CORROBORATION OF RECENT MODELS OF THE γG IMMUNOGLOBULIN MOLECULE*

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Cleavage of the interchain disulfide bonds of γG immunoglobulins (1)[1] allows separation of two kinds of polypeptide chains by means of chromatography in urea (2, 3) or gel filtration in acidic solvents (4, 5). The light chains (κ or λ chains; molecular weight 20,000) and the heavy chains (γ chains; molecular weight 55,000) appear to be natural subunits under the control of two independent sets of structural genes (6). This interpretation is supported by the finding that Bence-Jones proteins are composed of light chains (7–9). Moreover, light chains are common structural units of all classes of immunoglobulins whereas the heavy chains are distinctive in each class (10–14). Structural analyses (5, 7, 11, 15) have indicated that the bivalent γG immunoglobulin molecule has the molecular formula $\gamma_2\kappa_2$ or $\gamma_2\lambda_2$ in all animal species so far examined.

Several models have been proposed to account for the gross arrangement of the light and heavy chains within the molecule (15–19). The models are based on results of immunologic and chemical studies (5, 20–22) in which the chains and the fragments produced by proteolytic digestion were compared (see Fig. 12). These studies clearly suggest that the Fab fragments consist of light chains and portions of heavy chains and that the Fc fragment consists of residual portions of heavy chains. Although this assignment is supported by many lines of evidence, it has been difficult to demonstrate directly.

The present investigation provides direct corroboration of the correctness of

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[1] A unifying nomenclature has been recently suggested (1). The three classes of immunoglobulins are γG (previous name 7S γ-globulin), γA (previous names γ_{1A}, β_{2A}) and γM (previous names γ_{1M}, β_{2M}, or 19S). The heavy chains, which are different in each class, are called γ, α, and μ, respectively. The letters κ (antigenic type I) and λ (antigenic type II) have been proposed for the light chains which are known to represent common elements in all classes of immunoglobulins. The fragments released after hydrolysis with papain have been designated Fab (S fragments or fractions I and II) and Fc (F fragments or fraction III) and the A piece (5) has become Fd. The above nomenclature will be used in the present communication. A depiction of the chains and fragments of γG immunoglobulin is given in Fig. 12.

current models. This has been accomplished in a specific manner by procedures of peptide mapping as well as by analysis of doubly labeled reconstituted molecules (23, 24).

Materials and Methods

Human γG Immunoglobulin and Myeloma Protein.—Lyophilized human γG immunoglobulin (Cohn fraction II from Lederle Laboratories Pearl River, New York, lot C 790) was used in the experiments. Human γG myeloma protein (antigenic group I) was isolated from fresh serum of a patient with multiple myeloma by zone electrophoresis on starch. Protein concentrations were determined by the modified Folin-Ciocalteu method (25) as well as by measuring absorbancies at 280 mμ.

Hydrolysis with Papain.—The hydrolysis of γG immunoglobulin, γG myeloma protein and reconstituted 7S material proceeded for 1 hour (26). The fragments were separated by zone electrophoresis on starch (27, 28). In some experiments, the γG immunoglobulin was hydrolyzed for only 5 minutes and the reaction was stopped with N-ethylmaleimide (26).

Hydrolysis with Pepsin.—γG immunoglobulin was digested with pepsin (Worthington Biochemical Corporation, Freehold, New Jersey, 2× recrystallized, lot 661) according to the procedure of Nisonoff *et al.* (29). Cysteine was omitted. The main 5S fragment was recovered from reaction mixture by gel filtration on sephadex G-25 (Pharmacia, Upsala, Sweden) in tris-HCl buffer (0.05 M, pH 8.0).

Reduction of γG Immunoglobulins and Their Fragments and Separation of the Polypeptide Chains.—γG immunoglobulins or Fab fragments were dissolved in 0.05 M tris-HCl buffer (pH 8.0) made 0.1 M in 2-mercaptoethanol. After 1 hour of reduction, the chains were alkylated by making the solution 0.2 M in iodoacetamide (2, 3). The polypeptide chains were separated by gel filtration on sephadex G-100 in 6 M urea and 1.0 N propionic acid. In some instances, a mixture of 6 M urea and 0.1 N propionic acid was used instead.

For peptide mapping, the polypeptide chains were prepared from fully reduced γG immunoglobulin, in order to minimize the contamination of heavy chains by material containing light chains. γG immunoglobulin was dissolved in 8.0 M urea and 0.1 M 2-mercaptoethanol. The reduction was allowed to proceed at room temperature for 4 hours. After making the solution 0.2 M in iodoacetamide, the reduced alkylated chains were separated by gel filtration on sephadex G-100, using a mixture of 6 M urea and 1.0 N propionic acid as solvent. The dimensions of the columns used for gel filtration were 1 cm × 100 cm.

Reconstitution of 7S Material from Isolated Polypeptide Chains Labeled with Radioactive Isotopes.—These procedures have been described in detail elsewhere (23, 24). Two batches of human γG immunoglobulin were separately labeled with I^{131} and I^{125}. After reduction and alkylation in tris-HCl buffer (0.05 M, pH 8.0), the chains were separated by gel filtration in 0.5 N propionic acid (4). Mixtures of the complementary chains having different labels were made in propionic acid, and dialyzed exhaustively against neutral buffer at 4° C. Reconstituted 7S material was isolated by gel filtration on sephadex G-200 in neutral buffers (24) and was then submitted to the same sequence of experiments as native molecules. It was possible to calculate the relative amount of heavy and light material at every step of the experiment, since the specific activities of the separated chains, expressed as CPM (counts per minute) per unit absorbancy at 280 mμ, were known.

Starch Gel Electrophoresis in Urea.—This method has been described previously (3). When material labeled with radioactive iodine was used, the gels were stained and the radioactivity was determined. Strips corresponding to each separate component were isolated and cut into ½ cm segments. The radioactivity of each segment was measured in a dual channel scintillation counter (23, 24). Corrections were made for background, decay, and counting efficiency.

Two-Dimensional High Voltage Electrophoresis of Tryptic Hydrolysates of Polypeptide Chains

and Fragments (Peptide Mapping).—The details of the procedure have been reported (9). All samples to be compared were hydrolyzed with trypsin at the same time, under the same conditions, using the same preparation of enzyme (2× recrystallized, lot 6223 from Worthington Biochemical Corporation). The products of hydrolysis were submitted to two-dimensional electrophoresis as previously described (9). After development with ninhydrin and fixation with copper nitrate, the patterns were traced and compared. All comparisons were made on patterns of samples separated simultaneously under the same conditions.

Estimation of the Sedimentation Coefficients.—Sedimentation coefficients were estimated after ultracentrifugation in linear gradients of sucrose (5 to 20 per cent in tris-HCl, pH 8.0), according to the general procedure of Martin and Ames (30) and modifications previously described in detail (23). The sedimentation coefficient was calculated by referring to the position of the peak of alkaline phosphatase used as a standard (sedimentation coefficient 6.1S). The enzyme was mixed with the sample to be studied before applying it to the gradient.

