Biochemistry

Volume 10, Number 1 January 5, 1971

Structure of Heavy Chain from Strain 13 Guinea Pig Immunoglobulin-G(2). I. Isolation of Cyanogen Bromide Fragments*

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ABSTRACT: The determination of the amino acid sequence of heavy chain (γ_2 chain) from the major immunoglobulin in the serum of strain 13 guinea pigs, IgG(2), was undertaken. The aim is to demarcate all sections of this chain having a single amino acid sequence and then to compare the remaining regions showing sequence variability from antibodies of different specificities. γ_2 chain from strain 13 guinea pig IgG(2) was purified by gel filtration after mild reduction and radioalkylation. Four half-cystines were thus radiolabeled. Isolated γ_2 chain was subjected to CNBr cleavage, and five fragments, accounting for 303 residues from the C-terminal three-quarters of the chain, were isolated by gel filtration. Among these fragments were the C-terminal octadecapeptide (C-5), the "hinge region" fragment (C-1-c), the predominant carbohydrate-containing fragment (C-3), and two others (C-1-b and C-4). Fragment C-1-c contains three of the four radiolabeled half-cystines. In addition to these five sections, other CNBr fragments have been found which seem to come from the N-terminal one-quarter of γ_2 chain. The fourth radioactive half-cystine is also found in this region.

Most primary structural analyses of immunoglobulins have been of myeloma proteins, each of which appears to have a single amino acid sequence and to be the product of a clone of cells. The general location of the antibody site within these molecules has been inferred by comparing sequences from proteins grouped according to polymorphic form. Sections of the molecule where members of the group have differing sequences may be involved in determining specificity. These sections have been called "variable" regions.[1]

Another approach for demarcating those sections of immunoglobulin chains which comprise the antibody site is to determine the amino acid sequence of molecules of a particular polymorphic form from normal animals. In this way, one can examine antibodies of a specificity determined by the immunogen selected. In addition, the choice of inbred animals as serum donors avoids that sequence variability due to known or unknown allotypes, which makes difficult the interpretation of data from human paraproteins.

These samples from normal serum are complex mixtures of many specificities. Consequently, those sections of the molecules permitting the elucidation of a single sequence, based on component peptides obtained in high yields, presumably come from constant regions of the molecule, which are not directly involved in forming the antibody site. Sections containing amino acid interchanges and yielding component peptides with mixed sequences could be concerned with specificity. Problems associated with this method include the difficulty of isolating a particular polymorphic form from serum and the problem of isolating cleanly sections bearing a constant sequence in the presence of those with variable sequences.

The heavy polypeptide chain, γ_2 chain, from normal IgG(2) has received the most attention from investigators seeking to localize the antibody site and to describe specificity in terms of primary sequence. This is because isolated heavy chains from specific antibodies have been found to retain specific binding sites (Utsumi and Karush, 1964; Weir and Porter, 1966) and the same relative association as the parent molecule with chemically related haptens (Haber and Richards, 1966). Most light chains from specific antibodies, although they increase the association constants of heavy chains for specific haptens upon recombination (Franěk

* From the Department of Biology, The Johns Hopkins University, Baltimore, Maryland. *Received July 14, 1970.* Supported by the National Science Foundation Grant GB 7852.

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[1] The nomenclature is in accord with that suggested in *Bull. W. H. O.* (1964), *30*, 447, and with that proposed by the Conference on Nomenclature for Animal Immunoglobulins, Prague, June 1969.

et al., 1964), show little or no affinity for antigen when this is measured in the absence of heavy chains.

The C-terminal half of normal rabbit heavy chain, the Fc fragment, has been sequenced (Hill *et al.*, 1967). In addition, the sequences of two large sections of the Fd fragment, the N-terminal half of the heavy chain, were reported (Cebra *et al.*, 1968a,b) which, together with additional partial sequences, accounted for the C-terminal half of the Fd and overlapped into the sequence of Fc. Recently, a set of alternative sequences for the N-terminal 35 residues of this γ_2 chain has been worked out (Wilkinson, 1969), and predominant sequences for other portions of Fd have been placed so that only residues ~N-35–84 and N-100–109 remain unaccounted for in terms of at least one predominant sequence (Fruchter *et al.*, 1970).

