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Heterogeneity of Rabbit γ-Globulin with Respect to Cleavage by Papain*

Joel W. Goodman

ABSTRACT: When γ-globulin from an immunized rabbit was treated with papain for short periods of time, three fractions were recovered by sieving through Sephadex G-200. These had sedimentation coefficients of 3.6, 5.0, and 6.9 S. The 5.0-S fraction is an intermediate in the degradation which, based upon immunologic characterization, appears to consist of one fragment I or II and one fragment III. It lacks the ability to precipitate with antigen but specifically coprecipitates in the presence of undegraded antibody and antigen rather than producing inhibition as do the univalent 3.6-S antibody fragments. The 5.0-S intermediate is fully as active as 7-S antibody in eliciting passive cutaneous anaphylaxis in guinea pigs as only 0.025 μg of either antibody gave distinctly positive reactions. The residual 6.9-S fraction, upon redigestion, was more resistant to the action of papain than the starting material. This resistance did not seem to be related to the presence of fragments I or II in the molecule, but the resistant fraction possessed more hexose than the over-all γ-globulin. The additional hexose was associated with fragment I of the papain-digested molecule. The fragment of H polypeptide chain which is a component of the 5.0-S intermediate was isolated and was found to possess half of the hexose of fragment III, suggesting that the carbohydrate may be symmetrically distributed between the two H polypeptide chains of the γ-globulin molecule.

Rabbit γ-globulin is split by the proteolytic enzyme papain into functionally distinct fragments which have been designated I (or II) and III (Porter, 1959). Fragment I (or II) is composed of an L[1] polypeptide chain, which has a molecular weight of 20,000–25,000, and about half of an H[1] polypeptide chain. The intact H chain has a molecular weight of 50,000–55,000 (Fleischman *et al.*, 1962). Fragment III is composed of the remaining halves of two H chains. These elements fully account for the four polypeptide chains, two of each type, of which native γ-globulin is composed (Fleischman *et al.*, 1963).

During the course of investigations relating to the electrophoretic heterogeneity of fragment III (Paraskevas and Goodman, 1964), γ-globulin was exposed to papain for abbreviated periods of time, the reaction being aborted by addition of iodoacetamide in excess. The mixture was passed through Sephadex G-200 in order to resolve the digestion products from residual undegraded γ-globulin. Three protein peaks were obtained rather than the two which were anticipated. In addition to fractions with s_{20} values of 6.9 and 3.6 S, corresponding to native γ-globulin and its degraded products, respectively, a fraction with an s_{20} value of 5.0 appeared. This intermediate in the conversion of 6.9-S γ-globulin to 3.6-S fragments has recently been reported elsewhere and was characterized as consisting of one fragment I and one fragment III by means of peptide profiles (Nelson, 1964).

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[1] An alternate nomenclature in which the designations H and L are replaced by A and B, respectively, is also in use.

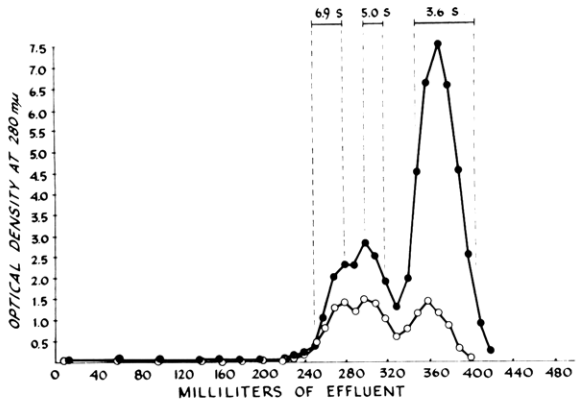

FIGURE 1: Chromatography of γ-globulin digested with 1% papain for 10 min on Sephadex G-200. Solid circles, pool of γ-globulin without prior digestion; open circles, 6.9-S fraction from pool of γ-globulin previously digested for 10 min with 1% papain.

The present investigation is concerned with an immunologic characterization of the 5.0-S intermediate as well as a study of the residual 6.9-S γ-globulin which remains after short periods of exposure to papain.

Materials and Methods

Rabbit γ-Globulin. γ-Globulin was prepared from the serum of a rabbit which had received repeated injections of bovine serum albumin in Freund's adjuvant by precipitation with sodium sulfate (Kekwick, 1940). The protein was purified further by ion-exchange chromatography on carboxymethylcellulose (CM-cellulose, Schleicher and Schuell Co.). After removal of serum proteins other than γ-globulin with 0.01 M potassium phosphate buffer, pH 6.0, the γ-globulin was eluted with 0.01 M phosphate buffer, pH 8.0, $\mu = 0.35$, by the addition of sodium chloride. γ-Globulin prepared in this manner appeared to be pure γG (Ceppellini *et al.*, 1964) as determined by immunoelectrophoresis (Scheidegger, 1955) using a goat antiserum against rabbit serum (Hyland Laboratories). It contained approximately 125 μg of antibody/mg of protein.