Double Diffusion in Agar and Immunoelectrophoresis.—These procedures have been described before (20).

RESULTS

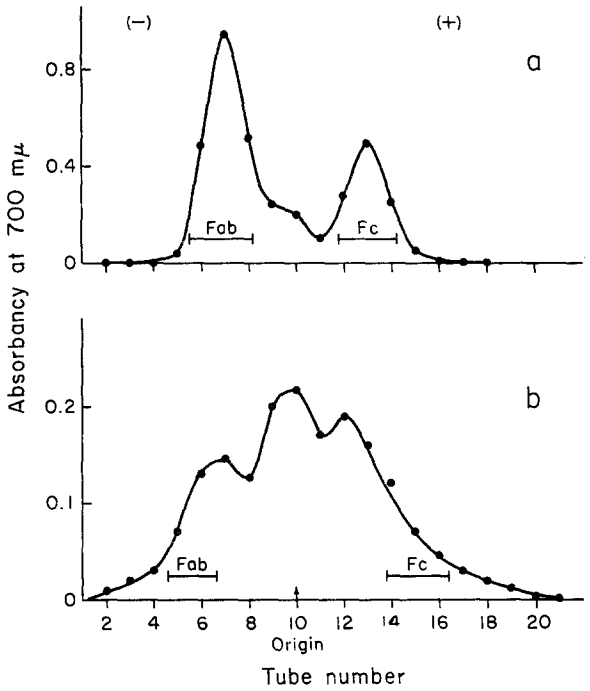
Isolation and Reduction of Fragments Obtained from Native and Reconstituted Molecules—The hydrolysis of human γG immunoglobulin or of human γG myeloma protein with papain cleaves the molecule into two types of fragments, which can be fractionated (Fig. 1 *a*) by starch zone electrophoresis (27, 28). The material studied in this case was a γG myeloma protein; similar results were obtained with human γG immunoglobulin. The slowest (cathodal) fraction consisted of Fab fragments as shown by immunoelectrophoresis and immunodiffusion in agar, using rabbit antisera against fragments and whole γG immunoglobulin. The fast moving (anodal) fraction was shown by the same techniques to consist of Fc fragments. The ratio of the yields of Fab to Fc fragments was 2/1, as expected from previous reports (26, 27).

In order to minimize possible heterogeneity resulting from the relative lack of specificity of papain, fragments were also isolated after hydrolysis for 5 minutes (Fig. 1 *b*). γG immunoglobulin was incompletely digested as indicated by the presence of a middle peak of protein with a range of mobility similar to that of the native protein. As shown by immune diffusion, the two types of fragments obtained from the initial portions of the first and the trailing portions of the third peaks were free of this material.

An aliquot of the solution containing the Fab fragments was reduced and alkylated. The entire mixture was immediately applied over a column of sephadex G-100 in 6 M urea and 0.1 N propionic acid. Two fractions were obtained as shown in Fig. 2. The first peak, containing heavier material, represented 15 per cent of the total mass. The positions at which the two peaks emerged were compared to those of light polypeptide chains and intact Fab fragments which had been applied over the same column in similar but separate operations. The first peak and the untreated Fab fragment emerged at the same volumes. The position of the second peak was identical with that of the light chain peak. When a buffer containing 6 M urea, 1.0 N propionic acid was used

for gel filtration, a similar pattern of separation was observed, except that the fraction recovered from the first peak represented 30 to 33 per cent of the total mass of reduced alkylated Fab fragments.

Since it was found that the relative amount of material present in each peak depended upon the conditions of separation, definitive names were not assigned



FIGS. 1 *a* and 1 *b*. Isolation by zone electrophoresis on starch of Fab and Fc fragments from γG molecules hydrolyzed with papain.

FIG. 1 *a*. γG myeloma protein after 1 hour of hydrolysis.

FIG. 1 *b*. Normal γG immunoglobulin after 5 minutes of hydrolysis. Absorbancy at 700 mμ: Absorbancy of Folin reaction at 700 mμ. (+) anode and (−) cathode.

to the fractions of reduced alkylated Fab fragments. For descriptive purposes we shall refer to the first peak and second peak as Fab/1 and Fab/2, respectively.

Evidence for the presence of a portion of heavy chains in the Fab fragments of human γG immunoglobulin has already been obtained for molecules reconstituted from light and heavy chains labeled with different isotopes (24). The partition of heavy and light material between Fab/1 and Fab/2 fractions prepared from such reconstituted molecules is presented in Fig. 3 *a*. As a control, 10 mg of the Fab fragments from native γG immunoglobulin was reduced

and alkylated, and applied over the same column under the same conditions (Fig. 3 *b*). The reconstituted material emerged in two peaks localized at the same position as the Fab/1 and Fab/2 fractions obtained from native material. In view of the small amount of reconstituted material used for the experiments (approximately 1 mg), the absorbancy was not measured directly but was estimated from the specific activity, as previously described (24). Four different

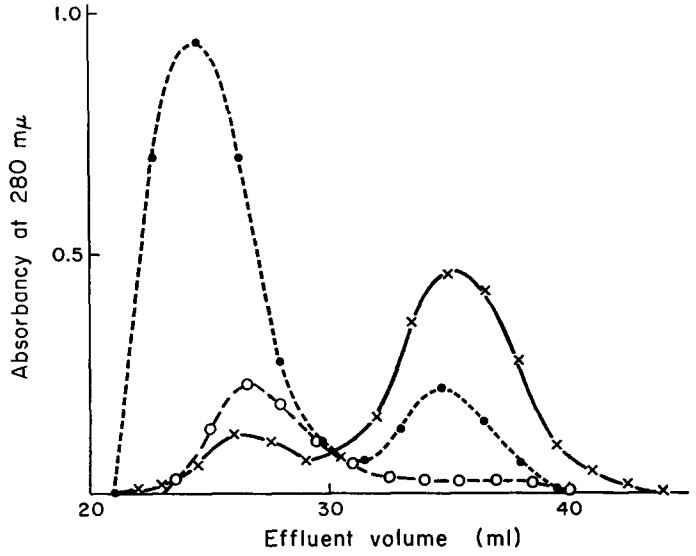
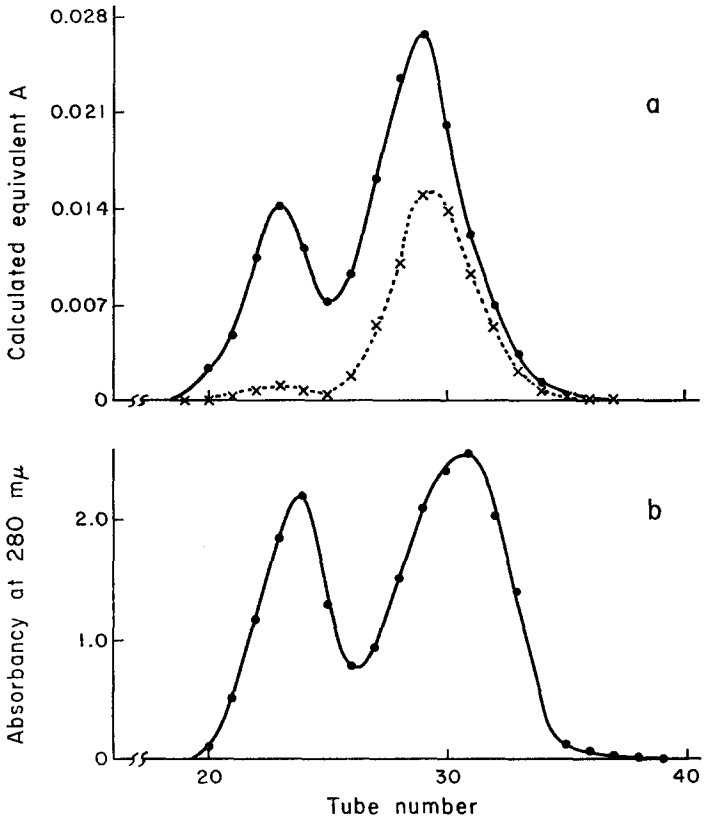


