of native material which was separated from the 3 S Pep-I, after reduction of the mixture, by passage through Sephadex G-200. A digestion period of 8 hours yielded a recovery of 4-5% in Fr II. With 18-24 hours' digestion, the recovery of Fr II reached its minimum, about 2%, and maximum recoveries of Fr III and Fr V were obtained. The average recovery of optical density in each fraction was 60.5, 2, 16, and 22%, respectively. Prolonged digestion of γ G-globulin by pepsin up to 96 hours again gave rise to a slight increase of Fr II, 5%. In the agar-diffusion plate (Figure 5), where fractions obtained from 20 hours' digestion were used, Fr II contained two types of antigens, one of which formed a spur relative to Pep-III' (Fr III) and another which was antigenically deficient compared with Pep-I (Fr I) but which nevertheless reacted well with anti-papain fragment I absorbed with the light chain. The structural significance of the latter antigen and the slight increase of Fr II on prolonged digestion are not readily explained. However such a further degradation product, if any, was a minor constituent.

The results indicate that pepsin initially split γ G-globulin into two major fragments, Pep-I (5 S) and a fragment which was antigenically indistinguishable from papain fragment III and which migrated with a sedimentation coefficient of 3.3 S in the ultracentrifuge. The fragment similar to papain fragment III was designated as Pep-III (Fr II). Upon the further action of pepsin,

Pep-III was degraded further into the Pep-III' fragment and the smaller peptides (Fr IV, V). Fragments Pep-I and Pep-III' did not seem to be broken down further by prolonged incubation with pepsin at pH 4.5.

Further experiments were carried out to determine if mild, prior reduction of γ G-globulin would alter the subsequent pattern of peptic fragmentation (see Palmer and Nisonoff, 1963). The protein was treated with 0.2 M β -mercaptoethanol with the cleavage of six disulfide bonds and then alkylated with iodoacetic acid. The reduced and alkylated γ G-globulin was digested with pepsin after removal of the former reagents by dialysis. Subsequent fractionation of the digest on the Sephadex G-75 column showed a shift of the position of Pep-I from Fr I to Fr II and the same position for Pep-III' (Figures 5, 6). Since the recoveries of Pep-I, consisting of Fr I plus the major peak Fr II, and Pep-III' were 63–64% and 16–17%, respectively, there was no evidence indicating additional proteolytic cleavage of the molecule or further liberation of smaller fragments associated with the original reduction.

Molecular Weight of the Fragments. When the fragment Pep-I, obtained by the Sephadex G-75 gel filtration of pepsin-digested γ G-globulin, was reduced and alkylated as described for whole γ G-globulin, the resulting product, designated MRA (mildly reduced and alkylated)-Pep-I, showed a single symmetrical peak of $s_{20,w} = 3.4$ S in the ultracentrifuge with small concentration dependence while the untreated Pep-I was 4.5 S at a protein concentration of 0.5%. The MRA-Pep-I, however, gave rise to a small amount of aggregate which was seen as a faster-moving peak with a Sephadex G-75 column. On the other hand no smaller fragment or peptide was liberated. After the aggregated material was removed by gel filtration, the molecular weight of the 3 S component was measured by sedimentation equilibrium in a short column of 0.17 cm height in a 12-mm double-sector cell at 11,272 rpm. The average molecular weight obtained in the range of protein concentration between 0.3 and 0.5% was $M_z = 81,500$ and $M_w = 55,000$. The latter value is in good agreement with that calculated from the sedimentation velocity and diffusion coefficient by Nisonoff *et al.* (1960a). When the data obtained from the sedimentation equilibrium runs were analyzed by plotting z/r versus $\int_a^r z dr$ an upward curvature of the points was noted. This indicated the presence of aggregate which probably had been formed after the treatment with Sephadex. The wide variation of the values obtained from different preparations also indicated the possible overestimation of the molecular weight of the fragment owing to contamination by variable amounts of the aggregate.

In view of these difficulties a more reliable value was sought by analysis of the unreduced fragment (Pep-I). Gel filtration, instead of prolonged dialysis, was used for equilibration of the protein with the solvent. A freshly prepared peptic digest of γ G-globulin at a protein concentration of 4% was passed through three 3×60 -cm columns of Sephadex G-75 connected in tandem. The columns had previously been equilibrated

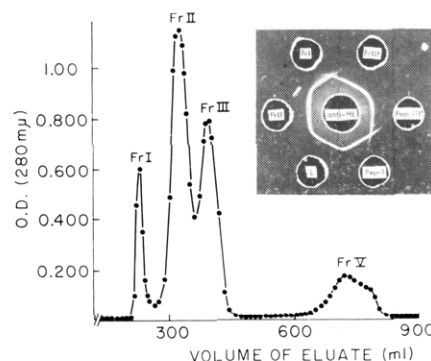


FIGURE 6: Gel filtration of peptic digest of MRA- γ G-globulin with Sephadex G-75 in 0.2 M NaCl–0.01 M sodium phosphate, pH 8.2, and 0.002 M EDTA. Insert: Gel-diffusion pattern of gel-filtration fractions with anti-HL serum (see Figure 3).

with the solvent, containing 0.2 M sodium chloride–0.01 M sodium phosphate buffer, pH 7.8. A fraction was then obtained at a protein concentration of 4 mg/ml, free from both aggregates and small fragments, which was directly subjected to the equilibrium run. The equilibrium was established in a short column of 2.2 mm in a double-sector cell at 10,589 rpm. The determination of the refractive increment of the solution was carried out in a 12-mm double-sector synthetic-boundary cell using 0.10 ml of the protein solution at 5227 rpm. The partial specific volume, \bar{v} , was determined in a 5-ml pycnometer using several preparations of the fragment Pep-I in the same solvent. A value of 0.728 ± 0.003 was obtained. The resulting apparent molecular weight was $M_z = 91,300$ and $M_w = 91,900$, using $\bar{v} = 0.730$. The graph of z/r versus $\int_a^r z dr$, plotted in Figure 7, was a straight line throughout the column and indicated little or no aggregate present in the solution. This result was not corrected by extrapolation of the protein concentration to zero although the concentration dependence of the fragment seems to be small. It appears, therefore, in view of the molecular weight (40,700) of papain fragment I of γ G-globulin obtained by Pain (1963), that there is a difference of about 5000 in the molecular weights between papain fragment I and the corresponding peptic fragment.