Antisera. Goat antisera against fragments I and III of rabbit γ-globulin were kindly provided by Dr. Melvin Cohn. The characteristics of these antisera have been reported (Goodman and Gross, 1963). A goat antiserum against light polypeptide chains of rabbit γ-globulin was furnished by Dr. Fred Karush. The fragment I and fragment III antisera were each absorbed with the corresponding heterologous antigen and the antiserum against L polypeptide chains was absorbed with H polypeptide chains. A rabbit antiserum against type IX pneumococcus polysaccharide was obtained from Dr. Jessie L. Hendry of the New York State Department of Health, and a sample of the homologous polysaccharide was generously donated by Dr. E. A. Kabat. By quantitative precipitin tests this antiserum was found to contain about 850 μg/ml of antibody N (S. B. Zolla and J. W. Goodman, unpublished data).

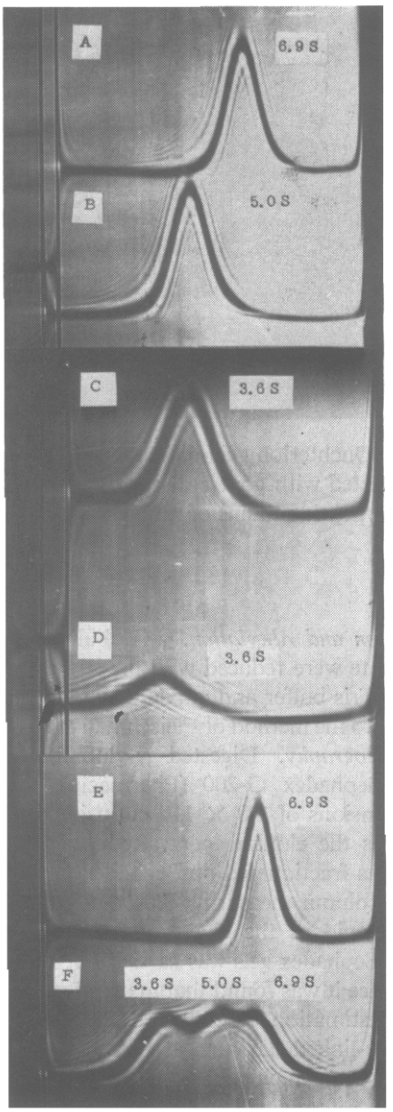

FIGURE 2: Photographs of schlieren patterns taken in the Spinco Model E analytical ultracentrifuge 80 min after the rotor reached 59,780 rpm, except A and B which were photographed after 64 min. A, 6.9-S fraction from G-200 column; B, 5.0-S fraction from G-200 column; C, 3.6-S fraction from G-200 column; D, 5.0-S fraction exhaustively digested with papain; E, γ-globulin used as starting material for digestion; F, 6.9-S fraction redigested with papain for 10 min.

Enzymatic Digestion. γ-Globulin was mixed with 1% mercuripapain (Worthington Biochemical) as described by Porter (1959). Ten minutes after mixing enzyme and substrate, a 1.5 molar excess of iodoacetamide over the cysteine in the buffer was added to abort digestion.

γ-Globulin was digested with crystalline pepsin (Mann Research Laboratories) according to the method of Nisonoff *et al.* (1960). The buffer contained 0.01 M cysteine.

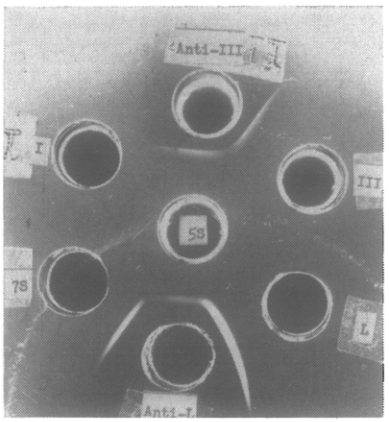

FIGURE 3: Ouchterlony gel-diffusion plate of 5.0-S intermediate tested with goat antisera against fragment III and L polypeptide chains of rabbit γ-globulin.