FIG. 2. Separation of polypeptide chains, Fab fragments, and reduced alkylated products of the Fab fragments by means of gel filtration on sephadex G-100 in 6 M urea, 0.1 N propionic acid. The figure summarizes the results of three separate experiments on: —X— reduced alkylated Fab fragments; ---●--- reduced alkylated human γG immunoglobulin; and ---O---O--- Fab fragments. No protein was present in the first 20 ml. of effluent.

preparations were submitted to the same sequence of experiments and the patterns of elution were similar. The first peak represented 20 to 25 per cent of the total material, and consisted mainly of label originally associated with heavy chains. Ninety-two to 95 per cent of the label associated with light chains emerged in the second peak. As estimated from the specific activities, both heavy and light material were present in approximately equal amounts in this second peak. Typical quantitative results are given in Table I.

The Fab/1 and Fab/2 fractions from native γG immunoglobulin were compared with unreduced Fab fragments and light chains by means of starch gel electrophoresis in urea formate buffer (Fig. 4). The Fab/1 fraction and the Fab fragments migrated in the same position. The mobility of the Fab/2 fraction was similar to that of light chains (Fig. 4 *a*). Identical results were



FIGS. 3 *a* and 3 *b*. Gel filtration of reduced and alkylated Fab fragments from native and reconstituted doubly labeled molecules (sephadex G-100 in 6 M urea, 1.0 N propionic acid).

FIG. 3 *a*. Fractions obtained from reconstituted and labeled molecules; —●— heavy chain material labeled with I^{131}, and —X— light chain material labeled with I^{125}. Calculated equivalent A.: Absorbancy at 280 mμ as estimated from specific activities of the chains.

FIG. 3 *b*. Fractions obtained from native γG immunoglobulin.

obtained with fragments and chains of the γG myeloma protein (Fig. 4 *b*). The bands were sharper, however, because of the relative homogeneity of this protein.

Fractions obtained from reconstituted molecules were also submitted to starch gel electrophoresis, and the partition of labels originally associated with heavy and light chains was determined. The results are presented on Fig. 5. The Fab/1 fraction migrated as a single band slightly faster than the heavy chains and in this respect it resembled the fraction obtained from native γG immunoglobulin. The Fab/2 fraction showed a mobility similar to that of light chains. The portion of the Fab/2 fraction derived from labeled heavy

chains migrated slightly faster than that derived from labeled light chains. The fast material containing label that was associated with heavy chains might have come from light chains originally contaminating the heavy chain preparations used to reconstitute the molecule. To check this possibility, an aliquot of the labeled heavy chains used for reconstitution was reduced and alkylated in 8 M urea and examined on the same gel. No radioactivity appeared in the

TABLE I

Contribution of Heavy and Light Chains to Whole Reconstituted 7S γG Immunoglobulin Molecules and to Fractions Derived from Reduction of Fab Fragments

Heavy chain / Light chain mass ratio in original mixtures	Heavy chain / Light chain mass ratio in 7S reconstituted molecules	Contribution to Fab/1 of			Contribution to Fab/2 of		
		Heavy chain material	Light chain material	Total	Heavy chain material	Light chain material	Total
6/1	3.9/1	16	4	20	37	43	80
1/1	2.95/1	16.6	2.1	18.7	42.7	38.6	81.3

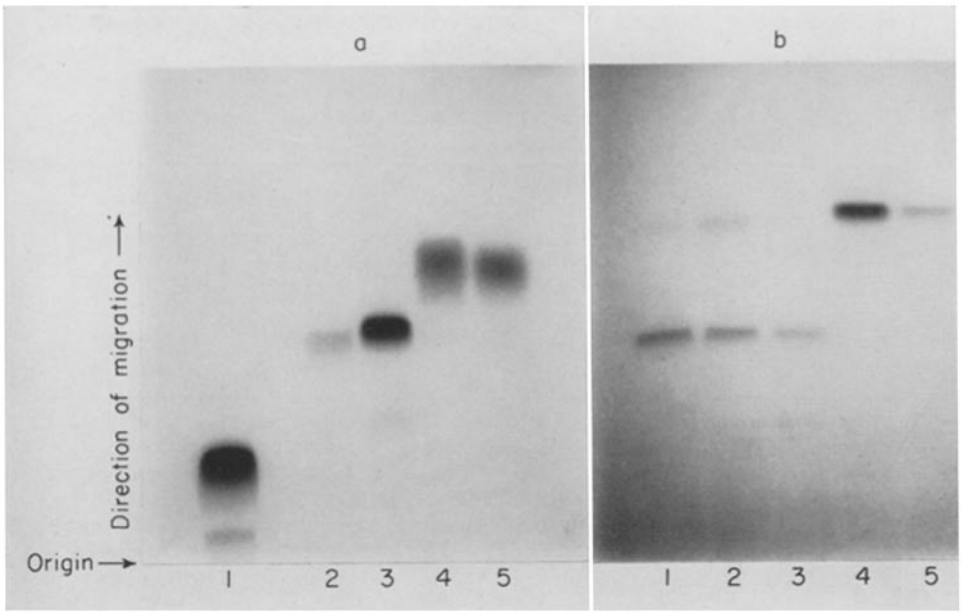
Heavy chains labeled with I^{131} and light chains labeled with I^{125} were mixed and dialyzed as described in Materials and Methods. Reconstituted 7S material was isolated by gel filtration on sephadex G-200 in tris-HCl buffer (0.05 M, pH 8.0), and hydrolyzed with papain for 1 hour. After isolation by zone electrophoresis on starch, the Fab fragments weree rduced in 0.1 M mercaptoethanol and the Fab/1 and Fab/2 fractions were separated by gel filtration on sephadex G-100 in 6 M urea, 1.0 N propionic acid (see Fig. 3). Each fraction was assayed for radioactivity in a dual channel scintillation counter and the numbers of counts per minute were converted into absorbancy units from the known specific activities of the chains. The heavy chain/light chain mass ratio in the original mixture was determined by reading the absorbancy of the separated chains prior to mixing. The contributions of heavy chain and light chain material to the fractions are expressed as the per cent of the total material recovered after gel filtration of reduced Fab fragments.

region of light chains. The results of the starch gel electrophoretic experiments were in complete agreement with those in which gel filtration was used.