In contrast, the second fragment derived from peptic digestion of γ G-globulin, Pep-III', was found to be considerably smaller than papain fragment III, based on a minimum molecular weight of 48,000 (Marler *et al.*, 1964; cf. Pain, 1963). The molecular weight of Pep-III' was determined as before in a 1.7-mm column at 19,160 rpm. The apparent molecular weight, $M_z = 28,700$ and $M_w = 27,500$, was evaluated using $\bar{v} = 0.725$, and the graph of z/r versus $\int_a^r z dr$ indicated a small amount of aggregate.

TABLE I: The N-Terminal Amino Acids of γ G-Globulin Fragments.^a

DNP-Amino Acid	Moles of N-terminal Residues per Mole Protein				
	Intact γ G (150,000 g)	Pep-I (92,000 g)	Pep-I from MRA ^b (92,000 g)	Pep-III' (27,000 g)	Pap-III (60,000 g)
DNP-aspartic acid	0.4	0.38	0.36	0	0.07
DNP-glutamic acid	Trace ^c	Trace	Trace	0	0.07
DNP-serine	0.1	0.13	0.08	0.15	0.3
DNP-glycine	Trace	Trace	Trace	0	Trace
DNP-threonine	0.1	0.06	0.10	0	0.07
DNP-alanine	0.95	0.82	1.1	1.55	0.3
DNP-valine	Trace	Trace	Trace	0.10	Trace

^a The assumed molecular weights are shown in parentheses. Results include only ether fraction of DNP-amino acids.

^b Pep-I (3 S) obtained from MRA- γ G-globulin; see text. ^c Less than 0.05 mole.

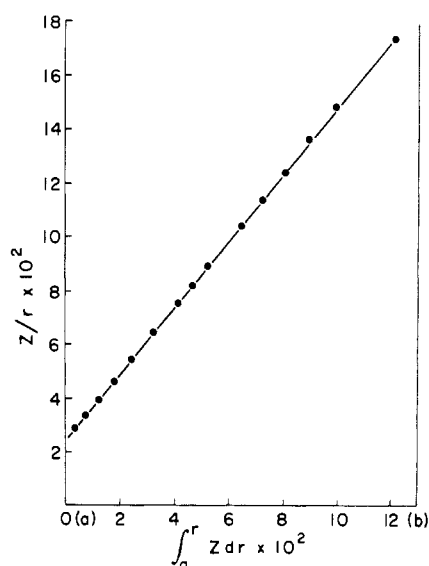


FIGURE 7: Test for presence of aggregates in sedimentation equilibrium of 5 S Pep-I (Van Holde and Baldwin, 1958). Meniscus is at 0 (a) and bottom of cell is at (b).

N-Terminal Residues of Pepsin-digested γ G-Globulin. The possibility that the difference between papain fragment I (II) and Pep-I is due to the presence of a non-covalently linked fragment in the latter material was evaluated by N-terminal amino acid assays. The results of such analysis of peptic and papain fragments and of the intact γ G-globulin are shown in Table I. Although the γ G-globulin preparation used here comprised only the slow-moving portion of the total γ G-globulin population, the N-terminal residues found

agree very well with those described previously (Porter, 1950; Orlans, 1955; Cebra *et al.*, 1961).

Of principal interest are the findings that Pep-I contains as its major N-terminal residues alanine and aspartic acid with small amounts of serine and threonine. These results are in accord with the N-terminal residues of intact γ G-globulin (see Table I) and with those of papain fragment I (Porter, 1959; Cebra *et al.*, 1961; Putnam *et al.*, 1962). No significant differences in the quantity of the N-terminal residues were noted, nor did the peptic treatment lead to the appearance of additional residues. Similar results were obtained with the 3 S fragment, corresponding to Pep-I, which was derived by peptic digestion of MRA- γ G-globulin. The implication of these findings is that peptic digestion, under the conditions employed here, did not split any peptide bond within Pep-I. Furthermore, it is unlikely that there is present in Pep-I a noncovalently linked fragment.

Comparison of the N-terminal residues of Pep-III' and papain fragment III (Table I) reveals a substantial difference between them. In particular the major residue of Pep-III' is alanine, which is present in fragment III to a much lesser extent. The latter possesses no dominant N-terminal residue and indeed behaves as though most of its terminal residues were blocked.