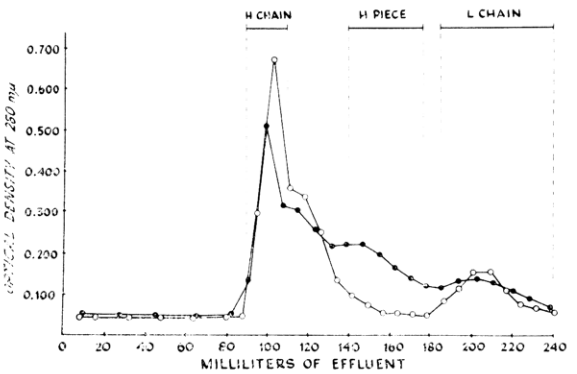

FIGURE 4: Reduced, alkylated γ-globulin chromatographed on Sephadex G-100 in 1.0 N propionic acid. Open circles, 7-S γ-globulin; solid circles, 5.0-S intermediate.

Reduction and Alkylation. γ-Globulin and the 5.0-S intermediate were reduced with 0.2 M mercaptoethanol in 0.55 M Tris buffer and alkylated with iodoacetamide according to the method of Fleischman *et al.* (1962).

Chromatography. Digested γ-globulin was sieved through Sephadex G-200 (Pharmacia) in a column with dimensions of 2.8 × 110 cm using 0.1 M sodium chloride as the eluting agent. Reduced and alkylated protein was fractionated on Sephadex G-100 (Pharmacia) in a column measuring 3.0 × 55 cm using 1.0 N propionic acid as the eluent. All of the chromatography on Sephadex gels was carried out in a cold room at 6–7° since it was found that material which interferes with the estimation of carbohydrate was eluted from these cross-linked dextrans at room temperature even with 0.1 M sodium chloride.

The 3.5-S fragments of papain- or pepsin-digested γ-globulin were fractionated on CM-cellulose as described by Porter (1959). Chromatographic fractions were concentrated by pervaporation and dialysis against water, followed by lyophilization. For calculating recoveries of protein from the columns, absorbancies at 280 mμ of 1.3, 1.4, 1.4, and 1.0 were employed for γ-globulin and papain fragments I, II, and III, respectively, at a concentration of 1.0 mg/ml (Porter, 1959). An absorbance of 1.3 was used for mixtures of the fragments. Calculated recoveries were in good agreement with the dry weights of the fractions.

Ultracentrifugation. Analyses were carried out in a Spinco Model E analytical ultracentrifuge at 20° at a speed of 59,780 rpm. The solvent was 0.1 M sodium chloride, and protein concentrations ranged from 5 to 10 mg/ml.

Carbohydrate Determinations. Hexose was determined by reaction with orcinol (Winzler, 1955) and colors were read at 505 mμ (Fleischman, *et al.*, 1963). A mixture consisting of two parts of mannose to one part of galactose was used as standard.

Immune Precipitation. Quantitative precipitin tests were set up at room temperature and incubated at 37° for 1 hr prior to refrigeration. After 6–7 days at 0–4°, the washed precipitates were dissolved in 0.25 N acetic acid and their optical densities were read in a spectrophotometer at 277 mμ (McDuffie and Kabat, 1956). For precipitation in agar the methods of Ouchterlony (1953) and Scheidegger (1955) were employed.

Passive Cutaneous Anaphylaxis (performed as described by Ovary (1958)). The abdomens of guinea pigs weighing about 350 g were shaved 24 hr prior to experiments. The animals were injected intracutaneously with 0.1-ml volumes containing various quantities of antibody. A total of four injection sites were used on each guinea pig. One of these received only saline and served as a negative control. Three hours later the guinea pigs were injected intravenously with 15 mg of bovine serum albumin in 0.5 ml of 1% Evans blue dye. After another period of 30 min the skins were retracted and inspected for the presence or absence of dye in the injected areas. The saline control areas showed uniformly negative reactions.

Results

γ-Globulin was digested with papain for 10 min at which time digestion was terminated with iodoacetamide. The protein was immediately applied to a column of Sephadex G-200 and the resultant chromatogram is shown in Figure 1. Three peaks were obtained which constituted, in order of elution from the column, approximately 10, 15, and 75% of the total recovered protein. The three fractions were each recycled through the G-200 column and were subsequently recovered in ultracentrifugally homogeneous form as seen in Figure 2A, B, and C. The respective s_{20} values of these fractions were 6.9, 5.0, and 3.6 S, which correspond to intact γ-globulin, the end products of the papain-digested molecule and an intermediate in the cleavage process.