Reduction of the Fragments Obtained by Hydrolysis of the γG Immunoglobulin with Pepsin.—Human γG immunoglobulin was incubated with pepsin for a period of 16 hours (29). Peptides of low molecular weight were removed by gel filtration through a column of sephadex G-25. The heavy component had a sedimentation coefficient of 5.1S as determined by ultracentrifugation in a linear gradient of sucrose (23). This material had the same antigenic determinants as the Fab fragments released by hydrolysis with papain (Fig. 6), and it showed no cross-reaction with the Fc fragments.

The peptic fragments were then submitted to the same sequence of experiments employed for the Fab fragments. Reduction with 0.1 M 2-mercaptoethanol followed by gel filtration under dissociating conditions led to the

separation of two components which corresponded in position of elution to the Fab/1 and Fab/2 fractions (Fig. 7). The two fractions obtained from the peptic fragments were prepared for peptide mapping in the same way as Fab/1 and Fab/2 fractions.



FIGS. 4 *a* and 4 *b*. Starch gel electrophoresis in urea formate buffer of Fab fragments and fractions obtained after reduction and alkylation.

FIG. 4 *a*. Fractions obtained from normal human γG immunoglobulin: 1. native human γG immunoglobulin; 2. Fab/1 fraction; 3. unreduced Fab fragment; 4. Fab/2 fraction; 5. light chains.

FIG. 4 *b*. Fractions obtained from γG myeloma protein: 1. unreduced Fab fragments; 2. reduced and alkylated Fab fragments; 3. Fab/1 fraction; 4. Fab/2 fraction; 5. light chains.

Peptide Maps of Tryptic Hydrolysates.—Structural relationships among the various fragments and chains were investigated by comparing patterns obtained by means of two-dimensional high voltage electrophoresis of tryptic hydrolysates. In preliminary experiments, it was found that the peptide maps of the Fab/1 fraction from γG immunoglobulin hydrolyzed with papain for 1 hour or longer were variable and were, therefore, not comparable with peptide maps of the polypeptide chains. For this reason, fragments isolated after 5 minutes of hydrolysis with papain were used. The reproducibility of the patterns was tested with several preparations and the number of ninhydrin-staining spots was determined. The colored spots will be referred to as peptide

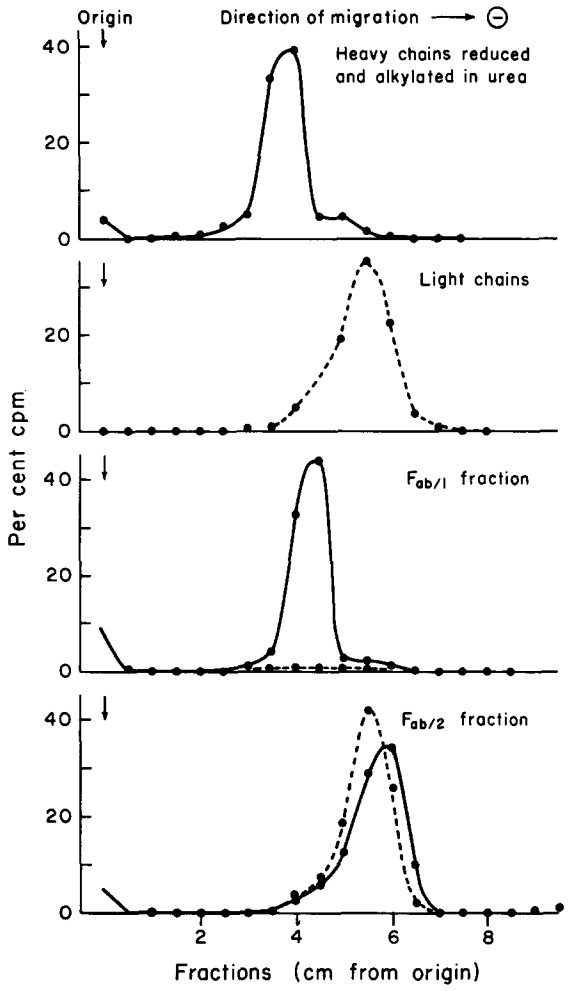


FIG. 5. Starch gel electrophoresis in urea formate buffer of chains and fractions labeled with radioactive iodine. Each strip was assayed separately for radioactivity (see Materials and Methods). —●— Material derived from heavy chains labeled with I^{131}, ---●--- material derived from light chains labeled with I^{125}. The heavy chains were fully reduced in 8 M urea as described in text. Per cent CPM.: per cent of total counts per minute recovered from each strip. ↓ Origin.

spots, although it is possible that some spots represented amino acids or amino sugars. Some spots were poorly resolved, probably because of the heterogeneity of γG immunoglobulins.

As shown in Fig. 8, the Fab/2 fraction and the homologous fraction obtained after peptic hydrolysis had similar peptide maps. Although these maps closely

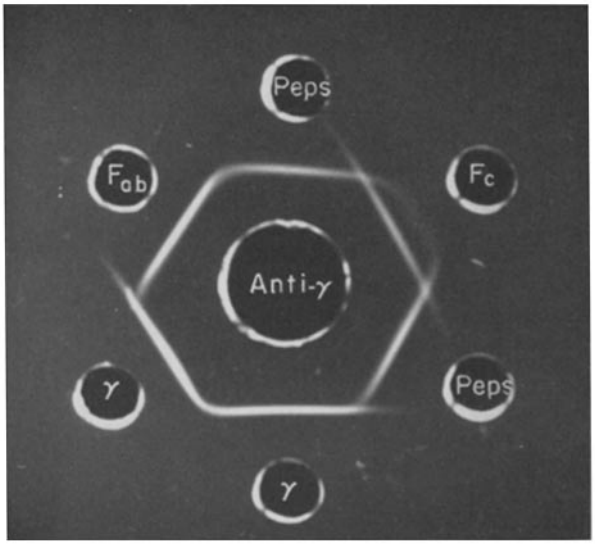


FIG. 6. Comparison by immune diffusion of fragments released after proteolysis of normal human γG immunoglobulin with papain and pepsin: Fab, Fab fragments, and Fc, Fc fragments obtained by hydrolysis with papain. Peps, Fragments obtained after digestion with pepsin in the absence of cysteine. γ, Human γG immunoglobulin. Anti-γ, Rabbit antiserum against human γG immunoglobulin.