Antigenic Properties of the Peptic Fragments. An antigenic comparison between Pep-I and papain fragments I (II) was carried out with antiserum prepared in the goat. Hyperimmune sera were obtained from a pair of goats by repeated injections of reduced and alkylated Pep-I (MRA-Pep-I). The reduction and alkylation were performed as described under Materials and Methods. Quantitative precipitin curves were determined for Pep-I, MRA-Pep-I, and papain fragment I. It was found in each case that the maximum amount of precipitable antibody was 8.0 mg protein

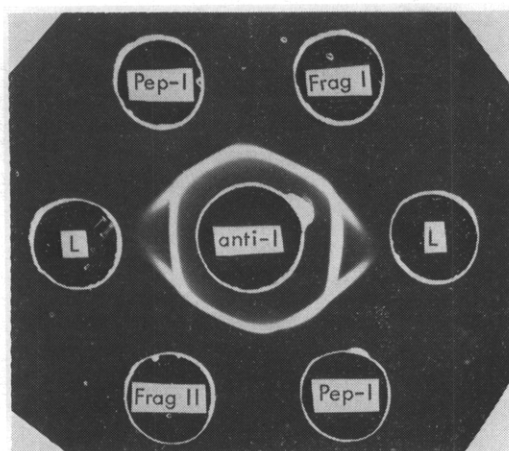


FIGURE 8: Gel-diffusion pattern of goat antiserum to Pep-I (anti-I) with Pep-I, papain fragment I (Frag I), light chain (L), and papain fragment II (Frag II). Note absence of spurs between Pep-I and papain fragments.

per ml of antiserum and the quantity of antigen needed at equivalence was approximately 0.80 mg protein. On the other hand the light chain of γ G-globulin precipitated a maximum of 5.4 mg of antibody per ml of serum with 0.34 mg of antigen. These antigenic relationships can be seen qualitatively by the Ouchterlony method as shown in Figure 8. The papain fragments I and II appear identical with Pep-I whereas the light chain of γ G-globulin is antigenically deficient. Antigenic identity between the papain and peptic fragments was also observed when a goat antiserum to papain fragment I was employed. The reduced quantity of antibody precipitated by the light chain and the heavy spur seen in Figure 8 are not accounted for simply by the presence of antibody specific for the portion of the heavy chain contained in papain fragment I, the A-piece of Fleischman *et al.* (1963). The anti-MRA-Pep-I serum did not precipitate either with the heavy chain or with the A-piece. This behavior was not limited to the antiserum induced by MRA-Pep-I but was also observed with antiserum to papain fragment I. Furthermore it was found that whereas Pep-I showed a precipitin reaction with the allotypic antisera anti-A₁, A₂, and A₃, the isolated heavy chain and A-piece did not do so. Evidently the phenomenon under consideration is associated with the separation of the light chain from the heavy chain and the A-piece.

The second major antigenic fraction of the peptic digestion of γ G-globulin, Pep-III', was further compared with papain fragment III with hyperimmune goat antiserum to the heavy chain of γ G-immunoglobulin (Utsumi and Karush, 1964). The quantitative precipitin curves in Figure 9 show that Pep-III' precipitated about 40% of the amount of antibody precipitated by papain fragment III. The fact that the latter removed all of the anti-heavy-chain antibody is in accord with the observation that despite intensive immunization with the heavy-chain antigen the goats

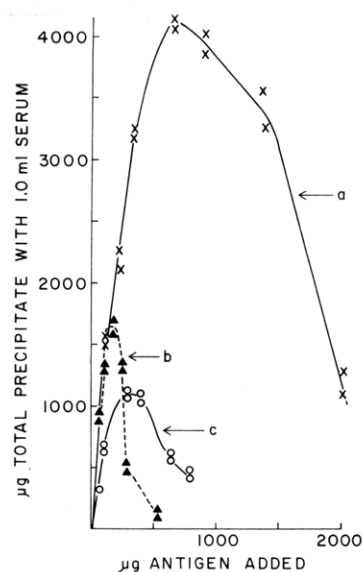


FIGURE 9: Precipitin curves of goat antiserum to heavy chain with papain fragment III (a) and Pep-III' (b). Curve (c) was obtained with antiserum absorbed with Pep-III' and papain fragment III.

employed did not produce detectable antibody against that portion of the heavy chain not contained in papain fragment III (Utsumi and Karush, 1964). The failure of Pep-III' to precipitate all of the antibody is reasonable in view of its smaller molecular weight relative to papain fragment III and its antigenic deficiency as seen by immunodiffusion (Figure 3). A discordant feature in the behavior of the antiserum arose from the observation that after its absorption with Pep-III' the supernatant antiserum did not yield the expected quantity of precipitated antibody on addition of papain fragment III (Figure 9c). This anomalous behavior could arise if the antibody specific for the antigenic determinants not contained in Pep-III' were of relatively low affinity, or as a consequence of the antigenic heterogeneity of that portion of papain fragment III which corresponds to Pep-V.

A third antigenic fragment resulting from the peptic digestion of γ G-globulin was found in Fr V (Figure 5). This is the fraction which is able to penetrate the gel matrix of Sephadex G-25. Its antigenic activity resides in its capacity to inhibit the precipitation of anti-heavy-chain antiserum by papain fragment III. The preparation of a sufficient quantity of this fraction for quantitative inhibition experiments required a modification of the original procedure. For the present purpose the peptic digest of γ G-globulin was simply passed through a column of Sephadex G-25 in a solvent containing 0.001 M sodium phosphate buffer, pH 8. A low salt concentration was desirable since the relevant fraction (Fr B, Figure 10a) was subsequently concentrated for use in the inhibition studies. The identification of Fr B as the same material as that contained in Fr V of the Sephadex G-75 filtration rests on the

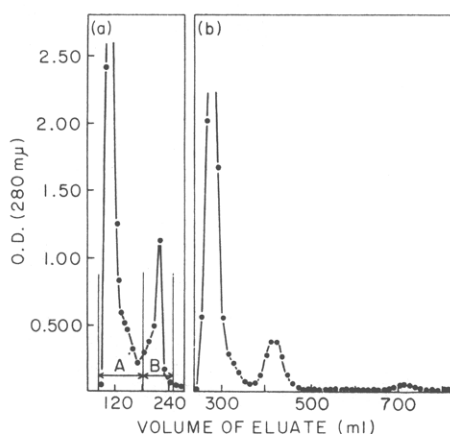


FIGURE 10: Gel filtration of peptic digest of γ G-globulin with Sephadex G-25 (a), and gel filtration of fraction A with Sephadex G-75 (b).