The 5.0-S intermediate was analyzed by immunodiffusion with goat antisera against L polypeptide

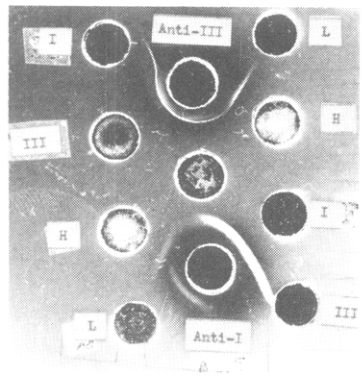

FIGURE 5: Ouchterlony gel-diffusion plate of 5-S piece tested with goat antisera against fragment I and fragment III of papain-digested γ-globulin. The 5-S piece is in the central well.

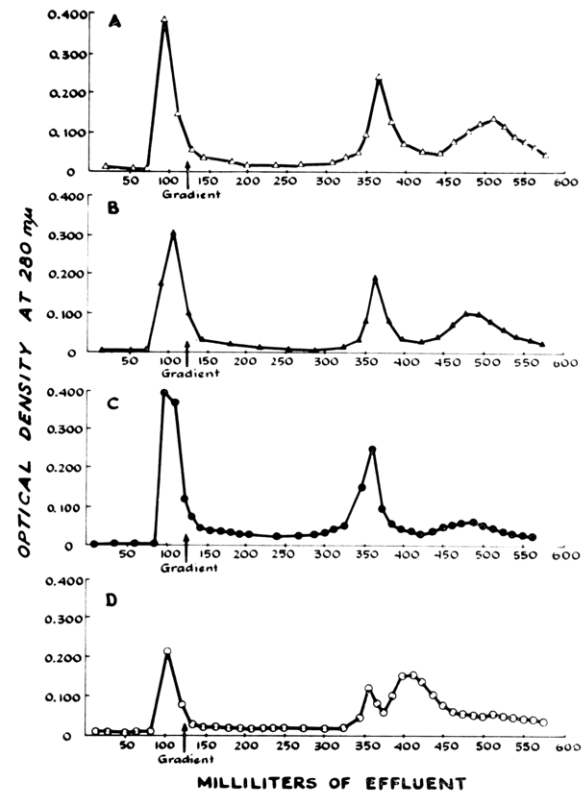

FIGURE 6: Chromatography on CM-cellulose using 0.01 M acetate, pH 5.5, as the initial buffer with a gradient to 0.9 M acetate, pH 5.5 A; γ-globulin digested with papain for 17 hr; B, 6.9-S fraction from G-200 column digested with papain for 17 hr; C, 3.6-S fraction from G-200 column; D, 5.0-S fraction from G-200 column digested with papain for 17 hr.

chains and papain fragment III of γ-globulin. It gave a pronounced band of precipitate with each of these antisera (Figure 3) and therefore appeared to possess at least one L polypeptide chain and a portion of the molecule corresponding to fragment III. Although it is not shown in the photograph, the anti-L chain serum did not give a precipitin band with fragment III.

The 5.0-S intermediate was reduced, alkylated, and passed through Sephadex G-100 in 1.0 N propionic acid in order to separate its component polypeptide chains. The chromatogram is shown in Figure 4 together with that of reduced, alkylated 7-S γ-globulin. Chromatographic peaks corresponding in position to those of H and L polypeptide chains of 7-S γ-globulin were obtained from the 5.0-S intermediate. In addition, a third broad fraction was eluted from the column between these two and will be designated as the 5-S piece. The total protein recovered from the column was about equally distributed between the first fraction and the other two fractions.

The 5-S piece was passed through the G-100 column a second time and was subsequently studied by immunodiffusion in agar using goat antisera against papain fragments I and III, each of which had been absorbed with the other fragment (Figure 5). Antifragment III serum gave precipitin lines with its homologous antigen and with H polypeptide chain but not with fragment I or L polypeptide chain. Antifragment I serum reacted strongly with fragment I and with L polypeptide chain, weakly with H polypeptide chain, and not at all with fragment III. The 5-S piece reacted strongly with antifragment III but gave a negative or at most questionable reaction with antifragment I serum. This very slight degree of reactivity could have been caused by contamination with either L or intact H polypeptide chain material. It thus appeared likely that the 5-S piece was identical with the segment of H polypeptide chain corresponding to fragment III. These findings are consistent with a composition for the 5.0-S intermediate of one H and one L polypeptide chain and a segment of a second H chain from the fragment III portion of the γ-globulin molecule.

Chromatography of the 3.6-S fraction from 10-min papain-digested γ-globulin on CM-cellulose gave a low yield of fragment III relative to fragments I and II (Figure 6c), consistent with a conversion of 7-S γ-globulin to a 5-S intermediate *via* the loss of one fragment I or II but no III. The 5.0-S intermediate was reexposed to papain for 17 hr which resulted in degradation to 3.6-S fragments (Figure 2D). These were chromatographed on CM-cellulose and an atypical pattern was obtained (Figure 6D). Fragment III appeared earlier than it normally does and was very poorly resolved from fragment II. When the third peak was tested in immunodiffusion with goat antisera against fragments I and III, it reacted with both. However, it was the largest fraction eluted from the column and accounted for approximately 58% of the total protein recovered. On the basis of the 5-S intermediate consisting of one fragment I or II and one fragment III, the third chromatographic fraction should have comprised 50% of the total protein.