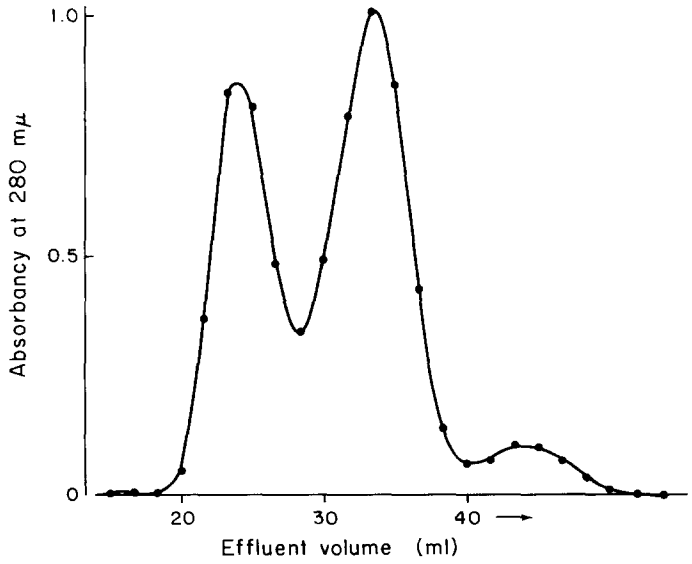
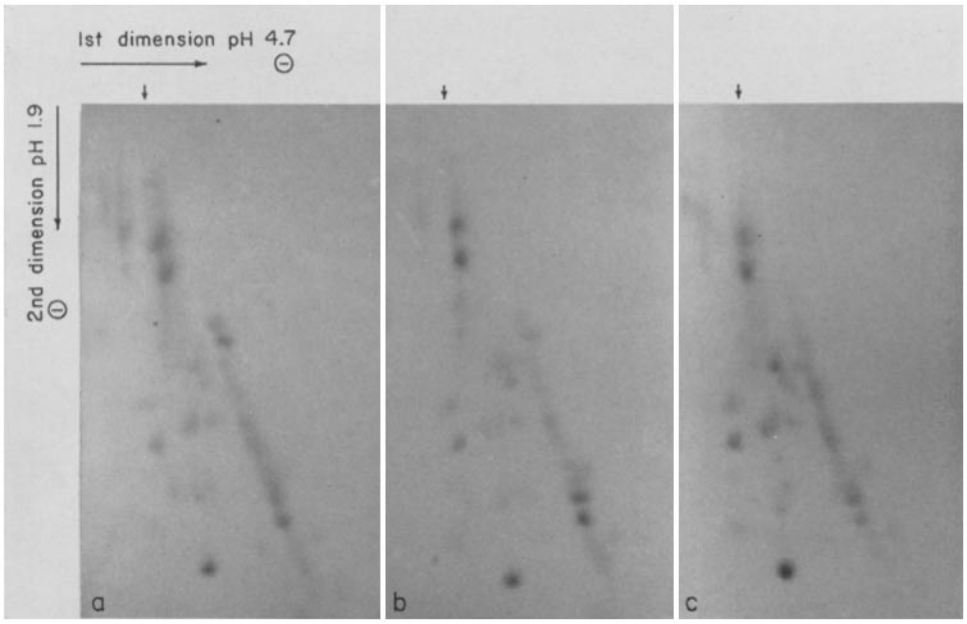


FIG. 7. Gel filtration (sephadex G-100 in 6 M urea, 1.0 N propionic acid) after reduction and alkylation of fragments obtained by hydrolysis of human γG immunoglobulin with pepsin. Compare positions of peaks with those of Fig. 2.

resembled those of light chains, several differences were observed. A total of 37 spots were identified in the hydrolysates of the Fab/2 fraction, whereas 34 spots were resolved in the patterns of light chains. Eight spots were present in the patterns of Fab/2 fractions and missing in those of light chains. All 8 of these spots were identified in the map of heavy chains and 4 of the 8 were found in the Fab/1 fraction. The peptide maps of light chains contained 5 spots not present in the maps of the Fab/2 fraction or any other fraction.

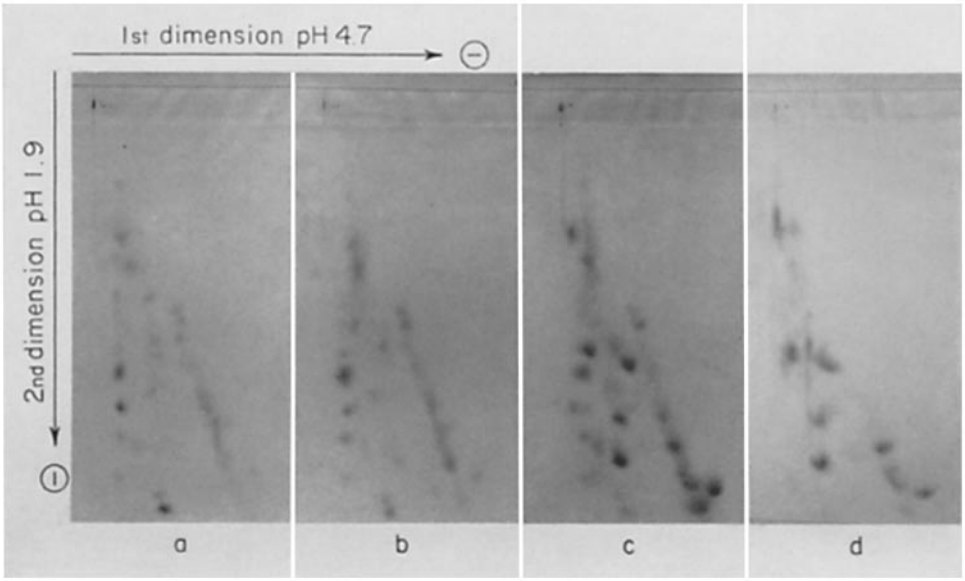


FIGS. 8 *a* to 8 *c*. Two dimensional high voltage electrophoresis of tryptic hydrolysates of Fab/2 fractions and light chains of human γG immunoglobulin. (*a*) Fab/2 fraction. (*b*) Fraction derived from peptic fragments, homologous to Fab/2 fraction. (*c*) Light chains. The vertical arrows indicate the positions of the origins.