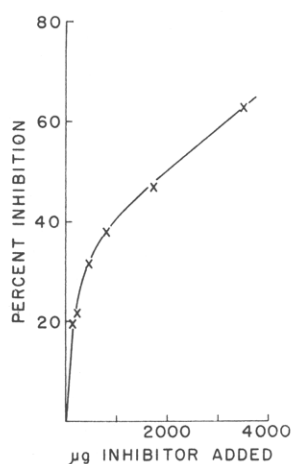


FIGURE 11: Inhibition by Pep-V of precipitin reaction between papain fragment III and goat antiserum.

behavior of Fr A and Fr B (Figure 10a) in gel filtration. In addition to its penetration of the Sephadex G-25 gel, the almost complete absence of Fr V from Fr A (Figure 10a) when the latter was passed through Sephadex G-75 (Figure 10b, cf. Figure 5) supports the equivalence of Fr B and Fr V.

The inhibition experiment was carried out with anti-heavy-chain antiserum absorbed with Pep-III' and papain fragment III as the precipitating antigen. The extent of inhibition as a function of the quantity of inhibitor added is shown in Figure 11. The reaction mixtures contained 1 ml of absorbed serum and 240 μ g papain fragment III, corresponding to equivalence, in a total volume of 2 ml. After incubation for 1 hour at 37° the mixtures were stored at 4° for 3 days. The precipitates were then washed three times with cold saline and dissolved in 0.1 M SDeS-0.02 M sodium phosphate buffer, pH 8, and the optical density was

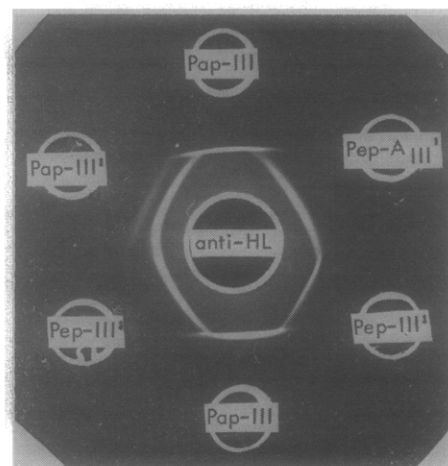


FIGURE 12: Gel-diffusion pattern of type-III fragments derived from the heavy chain by different procedures with anti-HL serum (see Figure 3). Pap-III, papain fragment III; Pap-III', papain fragment III'; Pep-A_{III}', peptic fragment from digestion of heavy chain; H, heavy chain.

read at 280 mμ. The quantity of the inhibitor required to yield 50% inhibition was equivalent to 10 mg of the original γ G-globulin. On the basis of a molecular weight of 50,000 for papain fragment III, the molar ratio of inhibitor to antigen at 50% inhibition was 13.9.

Several precipitating antigenic fragments associated with the heavy chain have been prepared by different procedures. Their antigenic relationship as revealed by immunodiffusion with goat anti-heavy-chain antiserum is shown in Figure 12. Papain fragment III' was prepared by chromatography on DEAE-cellulose of a papain digest of γ G-globulin after removal of the crystalline fragment III (Figure 4c). A fragment similar to Pep-III' was obtained by peptic digestion of the isolated heavy chain of γ G-globulin and gel filtration through a column of Sephadex G-75. The three kinds of type III' fragments exhibited antigenic identity with respect to anti-heavy-chain antiserum and antigenic deficiency relative to papain fragment III and heavy chain itself.

The occurrence of papain fragment III' is suggestive of a further breakdown of papain fragment III although its yield is only 3–5%. Its antigenic identity to Pep-III' and the stoichiometric recovery of the latter indicates that it does not result from the anomalous breakdown of a small fraction of the population of γ G-globulin molecules. Rather it appears that a portion of papain fragment III can undergo further proteolysis by papain. The molecular weight of papain fragment III' could not be measured because of the heterogeneity of the material. However its sedimentation coefficient, $s_{20,w} = 3.1$ S, suggests a larger molecular weight than that of Pep-III', 27,000, with $s_{20,w} = 2.4$ S.

Dissociation of Pep-III'. Since the present studies demonstrate that Pep-III' arises from the heavy chain the possibility was examined that this fragment exists in a dimeric form derived from the two heavy chains in

the original molecule. The N-terminal amino acid analysis (Table I) is compatible with a dimeric form since there is a total of 1.80 residues per 27,000 g. In view of the recent demonstration that γ G-globulin can be split into two identical halves by the reduction of a single susceptible disulfide bond and acidification (Palmer and Nisonoff, 1964; Nisonoff and Dixon, 1964), the participation of an interchain disulfide bond in Pep-III' was also evaluated.

Sedimentation analysis was carried out in the Spinco Model E ultracentrifuge comparing untreated Pep-III' with a reduced and alkylated preparation of Pep-III' (Figure 13). Three solvents were employed, containing 0.2 M NaCl-0.01 M sodium phosphate buffer, pH 7.8, and no SDeS (I), 0.0125 M SDeS (II), and 0.04 M SDeS (III). For the calculation of $s_{20,w}$ the correction factors used were 1.067 and 1.105 for solvents II and III, respectively (Utsumi and Karush, 1964).