The 5.0-S intermediate was assayed for its capacity

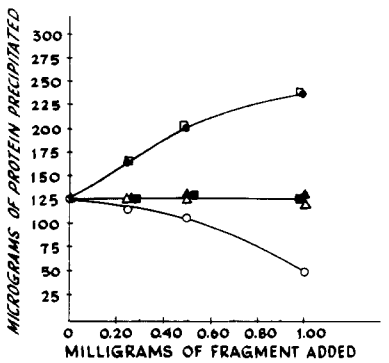

FIGURE 7: Effect of 5.0- and 3.6-S antibody fragments on immune precipitation. Each of the fragments or intact γ-globulin was added to quantities of antibody and antigen which gave a precipitate containing 125 μg of protein. ○, ●, and □: 3.6-, 5.0-, and 6.9-S antibovine serum albumin γ-globulin, respectively, in bovine serum albumin–rabbit antibovine serum albumin system; △, ▲, and ■: 3.6-, 5.0-, and 6.9-S antibovine serum albumin γ-globulin, respectively, in pneumococcus-type IX–rabbit antipneumococcus type IX system.

to combine with antigen (bovine serum albumin), and it proved to be unable to form specific precipitates either in saline or in semisolid media. However, in the presence of intact antibody and antigen it added to the precipitate (Figure 7), in contrast to the inhibition produced by 3.6-S univalent protein from the Sephadex G-200 column. It was fully as effective in this respect as 7-S antibody on a weight basis. The coprecipitation was specific since the 5.0-S intermediate had no effect on the quantity of precipitate formed by an unrelated immune system consisting of rabbit type IX pneumococcus antiserum and its homologous antigen.

The 5.0-S intermediate was tested for its ability to elicit passive cutaneous anaphylaxis reactions in guinea pigs, and the results are shown in Table I. On a weight basis, the 5.0-S intermediate was fully as active as native antibody in producing this reaction, only about 0.025 μg of antibody protein being required for a positive test with either 5.0- or 7-S antibody.

TABLE I: Passive Cutaneous Anaphylaxis in Guinea Pigs.

Type of Antibody	Reaction: Quantity of Antibody (μg) 2.5	0.25	0.025	0.0025
6.9 S undigested	+	+	+	−
5.0-S papain intermediate	+	+	+	−

The 7-S fraction obtained from the Sephadex G-200 column following digestion of γ-globulin by papain for 10 min was subjected to the enzyme for another 10-min period under conditions which were identical with those employed for the initial treatment except that the 7-S fraction had been reduced and alkylated following the initial 10-min exposure to enzyme. Other experiments have indicated that degradation of rabbit γ-globulin by papain proceeds more rapidly following reduction and alkylation of the substrate (J. W. Goodman, unpublished observations). When the products were sieved through Sephadex G-200 three fractions were again obtained, but now the total protein was approximately equally distributed between the three (Figures 1 and 2F). These results suggested that this 7-S protein was intrinsically more resistant to papain than the over-all γ-globulin.

Since γ-globulin is composed of populations of molecules containing either two fragment I or two fragment II pieces (Palmer *et al.*, 1962), the relationship

TABLE II: Hexose Contents of γ-Globulin and Fractions after 10 Min of Digestion with Papain.

Sample	Fraction of Total Protein Digested	Moles of Hexose per Mole of Protein
Native γ-globulin[a]		9.6
H chains[a]		4.4
L chains[a]		0.3
Papain I[a]		2.7
Papain III[a]		7.2
Pepsin I[a]		2.9
1% Papain		
6.9-S fraction[a]	0.05–0.10	17.4
5.0-S fraction[b]	0.10–0.15	12.4
3.6-S fraction[c]	0.75–0.85	2.8
0.2% Papain		
6.9-S fraction[a]	0.20–0.25	12.8
H chains[a]		6.0
L chains[a]		0.4
Papain I[a]		5.4
Papain III[a]		7.2
5.0-S fraction[b]	0.20–0.25	10.3
H chain[a]		5.7
L chain[a]		0.3
5.0-S piece[d]		3.7
3.6-S fraction[c]	0.50–0.60	2.0

[a] The assumed molecular weights of whole γ-globulin, H chains, L chains, and fragments I and III are 140,000, 50,000, 20,000, 42,000, and 50,000, respectively (Fleischman *et al.*, 1963). [b] Assumed molecular weight of 100,000 (Nelson, 1964). [c] Molecular weight assumed to be 44,000 based on mixture of fragments I and III [d] Assumed molecular weight of 25,000 based on one half of fragment III.