Encouraged by the progressive elucidation of much of the sequence from normal rabbit γ_2 chain, we have selected strain 13 inbred guinea pigs (Wright, 1922) as serum donors for the further structural analysis of normal immunoglobulins and purified antibodies. The primary reason for this selection was to avoid the variability in sequences due to differences in genotype found in outbred populations (Wilkinson, 1969; Prahl *et al.*, 1969). Other reasons for choosing the inbred guinea pig as immunoglobulin donor included the availability of methods to separate and distinguish some subclasses of IgG (Oettgen *et al.*, 1965; Osler *et al.*, 1969), the association of distinct secondary biologic activities with different subclasses of IgG (Ovary *et al.*, 1963; Bloch *et al.*, 1963; Oliveira *et al.*, 1970), and the marked differences in the ability of the two available inbred guinea pig families, strain 2 and strain 13, to respond to various immunogens (Levine *et al.*, 1963; Pinchuck and Mauer, 1968).

Materials and Methods

Animals. Our colony of Wright strain 13 inbred guinea pigs (Wright, 1922) was initiated by gifts from Professor Merrill Chase, Rockefeller University, Dr. Gerald Goldstein, University of Virginia, and Dr. Charles Todd, City of Hope, Calif. Breeding has been carried on in our animal facilities.

Preparation of IgG(2). Serum was collected by cardiac puncture from both naive guinea pigs and from those which had been immunized with a variety of hapten–protein conjugates. In general, each guinea pig was bled three times before exsanguination, with a total yield of serum per animal of about 30–35 ml. Specific antibody for use in other studies was removed from the immune sera by stepwise precipitation with hapten coupled to a heterologous carrier protein. The sera were then pooled and dialyzed at 5° for 2 days against 0.01 M sodium phosphate buffer (pH 8.0) to each liter of which was added 60 g of urea. The dialyzed serum (205 ml) was applied to a column of DEAE-cellulose (4 × 48 cm), equilibrated in the same buffer. All serum components, with the exception of IgG(2), were retained by the ion-exchange column. The IgG(2) directly eluted (3.1 μmoles) was then concentrated by dialysis *in vacuo* to a concentration of 20 mg/ml and dialyzed exhaustively against 0.1 M Tris-acetate (pH 8.0). The yield of IgG(2) from naive animals was ~2 mg/ml. Preimmunization increased the yield of IgG(2) to ~4 mg/ml even after the removal of specific antihapten antibody.

Mild Reduction and Radioalkylation of IgG(2). To a solution of IgG(2) (20 mg/ml in 0.1 M Tris-acetate, pH 8.0), solid dithiothreitol was added to make a final concentration of 0.01 M. The reaction vessel was covered, and the reduction was allowed to proceed for 2 hr at 37°. Radiolabeling of the easily reduced disulfide bonds was carried out by alkylation with iodoacetic-1-^{14}C acid. An amount of carrier iodoacetic acid, calculated to make the final reaction mixture 0.02 M, was dissolved and neutralized with 0.0041 ml of 2 M Tris/mg and was mixed with 0.15 mCi of iodoacetic-1-^{14}C acid. This solution was added dropwise to the constantly stirred reaction mixture. The pH was maintained at 8.0 by the continuous addition of 2 M Tris. After 20 min, the reaction mixture was dialyzed at 5° in dialysis tubing from which free thiol groups have been removed, against two 4-l. changes of each of the following: 0.1 M Tris-acetate (pH 8.0) for 5 hr; distilled water for overnight; and lastly, 1 M propionic acid for 24 hr. Up to 350 mg of mildly reduced and radioalkylated γ-globulin was applied to a column of Sephadex G-75 (4.5 × 118 cm) equilibrated in 1 M propionic acid (Fleischman *et al.*, 1962). The column effluent was monitored by reading the absorbance at 280 mμ in cuvets of 1-cm path length in a Zeiss M4QIII spectrophotometer. A typical pattern is shown in Figure 1. Pools were made as indicated: pools I and II contained the heavy chain (Harrington and Fenton, 1970); pool III, the light chain. The heavy- and light-chain pools were freeze-dried directly from propionic acid. A typical yield was 4.8 μmoles of heavy chain from 3.1 μmoles of IgG(2).

Cyanogen Bromide Cleavage of γ_2 Chain. Heavy chain (7.5 μmoles, 375 mg), mildly reduced and radioalkylated, was dissolved in a freeze-drying flask in 88% formic acid and enough water to make the final formic acid concentration 70% and the protein concentration 20 mg/ml. Quantitative cleavage of the heavy chain at methionine residues (Gross and Witkop, 1961) was achieved by adding enough solid CNBr to make a concentration of 100 mg/ml and allowing the reaction mixture to stand at 5° in the glass-stoppered flask for 24 hr. The reaction was terminated by the addition of 10 volumes of water/volume of reaction mixture and immediate freezing. The reaction mixture was then freeze-dried to remove excess reagents.