The evidence obtained in previous studies suggested that the peptide map of the heavy chains would consist of the sum of the peptides found in the patterns of the Fab/1 fraction and Fc fragment. Comparisons among these fractions are presented in Fig. 9. All 23 peptide spots of the Fab/1 fraction were present in the peptide maps of the heavy chains. The patterns of the corresponding fraction of the 5S peptic fragment contained only 3 spots not found in the maps of Fab/1 fractions obtained from Fab fragments. All of the 19 spots resolved in maps of the Fc fragment were present in maps of heavy chains. There were 12 spots shared by the patterns of the Fab/1 fraction and

the Fc fragment. In most cases, these spots were less intensely stained in maps of the Fab/1 fraction.

In addition to the above studies, comparisons were made between the peptide maps of light chains and heavy chains. The patterns differed as was expected from the results of previous studies (9) of γG myeloma protein. There were, however, 5 ninhydrin-positive spots in the same position in the maps of



FIGS. 9 *a* to 9 *d*. Two dimensional high voltage electrophoresis of tryptic hydrolysates of Fab/1 fractions, heavy chains, and Fc fragments of normal human γG immunoglobulin. (*a*) Fab/1 fraction derived from peptic fragments. (*b*) Fab/1 fraction derived from papain fragments. (*c*) Heavy chains. (*d*) Fc fragments. The origins may be seen at the upper left hand corners in each of the patterns.

both types of chains. This was a reproducible finding in analyses of four different preparations. In addition to having been developed with ninhydrin, several preparations were stained specifically for arginine (31), tryptophan (32), and proline (31). The results agreed with those obtained with ninhydrin staining.

The data obtained from the peptide maps are summarized in Table II and in the composite drawings of Fig. 10. These drawings stress the comparison between light chains and the Fab/2 fraction (Fig. 10 *a*) and between the heavy chains, the Fc fragment and the Fab/1 fraction (Fig. 10 *b*).

Hydrolysis of Heavy Chains with Papain and Pepsin.—Prolonged hydrolysis of whole γG immunoglobulin with papain led to variations in the patterns of

tryptic hydrolysates of the Fab/1 fraction. This suggested that the portion of the heavy chain corresponding to this fraction might be particularly susceptible to proteolysis. In order to examine this possibility, isolated I^{131}-labeled heavy chains were incubated for 1 hour with papain. Approximately 50 per cent by weight of the products of digestion consisted of small peptides as shown by gel filtration on sephadex G-25 in tris-HCl buffer (0.05 M, pH 8.0). The remainder had a sedimentation coefficient of 3.8 S, estimated by ultracentrifugation in a linear gradient of sucrose. It was found that 85 per cent of this material behaved like the Fc fraction after starch zone electrophoresis (Fig. 11 *a*;

TABLE II

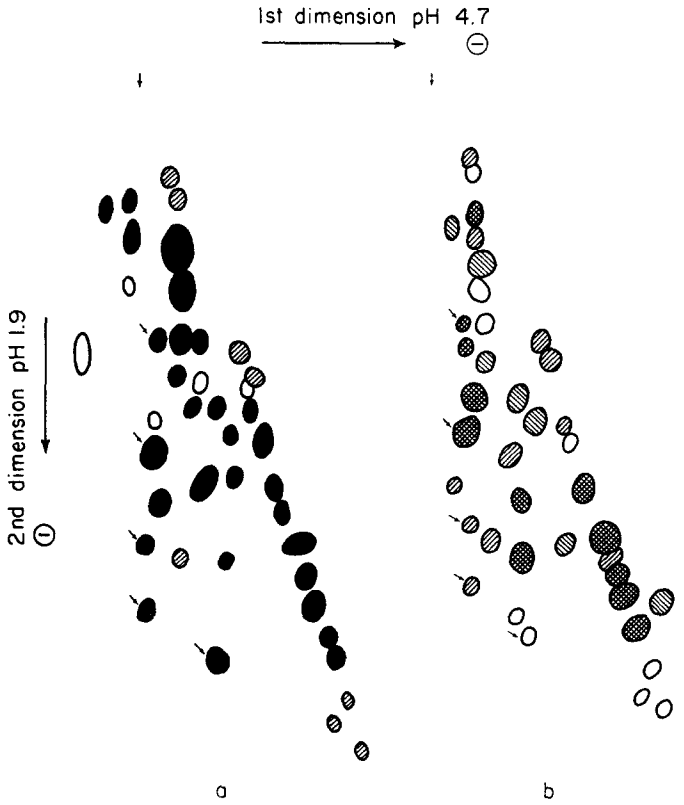
Correspondence of Ninhydrin-Positive Spots in Peptide Maps of Polypeptide Chains and the Various Fragments of Human γG Immunoglobulin

Patterns	Total no. of peptide spots	No. of peptides spots in the same position in maps of:				
		Heavy chains	Light chains	Fab/1 fraction	Fab/2 fraction	Fc fragment
Heavy chains	39	—	5	23	8	19
Light chains	34	5	—	0	29	0
Fab/1 fraction	23	23	0	—	4	12
Fab/2 fraction	37	8	29	4	—	0
Fc fragment	19	19	0	12	0	—

After development with ninhydrin and fixation with copper nitrate, the peptide maps were traced and the patterns so obtained were superimposed. The correspondence was then deduced from the position of the ninhydrin-staining spots on the patterns. Variations in intensity of staining were also taken into account. Very faint spots were not included. Several maps (3 or 4) obtained from different preparations of each chain or fraction were used for the comparisons.

compare with Fig. 1 *a*), and the two fractions showed reactions of antigenic identity (Fig. 11 *b*). The remaining 15 per cent of the 3.8 S material had a lower electrophoretic mobility and shared antigenic determinants with Fab fragments. When heavy chains were digested with soluble papain over a period of 16 hours, almost no slowly migrating material was recovered after electrophoresis on starch. This prolonged proteolysis of the heavy chains was associated with the appearance of several fast moving components.

Isolated heavy chains were also hydrolyzed with pepsin. The counterpart of the Fab/1 fraction appeared to be relatively resistant to further degradation. Components having higher electrophoretic mobilities were also isolated, in agreement with Goodman's observations (33). Their position on starch block corresponded to that of the fast components separated after hydrolysis of heavy chains with papain.