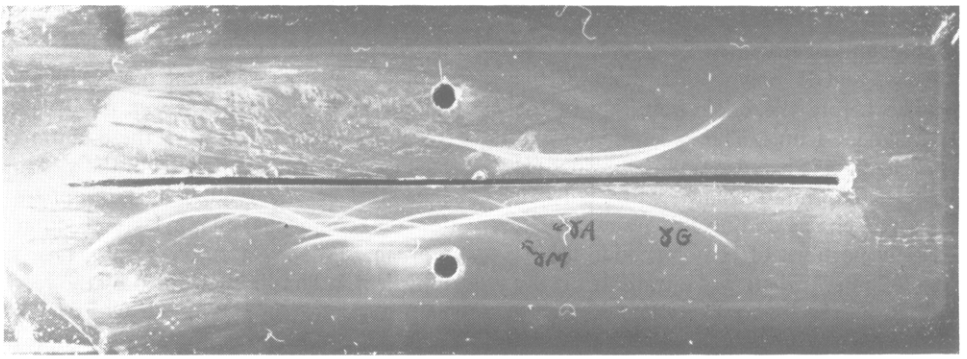

FIGURE 8: Immunoelectrophoresis of 6.9-S fraction from G-200 column. The 6.9-S fraction was placed in upper reservoir, whole rabbit serum in lower reservoir. Goat antirabbit serum was placed in central trough. The anode is to the left.

between resistance to papain and the presence of these fragments, if any, was determined by exposing the 7-S globulin which served as starting material and the residual 7-S fraction from the G-200 column to the enzyme for 17 hr in order to achieve complete breakdown to 3.5-S elements. Each of these preparations was fractionated on CM-cellulose and the chromatograms are shown in Figures 6A and 6B. The proportions of fragments I and II in the two 7-S globulins were identical, indicating that there is no relationship between susceptibility to papain and the presence of fragments I or II.

The 7-, 5-, and 3.5-S fractions from the G-200 column were analyzed for carbohydrate. To serve as a control, γ-globulin which had not been exposed to papain was passed through the same Sephadex G-200 column on which the digestion mixture had been fractionated. The results are shown in Table II. γ-Globulin was found to contain 9.6 moles of hexose/mole of protein, in good agreement with the results reported by Fleischman *et al.* (1963). The H and L chains of γ-globulin contained 4.4 and 0.3 moles of hexose, respectively/mole of protein. Since the assembled molecule is composed of 2 moles of each type of polypeptide chain, the sum of the hexose in this amount of chain material is 9.4 moles/mole of γ-globulin, in excellent agreement with the value for the intact molecule. Papain fragments I and III had 2.7 and 7.2 moles of hexose/mole of protein, respectively, while pepsin fragment I gave a value of 2.9 moles, within experimental error of the value given by papain fragment I. Fragment II from treatment of γ-globulin either with papain or pepsin showed a hexose content of 1.3 moles/mole. Other investigators have obtained lower values for the hexose of papain fragment II (Porter, 1959; Fleischman *et al.*, 1963). Since fragment III has been shown to be electrophoretically heterogeneous (Paraskevas and Goodman, 1964; Stelos *et al.*, 1964), the presence of carbohydrate in chromatographic fraction II could be the result of an artifact introduced by the fractionation procedure. This possibility is supported by the data in Table II since the sum of hexose in fragments I and III is 9.9 moles/mole of γ-globulin, consistent with the value of 9.6 moles/mole for the intact molecule. The values for fragment II have thus been omitted from the table.

After digestion of γ-globulin by 1% papain for 10 min, the residual 6.9-S fraction possessed 17.4 moles of hexose/mole of protein while values for the 5.0- and 3.6-S fractions were 12.4 moles/mole and 2.8 moles/mole, respectively. When γ-globulin was exposed to 0.2% papain for 10 min, the 6.9-S fraction comprised 20–25% of the total protein rather than 5–10% when 1% papain was employed. The hexose content of the 6.9-, 5.0-, and 3.6-S fractions in this instance was 12.8, 10.3, and 2.0 moles/mole of protein, respectively. Of particular interest was the finding that the hexose content of fragment III from native γ-globulin and from the residual 6.9-S fraction was identical, 7.2 moles/mole of fragment, while the entire difference between the hexose content of the two 6.9-S proteins was found in the carbohydrate associated with fragment I. Fragment I from native γ-globulin and from the residual 6.9-S fraction had 2.7 and 5.4 moles of hexose/mole of fragment, respectively.