Purification of the CNBr Fragments. Freeze-dried, mildly reduced and radioalkylated heavy chain (242 mg), after CNBr cleavage, was allowed to stand overnight at 5° in 15 ml of 8 M urea, 0.1 M in formic acid. The solution was then applied to a column of Sephadex G-100 (3.8 × 140 cm) equilibrated in the same solvent. Elution was monitored by reading the absorbance of effluent at 280 mμ (see Figure 2). Pools were freed of urea by passage through a column of Sephadex G-25, coarse (3 × 70 cm) in 0.05 M formic acid, and then freeze-dried. The desalting column was monitored by reading the absorbance at 230 mμ.

Individual pools, certain of which were subjected to complete reduction and alkylation of disulfide bonds, were then recycled through an appropriate column of one of the following types: (1) Sephadex G-75 (3.4 × 180 cm) in 0.05 M NH_4HCO_3; (2) Sephadex G-50, fine (2.2 × 170 cm), in 8 M urea, 0.1 M in formic acid; (3) Sephadex G-50, fine (3.4 × 180 cm), in 0.05 M NH_4HCO_3; and (4) Sephadex G-50, fine (3.4 × 180 cm), in 0.05 M NH_4OH. Details of the procedure for each pool are found in the text.

Effluents from columns in NH_4HCO_3 or NH_4OH were monitored by reading the absorbance at 215 mμ; those from columns in urea, at 280 mμ. Fragments were desalted either by freeze-drying from volatile buffers, by rotary evaporation, in the case of C-5, or by gel filtration through Sephadex G-25, as described above.

Complete Reduction of Disulfide Bonds and Carboxymethylation of Resulting Cysteine Residues. Enough 7 M

TABLE I: Amino Acid Compositions of γ_2 Chain.

	γ_2 Chain	γ_2 Chain (Ox)[a]
Lys	27	27
His	6	
Arg	14	17
CMCys	6	*b*
$CySO_3$	*b*	10–11
Met-sulfone	*b*	6–7
Asp	37	36
Thr	40	40
Ser	48	45
Glu	40	40
Pro	30	36–39
Gly	30	28
Ala	27	26
Val	44	38
Ile	15	16
Leu	29	30
Tyr	18	
Phe	17	14

[a] γ_2 chain was oxidized with performic acid by the method of Moore (1963), converting half-cystines to cysteic acid and methionine to methionine sulfone. Oxidation destroyed most tyrosine and histidine residues. [b] Not present.

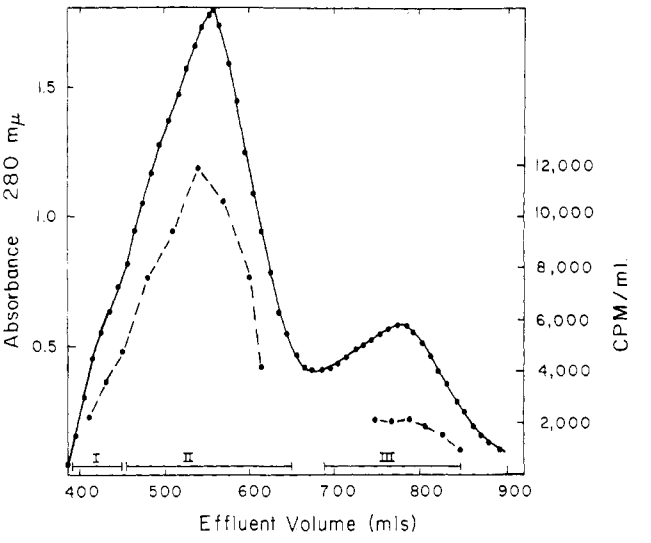

FIGURE 1: Elution profile of the separation of mildly reduced and radioalkylated heavy and light chains from 3.1 μmoles of IgG(2) on a column of Sephadex G-75 (4.5 × 118 cm) equilibrated with 1 M propionic acid. Details of the procedure are found in Materials and Methods. The solid line marks the absorbance at 280 mμ, while the dashed line marks the radioactivity. Fraction size is 10 ml.

guanidine hydrochloride, 0.1 M in Tris-acetate (pH 8.0), was added to the freeze-dried fraction to make the concentration of protein 10–20 mg/ml. Solid dithiothreitol was added to give a final concentration of 0.05 M. The reaction vessel was covered, and the reduction was allowed to proceed at room temperature for 2 hr. Iodoacetic acid, calculated to make the reaction mixture 0.11 M, was dissolved and neutralized in 0.0041 ml of 2 M Tris/mg. This solution was added dropwise to the reaction mixture, which was stirred constantly. The pH was maintained at 8.0 by the addition of 2 M Tris. After 20 min, the reaction mixture was applied to a column for separation of the components.