After reduction and alkylation the value of $s_{20,w}$ was the same in solvent I as that for the untreated sample, namely, 2.4 S. Nor was any change observed in solvent II even though the reduced sample was slightly aggregated. Similar results were obtained when both the reduced and original samples were first exposed to 0.05 M SDeS and then their sedimentation coefficients measured in solvent II. However, when the reduced and control samples were run in solvent III a substantial decrease in $s_{20,w}$ (to 1.7 S) was found (Figure 13c, d). In addition to the major peak observed for the reduced sample in solvent III there was a minor component which sedimented more rapidly than 2.4 S (Figure 13c). This phenomenon is similar to that observed in the sedimentation behavior of the heavy chain in solvents containing SDeS (Utsumi and Karush, 1964).

An effort was made to separate the 1.7 S component so that it could be compared antigenically with the original 2.4 S material. Untreated Pep-III' was exposed to 0.1 M SDeS and then passed through a column of Sephadex G-75 without detergent. Most of the protein appeared at the same position as the original Pep-III', but a variable and minor portion ($\sim 10\%$) appeared in

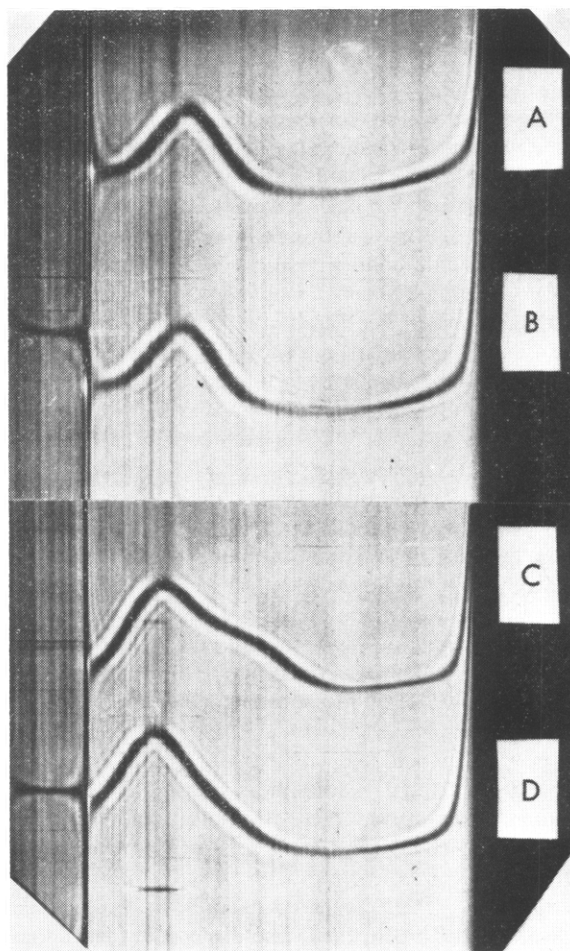


FIGURE 13: Sedimentation patterns of Pep-III' under different conditions. (A) Unreduced, solvent I (see text); (B) unreduced, pretreated with 0.05 M SDeS, solvent II (see text); (C) reduced and alkylated, solvent III (see text); (D) unreduced, solvent III. After 72 minutes at 59,780 rpm. Sedimentation is from left to right.

TABLE II: Carbohydrate Content of γ G-Globulin Fragments.

Sample	Calculated per:	Molar Content of Carbohydrate ^a		
		Hexose ^b	Hexosamine ^c	Fucose
Untreated	150,000 g	9.5 \pm 0.5	10.5 \pm 0.8	1.0
Heavy chain	110,000 g	9.9 \pm 0.1	9.2	1.2
Pep-I ^d	92,000 g	3.6 \pm 0.3	1.5 \pm 0.4	0.2
MRA-Pep-I ^d	92,000 g	4.0	1.3 \pm 0.4	^e
Pep-III'	27,000 g	<0.4	<0.1	
Pep-IV	mole of γ G	6.3 \pm 0.4	7.0	1.1
Pep-V	mole of γ G	<0.9	<0.5	
Total (Pep I + Pep-IV)	mole of γ G	9.9 \pm 0.7	8.5 \pm 0.4	1.3

^a The results do not include sialic acid content. ^b Determined as glucose, by reducing activity. ^c Determined as glucosamine. ^d Determined after treatment with Sephadex G-75 in the presence of 1 N propionic acid or 1 N acetic acid (see text). ^e Not determined.

a slower-moving peak. The material from both peaks gave a value of 2.4 S in solvent I and were indistinguishable antigenically.

The results of these experiments are compatible with the view that the fragment Pep-III' is a dimer which can be reversibly dissociated into two equivalent halves arising from the two heavy chains of the original molecule and that these subfragments are not cross-linked by any disulfide bonds.

Carbohydrate Content of Peptic Fragments. The results of the analysis of Pep-I and other peptic fragments for hexose and hexosamine are shown in Table II. There is a striking similarity between the carbohydrate content of Pep-I and that originally associated with papain fragment I (Porter, 1959). The latter contained 3.1 moles of hexose, 2.3 moles of hexosamine, 0.65 mole of sialic acid, and 0.2 mole of fucose per mole of fragment I. However it was subsequently found (Fleischman *et al.*, 1963) that the carbohydrate of fragment I was part of a small glycopeptide not covalently linked to the fragment. The attempt was made therefore to ascertain whether the carbohydrate of Pep-I was indeed covalently linked to the protein. This was done by the filtration through Sephadex G-75 of 50-mg portions of Pep-I as follows: (1) in 1 N propionic acid with a 2.8- × 110-cm column; (2) in 1 N propionic acid with two 3.5- × 58-cm columns in tandem; (3) in 1 N acetic acid with a 2.1- × 90-cm column. In no case was there any indication of a separation of the carbohydrate from the protein. The recovery of all of the carbohydrate of the original γ G-globulin in the heavy chain prepared as previously described (Utsumi and Karush, 1964) also provides evidence that the carbohydrate was not a contaminant of the γ G-globulin preparation.