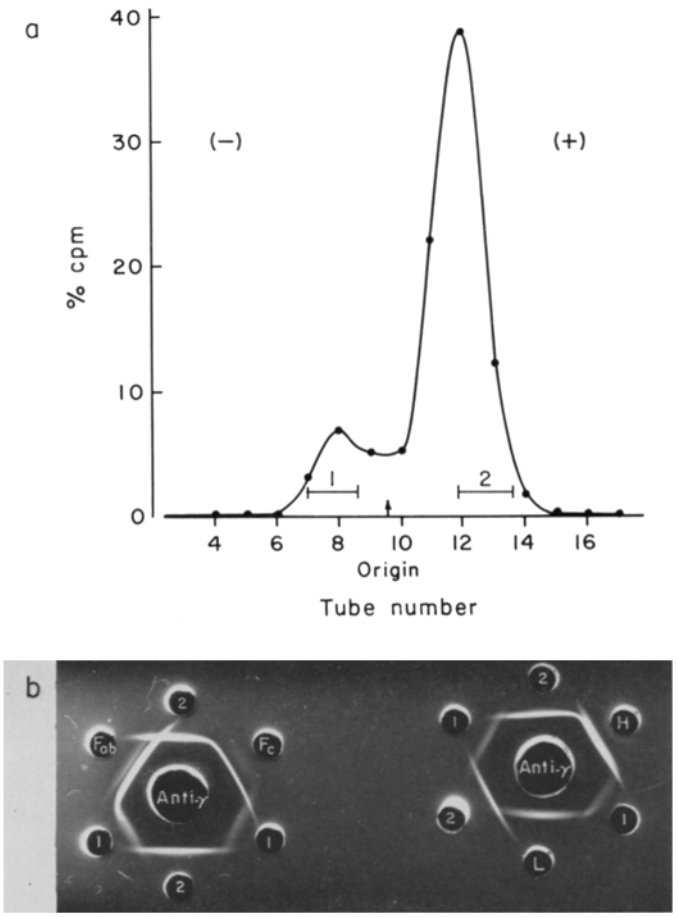
FIGS. 10 *a* and 10 *b*. Composite drawings of the patterns of peptide maps presented in Figs. 8 and 9.

FIG. 10 *a*. Patterns of the "light chain" type (composite drawing of maps of light chains and Fab/2 fraction): ■ Peptides present both in the light chains and in the Fab/2 fraction. ▨ Peptides of Fab/2 fraction also found in patterns of heavy chain type. □ Peptides of light chains not found in Fab/2 fraction.

FIG. 10 *b*. Patterns of the "heavy chain" type. (Composite drawing of maps of heavy chains, Fab/1 fraction and Fc fragments.) ▩ Peptides of the heavy chains present both in the Fab/1 fraction and in the Fc fragment. ▨ Peptides of the heavy chains present in the Fab/1 fraction and not in the Fc fragment. ▧ Peptides of the heavy chains present in the Fc fragment and not in the Fab/1 fraction. □ Peptides present in the hydrolysates of the heavy chains and neither in Fab/1 fraction nor in Fc fragment. The diagonal arrows indicate ninhydrin-positive spots which were common to patterns of both light and heavy chains. The vertical arrows indicate the positions of the origins.

DISCUSSION

Several models of the γG immunoglobulin molecule have been suggested (15–19). Although they differ in several respects, they all agree on the assignment of relationships among the polypeptide chains and the fragments produced by proteolytic hydrolysis. A recent model (19) is depicted in Fig. 12. The



FIGS. 11 *a* and 11 *b*. (*a*) Isolation of the non-dialyzable products of I^{131}-labeled heavy chains hydrolyzed with papain for 1 hour (zone electrophoresis on starch). % CPM: per cent of total counts per minute recovered from starch block. (+) anode, and (−) cathode. (*b*) Immune diffusion of Fab and Fc fragments and of the products of hydrolysis of heavy chains by papain. 1—Slow moving fraction. 2—Fast moving fraction. Fab—Fab fragments and Fc—Fc fragments. L—light chains and H—heavy chains. Anti-γ: Rabbit antiserum against human γG immunoglobulin.

Fab fragments are composed of light chains and a portion of heavy chains (Fd fragments); the Fc fragments comprise the residual portions of the heavy chains.

This assignment has received support from numerous lines of evidence. Antigenic analyses (20, 5, 22) have indicated that the antigenic determinants of light chains are present in Fab fragments whereas the determinants of Fc fragments are present in heavy chains. Starch gel electrophoretic analysis