The hexose values of the component chains of the 5.0-S intermediate were 5.7, 0.3, and 3.7 moles/mole of protein for the H chain, L chain, and 5.0-S piece fractions, respectively. The figure of 3.7 moles/mole for the 5.0-S piece, which is one of the two fragments of H chains of which fragment III is composed, is almost exactly half of the 7.2-moles content of fragment III.

Since contamination with only 10–20% of γA or γM (Ceppellini *et al.*, 1964), which are much richer in carbohydrate than γG (Cohen and Porter, 1964), could have been responsible for the elevated hexose content of the residual 6.9-S fraction, the latter was analyzed by immunoelectrophoresis at a concentration of 50 mg/ml using goat antiserum against rabbit serum proteins. The pattern which was obtained is shown in Figure 8. The residual 6.9-S fraction gave only a γG precipitin arc in contrast to the complex pattern given by whole rabbit serum.

Discussion

The cleavage of rabbit γ-globulin to 3.6-S fragments

appears to proceed through a 5.0-S intermediate which is composed of one unit of fragment I and one unit of fragment III. Thus, one H polypeptide chain of the molecule is broken before the other, resulting in the release of one fragment I unit. The experimental evidence for this mechanism is based on the following: (1) reactivity of the 5.0-S intermediate with antisera against L polypeptide chains and against fragment III, which contains only portions of H polypeptide chains (Figure 3). The 5.0-S intermediate was ultracentrifugally homogeneous (Figure 2) so it is unlikely that reactivity with either antiserum was produced by contaminants. (2) Upon reduction and alkylation, the intermediate yielded chromatographic fractions corresponding to H and L chains plus a third fraction which was eluted between the former two from the Sephadex G-100 column. Based upon optical density at 280 mμ, the H chain fraction accounted for about 50% of the total protein, which would be the expected result if the molecule consisted of one H chain, half of a second H chain, and one L chain. (3) The 5-S piece, which is the portion of the broken H chain that is a constituent of the intermediate, reacted strongly with antifragment III serum which had been absorbed with fragment I and had a hexose content of 3.7 moles/mole of protein, half the amount found in fragment III. Fragment III would be composed of two 5-S pieces. (4) Indirect evidence derives from the low yield of fragment III in the 3.6-S fraction of the 10-min digest (Figure 6C). Fragment III normally constitutes about one-third of totally digested γ-globulin (Figure 6A or B). In this experiment, it comprised only about 20% of the total protein. Total digestion and chromatography of this 5.0-S intermediate gave an elevated fragment III peak, although it was displaced toward the fragment II peak (Figure 6D). The reason for this displacement is unresolved. These results provide immunologic confirmation of the structure of the intermediate proposed by Nelson (1964) based on peptide profiles.

The 5.0-S intermediate, on the basis of the structure proposed above, should contain a single antibody-combining site and, as expected, was unable to precipitate with antigen. However, it specifically coprecipitated with undegraded antibody and antigen rather than cause inhibition of precipitation as did the univalent 3.6-S fragments (Figure 7). Isolated H chains of antibodies also coprecipitate specifically but they aggregate in aqueous neutral solutions and thus effectively present more than a single partial combining site per particle (Porter, 1962). No aggregation of the intermediate was detectable in the analytical ultracentrifuge (Figure 2B). Univalent antibodies prepared by reassociation of half-molecules, using a large ratio of normal γ-globulin to antibody, block precipitation (Hong *et al.*, 1964) and thus differ in this respect from the 5.0-S univalent antibody described here.

The 5.0-S intermediate was found to be fully as effective as undigested γ-globulin in eliciting passive cutaneous anaphylaxis in guinea pigs (Table I). Previous studies with univalent fragments from pepsin-digested antibodies disclosed that they were totally ineffective in eliciting direct passive cutaneous anaphylaxis reactions, even when used at concentrations 1000-fold greater than that sufficient for positive reactions with native antibody (Ishizaka *et al.*, 1962; Ovary and Taranta, 1963). However, these antibody fragments lack the fragment III portion of the γ-globulin molecule (Nisonoff *et al.*, 1960) which is apparently essential for the cutaneous anaphylaxis reaction (Ovary and Karush, 1961). Recently, Ovary has reported that univalent 7-S antibody formed by the recombination of half-molecules of antibody with half-molecules of normal γ-globulin are as effective as bivalent 7-S antibody in eliciting passive cutaneous anaphylaxis reactions (Ovary, 1965). He postulated that the bridging of two antibody molecules, regardless of their valence, was necessary and sufficient to evoke reaction. The results presented here support this contention in that they indicate that univalency, *per se*, does not affect mediation of passive cutaneous anaphylaxis as long as the antibody possesses the equivalent of fragment III. Other reports have appeared which implicated nonprecipitating antibodies in the mediation of passive anaphylaxis (Kabat and Benacerraf, 1949; Ovary and Biozzi, 1954). However, the valence of these undigested antibodies is uncertain (Weigle and Maurer, 1957).