The monosaccharides which constitute the hexoses of rabbit γ G-globulin have been reported by Nolan and Smith (1962) and Fleischman *et al.* (1963) to consist of mannose and galactose in a ratio of 2:1. The determination of hexose content as glucose in this study does not introduce any serious error since the molar extinction coefficients appropriate for glucose, mannose, and galactose in the method employed here are in the ratio of 1:1:0.92. This method was furthermore found to give good reproducibility and freedom from complications owing to the presence of protein in the original sample. The use of the Dowex 50 columns as described also allowed for the complete separation of hexose and hexosamine. Finally, it is apparent from Table II that the mild reduction and alkylation of Pep-I did not affect the carbohydrate analysis. Thus the interchain disulfide bond is not associated with the binding of a glycopeptide.

The analysis of Pep-III' showed little or no carbohydrate in this fragment. In this respect also, as well as antigenically, it is distinguishable from papain fragment III which contains about two-thirds of the total carbohydrate of γ G-globulin (Fleischman *et al.*, 1963). The further breakdown of fragment III to Pap-III' rendered the latter antigenically identical to Pep-III'. Nevertheless Pap-III' retained 6.5 moles of hexose per 40,000 g protein, in contrast to the absence of carbohydrate in

Pep-III'. The small amount of carbohydrate found in the smallest fragment, Pep-V, was probably due to contamination with the carbohydrate-rich fragment Pep-IV.

The bulk of the carbohydrate of γ G-globulin was found associated with a fraction in the gel filtration (Fr IV in Figure 5a) corresponding to a relatively small fragment, designated Pep-IV. This fraction did not show absorption at 280 m μ but could be recognized by the ninhydrin reaction. The gel-filtration pattern obtained in the absence of EDTA by ninhydrin analysis is compared with the ultraviolet absorption profile in Figure 5a. The nonabsorbing fraction comprises two unequal peaks, IV-1 and IV-2, the second of which overlaps the ultraviolet-absorbing Fr V. Gel filtration in 1 N acetic acid provided a more convenient procedure for the preparation of the dialyzable fractions IV and V since they could be obtained free of salt. Although this solvent gave poor resolution of Fr I (Pep-I) and Fr III (Pep-III'), equally good separation of the remaining fractions was found. The subfractions of Fr IV were isolated separately or pooled, with some of Fr V as a contaminant of the pool. After removal of acetic acid by flash-evaporation the dried preparations were completely soluble in water. The carbohydrate content of Pep-IV shown in Table II represents the sum of the contents of Fr IV-1 and Fr IV-2 and was calculated on the basis of the quantity of γ G-globulin which yielded the amount of these fractions recovered. It may be seen that of the total carbohydrate content of γ G-globulin approximately 100% of the fucose and 70% each of the hexose and hexosamine were found in Pep-IV. The distribution of each of these between Fr IV-1 and Fr IV-2 was the same, namely, 40 and 60%, respectively. It is evident that the carbohydrate contents of Pep-I and Pep-IV account fairly well for the total carbohydrate content of the original γ G-globulin.

The localization of carbohydrate in Pep-I and Pep-IV demonstrates that there are two distinguishable regions of the heavy chain to which oligosaccharides of different composition are attached. In Pep-I the covalently bound carbohydrate contains approximately 4 moles of hexose, 2 moles of hexosamine, and probably no fucose. Its similarity to the noncovalently bound glycopeptide found in papain fragment I (Fleischman *et al.*, 1963) indicates that papain cleavage splits out a glycopeptide located between fragment I and III. Pepsin, on the other hand, does not appear to attack this region of the heavy chain. Because of the presence of two heavy chains in γ G-globulin it is likely that the glycopeptide exists as equal halves. Accurate estimation of the sialic acid content of the glycopeptide would be very useful in this connection.

The composition of the carbohydrate in Pep-IV is similar to that described by Fleischman *et al.* (1963) for the carbohydrate in papain fragment III and also corresponds to the glycopeptides studied by Nolan and Smith (1962). The molar content of carbohydrate in Pep-IV is greater than in Pep-I and differs in composition. If the carbohydrate of Pep-IV were symmetrically distributed between the two heavy chains, then each unit

would consist of 3-4 moles of hexose, 3-4 moles of hexosamine, and 1 mole of fucose.

It is of interest to note that neither of the two carbohydrate moieties appears to play any role in the antigenicity of γ G-globulin. This can be inferred from the fact that the antigenicity of the heavy chain can be accounted for by Pep-III' and Pep-V and from the observation that there is antigenic identity between Pep-I and papain fragment I.

Discussion

A diagrammatic representation of the relation of the peptic fragments to the natural subunits of γ G-immunoglobulin and to the papain fragments is shown in Figure 14. The four-chain structure proposed by Porter (1962) has been assumed and the disposition of the peptic fragments has been made on the basis of their antigenic properties.

The close similarity between Pep-I and papain fragment I (II) has been demonstrated by Nisonoff *et al.* (1960a,b) in terms of immunologic activity, sedimentation coefficients, and molecular weights. Furthermore their antigenic identity (Goodman and Gross, 1963), confirmed in the present study, revealed that a substantial portion of the fragments must be common. Other recent evidence has shown that the light chains are contained in papain fragment I (II) (Fleischman *et al.*, 1963). Since our observations (Figure 6b) indicate that the light chain is part of Pep-I, the common portion of the two kinds of fragments in all likelihood includes the entire light chain.