Amino Acid Analysis. Acid hydrolysis was carried out on 0.015–0.03 μmole of peptide in an acid-cleaned, evacuated (10–20 μ) sealed glass tube (Corning, Pyrex ignition tube, 14 × 100 mm) containing 0.5 ml of constant-boiling HCl and 10 μl of 0.1 M phenol to prevent destruction of tyrosine (Sanger and Thompson, 1963). Hydrolysis was performed in a boiling toluene bath (110°) for 16 hr. Conversion of homoserine from its lactone into the free carboxyl form was done by treatment of the hydrolysate, after removal of HCl by rotary evaporation, with 2 ml of pyridine acetate (pH 6.5) (pyridine–glacial acetic acid–water, 10:0.4:90, v/v) at 100° for 30 min (Ambler, 1965). The pyridine acetate was removed by rotary evaporation and the sample dissolved in 0.2 M sodium citrate (pH 2.2) immediately prior to its analysis.

Other Materials. Urea (8 M) for column chromatography was freed of electrolytes by passage through a column of Amberlite MB-3 (4 × 48 cm).

CNBr (Matheson Coleman & Bell) and Ultra Pure guanidine hydrochloride (Mann Research Laboratories) were used directly. Dithiothreitol (Calbiochem) was stored in a refrigerator.

Iodoacetic acid (Eastman Organic Chemicals) was recrystallized from warm diethyl ether by addition of petroleum ether (bp 20–40°).

Free thiol groups were removed from $^8/_{32}$-in. dialysis tubing (Union Carbide, Food Products Division) by heating it for 15 min at 80° in 0.05 M NH_4HCO_3, followed by its submersion in 0.05 M NH_4HCO_3, 0.01 M in *N*-ethylmaleimide, for 2 hr at room temperature, and lastly washing it in water for 15 min at 80°.

Iodoacetic-1-^{14}C acid (New England Nuclear) was obtained with a specific radioactivity of 7.6 mCi/mmole. Counting of ^{14}C-labeled peptides was done in modified Bray's solution (Segal and Harrington, 1967) or in the solvent system of Kinard (1957) with the use of a liquid scintillation counter (Nuclear-Chicago).

γ_2 chain was oxidized by the performic acid treatment of Moore (1963).

The presence of tryptophan in fragments was determined by the Ehrlich stain of Easley (1965).

Results

Heavy (γ_2) Chain from Guinea Pig IgG(2). The specific activity of heavy chain on mild reduction and radioalkylation was calculated for six points about the peak (Figure 1) and was averaged to be 8448 cpm/mg. The specific activity of light chain (Figure 1) was calculated for five points about the peak and was averaged to be 4203 cpm/mg. Assuming a molecular weight for heavy chain of ~50,000 and that for light chain of ~25,000, the ratio of the specific activities of heavy to light chains is 4:1. Assuming one easily reduced half-cystine on the light chain, we would expect to find four such residues on the γ_2 chain.

The isolated heavy chain gave amino acid compositions listed in Table I for samples either completely reduced and carboxymethylated, or mildly reduced and alkylated and oxidized with performic acid. The residue numbers were normalized to give a total of about 450. The overall analyses are in accord with those previously reported (Lamm, 1969).