There is a heterogeneity of rabbit γ-globulin with respect to susceptibility to papain, as shown by the observation that the 7-S globulin remaining after digestion for 10 min shows less degradation when re-exposed to the enzyme under conditions identical with those of the initial treatment (Figure 1). This heterogeneity does not appear to be a function of whether the molecule bears fragments I or II since the totally digested resistant fraction yields essentially the same ratio of fragment I:fragment II as the starting material (Figure 6A and B).

However, the hexose content of the 5–10% 7-S protein remaining after 10 min of digestion with 1% mercuripapain was almost twice that of the starting material (Table II). When only 0.2% enzyme was used for 10 min, 20–25% of the γ-globulin remained intact and the hexose content of this fraction was only about 30% greater than that of the over-all γ-globulin. Thus, the difference in hexose contents of γ-globulin appears to be real rather than an artifact due to handling. Further support for this derives from the fact that the γ-globulin pool was sieved through the same Sephadex G-200 column as the digested material. The hexose content of γ-globulin treated in this way was in excellent agreement with values reported elsewhere (Fleischman *et al.*, 1963). Furthermore, the 3.6-S protein, which should have been composed of fragments from γ-globulin which were relatively low in hexose, was indeed lower in hexose than the over-all γ-globulin in each case, but more strikingly in the instance where only 50–60% of the protein had been cleaved to 3.6-S fragments (Table II).

These findings suggest that rabbit γ-globulin consists of a heterogeneous population of molecules with respect to carbohydrate content. Susceptibility to cleavage

by papain appears to be inversely related to the carbohydrate content of the molecule.

The distribution of carbohydrate in the γ-globulin molecule is uncertain at present. Rosevear and Smith (1961) and Nolan and Smith (1962) found the carbohydrate of human and rabbit γ-globulin to be attached to a unique peptide, and thus it appeared to exist as a single moiety. Following papain digestion, however, about 70% of the total carbohydrate is firmly bound to fragment III while the remainder is loosely associated with fragment I (Fleischman *et al.*, 1963). These investigators postulated the existence of two distinct carbohydrate moieties in the molecule, the larger on fragment III and the other at a point on the H chain which is cleaved by papain, giving rise to the glycopeptide. An alternative to this proposal is that there is indeed a single carbohydrate moiety, but that there are peptides within this unit which can be cleaved by papain, causing the release of a glycopeptide. Pepsin fragment I was analyzed for hexose because it seemed that if a single carbohydrate moiety were present, then its susceptibility to the two enzymes might be quite different. On the other hand, pepsin probably splits the H chains of γ-globulin near the point of cleavage by papain but, if anything, farther toward the fragment III residue, so that a glycopeptide near the point of cleavage by papain should also be found in the pepsin fragment. The latter possibility proved to be the case since the hexose content of papain and pepsin fragment I were 2.7 and 2.9 moles/mole of protein, respectively. This finding would appear to support the existence of two distinct carbohydrate moieties but does not exclude the possibility of a single moiety with a peptide region which is susceptible to either enzyme.

Of considerable interest was the finding that the entire difference in hexose content of over-all and papain-resistant γ-globulin was found to be associated with papain fragment I, while the hexose content of the two fragment III portions was identical. If the carbohydrate is indeed present as two moieties in the intact molecule, then there may be one moiety on the region of the H chain contained in fragment III which is constant for all molecules and another moiety at or near the point of cleavage by papain which varies throughout the population of molecules. The more carbohydrate there is at this location, the more difficult it becomes for papain to attack the polypeptide chain. Again, these findings do not exclude the possibility of a single carbohydrate moiety with a core which is the same for all molecules and is found on fragment III after treatment with papain.

Finally, the finding that the hexose content of the 5-S piece is precisely half that of fragment III provides strong circumstantial evidence for a symmetrical distribution of carbohydrate between the two H chains of the γ-globulin molecule. The 5-S piece and the H chain fractions from the 5-S intermediate represent the individual H chains in this group of molecules. Both contain appreciable amounts of hexose and, while it might be argued that each fraction contains molecules which differ from one another in carbohydrate content, the value of 3.7 moles of hexose/mole of 5-S piece makes coincidence appear unlikely.

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