On the other hand the fact that Pep-I and papain fragment I (II) are not identical is evident from the difference in their molecular weights (46,000 versus 40,700). The additional portion of the heavy chain which is included in Pep-I carries with it one of the two carbohydrate moieties of γ G-globulin (Table II). This feature also distinguishes the two kinds of fragments, since recent findings by Fleischman *et al.* (1963) demonstrate the absence of carbohydrate in papain fragment I (II). It was observed by these investigators, however, that a glycopeptide was released by papain digestion. Because its carbohydrate composition is very similar to that found in Pep-I it may be inferred that papain digestion under the usual conditions causes peptide cleavage on both sides of this carbohydrate (C_1 in Figure 14). These considerations serve to localize this carbohydrate in the regions of the heavy chain from which the C terminus of papain fragment I (II) arises. It is of interest to note that the observed antigenic identity of Pep-I and papain fragment I (II) indicates that this carbohydrate moiety does not act as an antigenic determinant.

A further similarity between Pep-I and papain fragment I (II) is provided by the virtual identity in their N-terminal residues (Table I) which in turn are the same as those found with the intact γ G-globulin molecule. The molecular weight of 92,000 found for Pep-I by sedimentation equilibrium in this study is substantially lower, it may be noted, than the value of 106,000 reported by Nisonoff *et al.* (1960a) based on sedimenta-

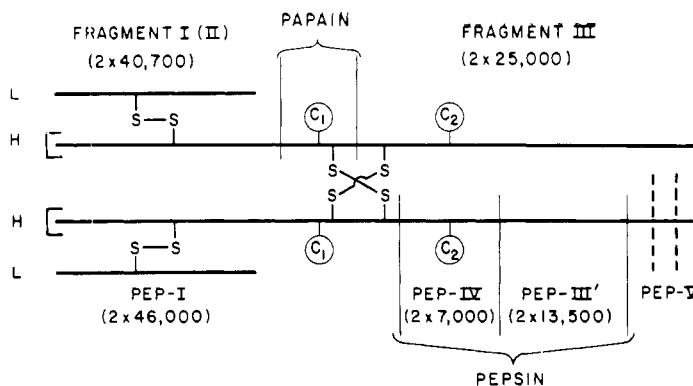


FIGURE 14: Diagram depicting the relationship of peptic fragments (Pep-I, Pep-IV, Pep-III', and Pep-V) to the papain fragments I and III and to the heavy (H) and light (L) chains. C_1 and C_2 are carbohydrate moieties. The inferred location of the papain and peptic cleavages are shown. Each interheavy-chain disulfide bond is asymmetric with respect to its half-cystine groups. Measured molecular weights are given in parentheses. The value for Pep-IV is approximate and based on the mass recovery from a known quantity of γ G-globulin; it does not include the contribution of the carbohydrate.

tion velocity and diffusion. The difference may be owing to aggregation in the earlier investigation.

The more extensive fragmentation by pepsin of the heavy chain than that effected by papain has allowed the recognition and separation of three additional fragments. Among these, Pep-III' constitutes the largest intact portion of papain fragment III with which these are associated. It is clear from its antigenic properties that Pep-III' does not possess all of the antigenic determinants of papain fragment III nor, therefore, of the heavy chain. This deficiency is in accord with its lower molecular weight of 27,000 versus about 50,000 for fragment III. In addition Pep-III' lacks carbohydrate and any interchain disulfide bond since its sedimentation coefficient ($S_{20,w}$) decreased from 2.4 S to 1.7 S in 0.04 M SDeS. The 2.4 S unit is probably made up of equivalent halves which exhibit a strong tendency toward dimerization. A recent study by Goodman (1963) of the peptic digestion at pH 4.0 of papain fragment III suggests that the further fragmentation reported by this author may parallel that observed in the present study. However the limited characterization of the fragments derived from papain fragment III does not allow a detailed comparison of the two procedures.

In contrast to Pep-III' the other two fragments, Pep-IV and Pep-V, or more precisely, two groups of fragments, consist of low-molecular-weight substances. The first of these, Pep-IV, is of interest because it carries practically all of the fucose of the intact γ G-globulin and 70% each of the hexose and hexosamine. This fragment possesses few if any aromatic amino acids since it was recognized only by the ninhydrin reaction. The similarity of its carbohydrate composition to that found for

papain fragment III indicates that it is the same moiety (C_2 in Figure 14) which is involved in both cases. It also appears that this carbohydrate unit, like the other, does not contribute to the antigenic specificity of the heavy chain. Indeed the entire Pep-IV seems not to be involved antigenically since the antigenic specificity of the heavy chain can be accounted for by the fragments Pep-III' and Pep-V.

The final group of fragments, Pep-V, is characterized by its small size, probably not exceeding 5000, since it can enter the matrix of Sephadex G-25, and by its virtual lack of carbohydrate. In view of the mass recovery of Pep-V (~15%), the figure of 5000 for the maximum molecular weight indicates that this group of fragments has arisen from the multiple cleavage of a substantially larger portion of papain fragment III. Unlike Pep-IV, however, it has retained much of the antigenic specificity of the original γ G-globulin molecule since it did effectively inhibit the precipitation reaction between papain fragment III and anti-heavy-chain serum absorbed with Pep-III'. The quantitative significance of the antigenic determinants of Pep-V may be judged from the fact that 60% of the original anti-heavy-chain antibody remained in the absorbed serum and the fact that this fragment can inhibit the precipitation of most, if not all, of this antibody.

Further examination of Pep-V has revealed that it is heterogeneous both with respect to its size and its chromatographic properties. Only the largest fragment within this population showed the inhibitory activity noted above. There is the interesting possibility that the antigenic heterogeneity of the heavy chain seen in Figure 1 may be localized in Pep-V. Our observation that Pep-V can be separated into at least seven fractions with Dowex 50 provides a basis for the attempt to separate and characterize the fragments associated with this antigenic difference.