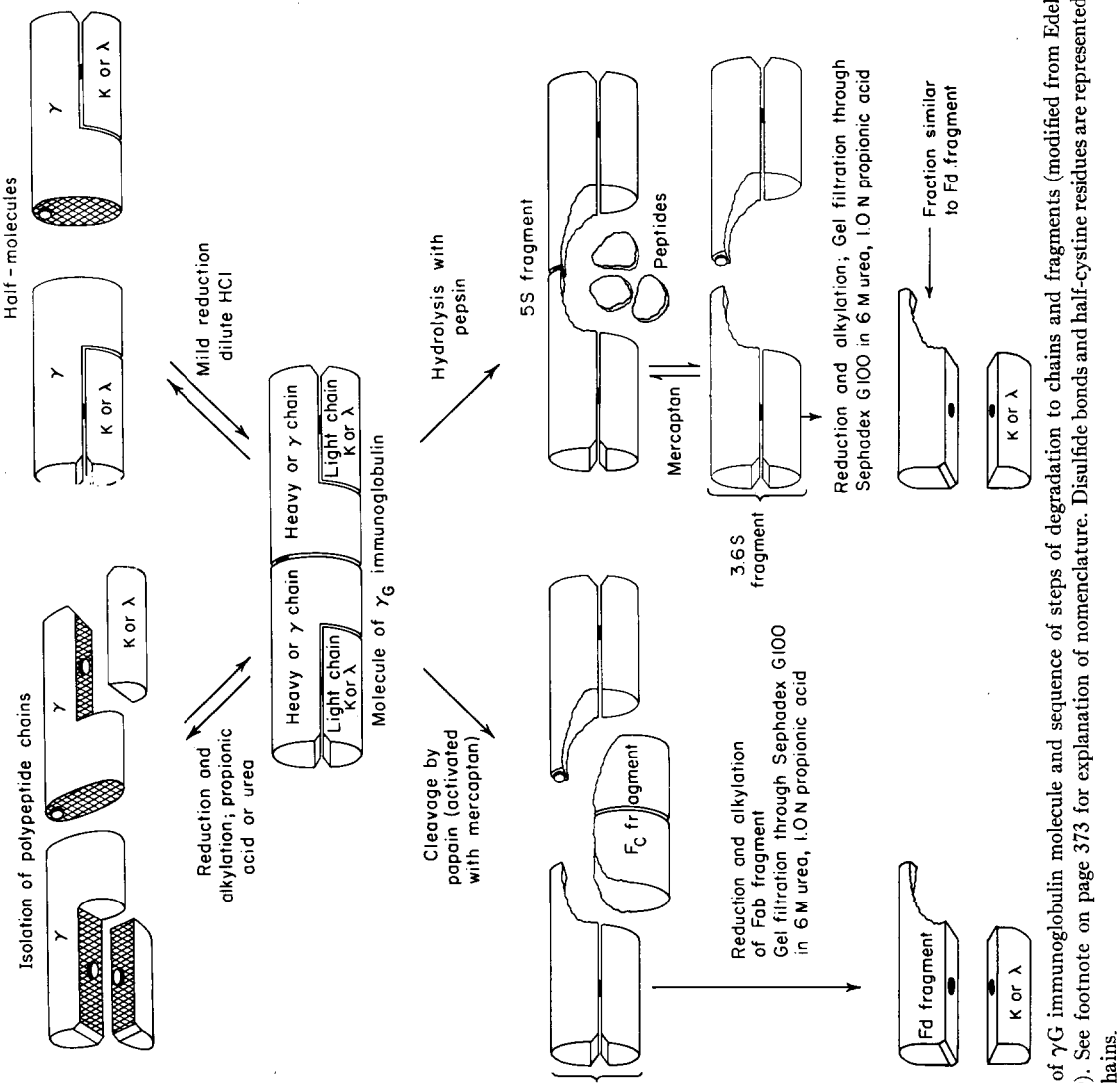


FIG. 12. Model of γG immunoglobulin molecule and sequence of steps of degradation to chains and fragments (modified from Edelman and Gally, reference 19). See footnote on page 373 for explanation of nomenclature. Disulfide bonds and half-cystine residues are represented as small discs between the chains.

suggested the presence of light chains in Fab fragments (15). Fleischman *et al.* (5), Pain (34), and Crumpton and Wilkinson (21) have provided data on the molecular weight and amino acid composition of the chains and fragments of rabbit γ-globulin and have suggested the same arrangement of chains in the molecule. Experiments on doubly labeled reconstituted molecules of γG immunoglobulins (24) and recent studies on mechanism of papain cleavage (35) also support this arrangement. The present studies provide a more direct and specific confirmation of the proposed arrangement of polypeptide chains in γG immunoglobulins. A complete proof, however, must await quantitative recovery and characterization of tryptic peptides both of chains and fragments.

After reduction and alkylation of the Fab fragments of human γ-globulin, Fab/1 and Fab/2 fractions were obtained by gel filtration or starch gel electrophoresis in urea. As discussed below, the Fab/1 fraction appears to consist of dimers of the Fd fragments and the Fab/2 fraction consists of slightly degraded light chains and a variable amount of Fd fragments. Thus, the Fab/1 and Fab/2 fractions corresponded only approximately to the "A piece" and light chain fraction of Fleischman *et al.* (5). In the present experiments, however, the separation of these components was found to be incomplete.

The gel filtration and starch gel electrophoresis indicated that the Fab/1 fraction was in the gross similar in size to the Fab fragment. These results are compatible with the suggestion of Fleischman *et al.* (5) that the Fd fragments may dimerize. The incomplete fractionation of Fd fragments and light chains encountered in the present experiments is attributable to the presence of undimerized material which emerges with the Fab/2 fraction. This was confirmed by the presence of material containing heavy chain label but migrating slightly ahead of light chains after starch gel electrophoresis in urea (see Fig. 5).

All of the tryptic peptides of Fab/1 fractions were present in the peptide maps of heavy chains. Analyses of the Fab/1 fraction prepared from labeled molecules also indicated that it consisted of a portion of heavy chains. The slight contamination with label corresponding to light chains may have resulted from dimerization of light chains (24). These results indicate that the Fab/1 fraction is made up of Fd fragments (Fig. 12).

The Fab/2 fraction resembled the light chains in its behavior upon starch gel electrophoresis and gel filtration. As pointed out above, however, the quantitative analysis of reconstituted doubly labeled molecules indicated an equal contribution of labeled material from heavy chains and light chains. These values were calculated from the specific activities of the chains assuming even distribution and accessibility to iodination of the tyrosyl residues of the chains. This assumption is not completely valid, since the specific activity of the light chains was found to be only 75 per cent as great as that of the heavy chains. It is therefore likely that the calculated values do not reflect precisely the actual mass of fragments of the heavy chains found in the Fab/2 fraction.

Peptide mapping of the Fab/2 fraction showed that most of the light chain peptides were present in that fraction. A small number of peptides of the heavy chains were also identified in the Fab/2 fraction; this is consistent with the presence of heavy chain fragments indicated in the experiments on labeled molecules.

Similar analyses of Fc fragments confirmed that they consist of residual portions of heavy chains. There was considerable overlap of the peptide patterns of Fc fragments and Fab/1 fractions, suggesting that a susceptible region of heavy chains is attacked by papain at multiple points. Hydrolysis of isolated heavy chains by papain indicated that the portion of the chains corresponding to the Fc fragment was relatively resistant to proteolysis, whereas that corresponding to the Fd fragment was cleaved to small peptides. The portion of the heavy chains corresponding to Fd fragments (see Fig. 12) seems less susceptible to attack when combined with light chains in the whole molecule or Fab fragment. It is still more susceptible than the Fc fragment however, as indicated by the variability of the peptide maps of Fab fragments obtained from γG immunoglobulin exposed to papain for 1 hour. In contrast to its behavior after hydrolysis with papain, the Fd fragment appears to be relatively resistant to hydrolysis with pepsin (36).

In agreement with the findings of Nisonoff and co-workers (29), pepsin attacked the Fc fragment preferentially, and left the portions of the molecules corresponding to Fab fragments relatively untouched. The close resemblance of Fab fragments and the 5S fragments (29, 37–39) has been confirmed by the present analyses of peptide maps. As pointed out by Nisonoff and Dixon (40), the sites of cleavage of whole γ-globulin by papain and pepsin may differ slightly. This is in accord with the present observation that an additional 3 peptide spots were resolved in maps of tryptic hydrolysates of Fab/1 fractions derived from the 5S peptic fragment.

The presence of peptide spots appearing in the same places in peptide maps of both heavy and light chains raises the possibility that the chains have certain amino acid sequences in common. Since some of these spots appeared in the region of neutral peptides, better and more quantitative procedures of analysis than that of peptide mapping would be required to decide this point. As emphasized above, although the present experiments provide strong support for the proposed models of the γG immunoglobulin molecule, a complete and rigorous proof will ultimately depend on quantitative recovery and analysis of the peptides.

SUMMARY

The relationships between the polypeptide chains of γG immunoglobulin and fragments of the molecule produced by papain and pepsin have been investigated. Specific procedures were employed including peptide mapping of

tryptic hydrolysates and analysis of molecules reconstituted from chains labeled with different iodine isotopes. By these means, the Fab fragment was shown unequivocally to consist of the light chain and a portion of the heavy chain, the Fd fragment. The Fc fragment was found to be comprised of the residual portions of the heavy chain. These findings support the gross arrangement of chains embodied in recent models of the γG immunoglobulin molecule.

The present studies have also provided additional information on the susceptibility of γG immunoglobulin to proteolytic cleavage. It was found that the portion of heavy chains corresponding to the Fd fragment was extensively cleaved by papain.

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