The relative positions of fragments Pep-III', Pep-IV, and Pep-V within the right half of the heavy chain shown in Figure 14 is largely arbitrary. However it may be reasonably inferred that Pep-IV and Pep-III' are contiguous from the observation that Pap-III' retains carbohydrate although it has lost the antigenic determinants associated with Pep-V (Figure 11).

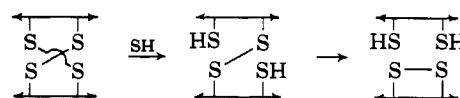
The relative significance of the two regions of papain cleavage shown in Figure 14 may be assessed from studies with water-insoluble papain (Cebra *et al.*, 1961; Cebra, 1964). In the earlier study it was shown that limited proteolysis, involving the split of three to five peptide bonds per molecule, was sufficient to lead to fragmentation to 3.3 S fragments following reduction. Furthermore this limited digestion did not produce any low-molecular-weight peptides. It appears therefore that only one of these regions was attacked by the insoluble papain during the limited reaction. For reasons to be presented shortly, the more vulnerable region is probably the one on the right side of carbohydrate C_1 (Figure 14).

In the later investigation Cebra (1964) found that, after limited proteolysis with insoluble papain, rabbit γ G-globulin could be dissociated into a soluble com-

ponent with a molecular weight of about 85,000 and an insoluble component. The soluble fragment retained the specific capacity to precipitate antigen and was clearly analogous to 5 S Pep-I. A significant property of this material was that it could be split by disulfide reduction into univalent fragments (Cebra *et al.*, 1961) similar to papain fragment I, with a decrease of the sedimentation coefficient from 5 to 3 S (Cebra, 1964). Thus the existence of at least one disulfide bond between the heavy-chain fragments in the 5 S component itself may be safely assumed. Furthermore our own studies on the papain digestion of Pep-I, which will be described elsewhere, indicate quite clearly that the half-cystines which participate in this disulfide are located at the right of the second cleavage region of papain. The basis for this conclusion is our observation that the 3 S fragments obtained by papain digestion of Pep-I in the presence of reducing agent (0.001 M mercaptoethanol) followed by alkylation did not contain any carboxymethylcysteine, whereas this constituent was present in the low-molecular-weight product. To summarize, therefore, the limited papain digestion of γ G-globulin cleaves the heavy chain at the right of carbohydrate C_1 with the appearance of an interheavy-chain disulfide located close to this point but within the 5 S component. Digestion with soluble papain in the presence of reducing agent proceeds beyond this stage and cleaves the heavy chain at a point left of both C_1 and the half-cystines of the disulfide bond.

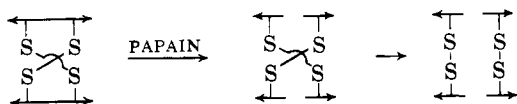
The existence of another interheavy-chain disulfide holding together the chains of papain fragment III is indicated by the decrease to one-half in molecular weight of this fragment following reduction (Marler *et al.*, 1964). In apparent conflict with the evidence for at least two interheavy-chain disulfides is the evidence provided by Palmer and Nisonoff (1964) that two-thirds of the γ G-globulin molecules can be dissociated into equal halves by the reduction of one very labile interheavy-chain disulfide bond. A further complexity in the interpretation of the structural role of disulfide linkages stems from the behavior of γ G-globulin in sodium dodecyl sulfate following limited digestion with insoluble papain (Cebra, 1964). The dissociation of the molecule was very slow, requiring about 16 hours for maximum yield of the 5 S component, and the process was inhibited by sulfhydryl reagents. This behavior suggested that an intramolecular disulfide interchange was occurring.

An attempt at a unified interpretation of the observations summarized is indicated in Figure 14. Two unsymmetrical interheavy-chain disulfide bonds are assumed to exist in the native molecule and to be located in sufficient proximity that interchange can occur between them. On this basis the reduction of one disulfide would be followed by an interchange such that one heavy chain would acquire an intrachain disulfide and the other a pair of sulfhydryl groups as follows:



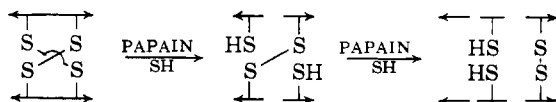
If the protein molecule is highly charged, e.g., by low pH, then this exchange would allow the separation of half-molecules.

In the case of cleavage by insoluble papain in the absence of a reducing agent the dissociation of fragments would require the following interchange:



In the presence of detergent, where the molecule is highly charged, the interchange would be thermodynamically favored by virtue of the separation of the fragments which would follow the interchange.

Finally, under the usual conditions of papain digestion, in which a reducing agent is present, the process of interchange would be greatly facilitated by the probable reduction of the labile disulfide bond and possibly the catalytic effect of the reducing agent. The combined effect of papain cleavage and reduction may be represented as follows:



In this case there are two cleavages of each heavy chain. The first of these may be reasonably assumed to occur between the half-cystines on each chain in accordance with the inferred behavior of insoluble papain. The second cleavage which then takes place at the left of the first may be facilitated by the conversion of the adjacent half-cystines to the sulfhydryl form.

In the foregoing discussion of the role of the inter-heavy-chain disulfides it has been assumed for purposes of discussion that the entire population of γ G-globulin molecules is uniform in terms of the number of such disulfides and their potentiality for interchange. It is not unlikely that a significant degree of heterogeneity exists with respect to these properties, as indeed has been suggested by the observation of Palmer and Nisonoff (1964). The elucidation of such heterogeneity as well as further evaluation of the validity of our proposed arrangement of the interheavy-chain disulfides (Figure 14) awaits completion of our studies on the fragmentation of Pep-I.

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