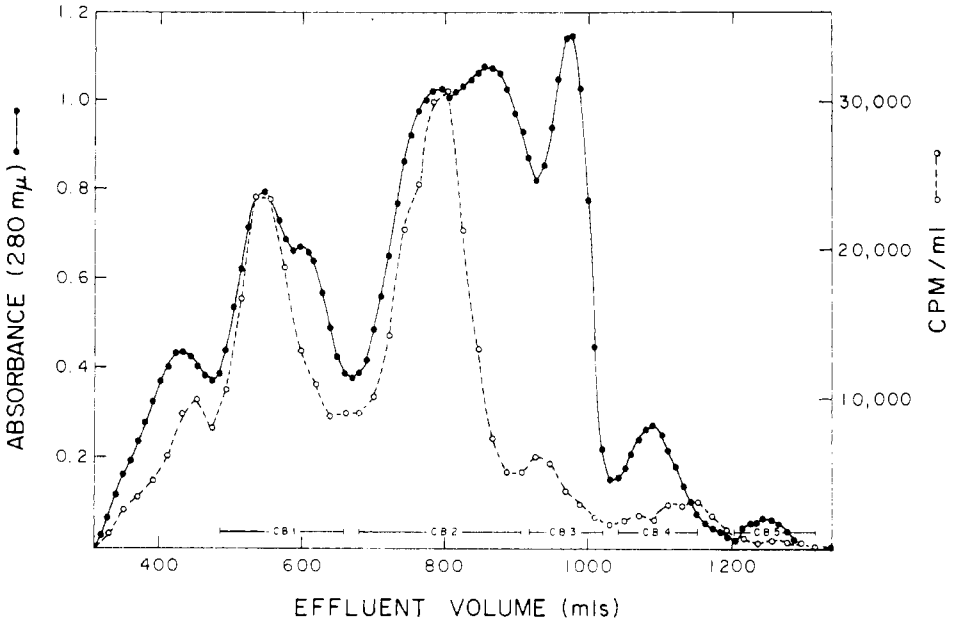

FIGURE 2: Elution profile of the initial separation of CNBr fragments from 4.8 μmoles of γ_2 chain on a column of Sephadex G-100 (3.8 × 140 cm) equilibrated with 8 M urea, 0.1 M in formic acid. Details of the procedure are found in the text. The solid line is the absorbance at 280 mμ; the dashed line is the radioactivity. Fraction size is 10 ml.

The oxidized samples contained six to seven methionine sulfone residues. Thus, in the simplest case, a CNBr digest would be expected to yield seven to eight fragments. Of course, the occurrence of contiguous methionines or of methionines as variable residues would alter the expected number of fragments.

Initial Fractionation of CNBr Digest of γ_2 Chain. Mildly reduced and radioalkylated heavy chain, after subjection to CNBr cleavage, was applied to a column of Sephadex G-100 in 8 M urea, 0.1 M in formic acid (Figure 2). Radioactivity marks the elution of peptides containing carboxymethylated half-cystines which participated in labile disulfide bonds. These labile disulfides of IgGs usually form interchain bridges (Cecil, 1963) but in some instances form intrachain bonds (O'Donnell *et al.*, 1970). In guinea pig IgG(2), they all appear to be involved in forming interchain bonds (Oliveira and Lamm, 1971).

Isolation of Fragments C-3, C-1-c, and C-1-b from Fraction CB2. Fraction CB2 contained the bulk of the radiolabel of the fragmented heavy chain (Figure 2). CB2 was filtered through a column of Sephadex G-75 on 0.05 M NH_4HCO_3, which separated a cold component (pool II) from two radioactive ones (pools I and III). The elution profile of this fractionation is shown in Figure 3. Recycling of the cold fraction (pool II) through a column of Sephadex G-50 in 0.05 M NH_4HCO_3 yielded C-3, whose analysis in Table II indicates

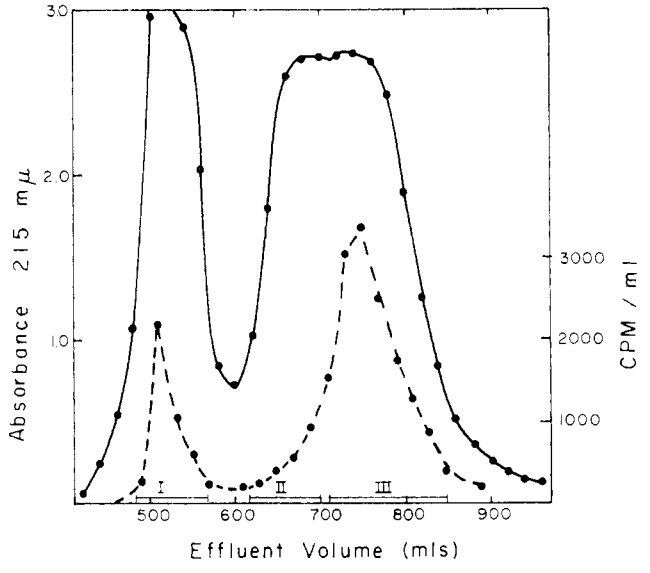

FIGURE 3: Elution profile of the resolution of CB2 on a column of Sephadex G-75 (3.4 × 180 cm) equilibrated with 0.05 M NH_4HCO_3. Details of the procedure are found in the text. The column was monitored by reading the absorbance at 215 mμ (solid line) and by measuring the radioactivity (dashed line). Fraction size was 10 ml.

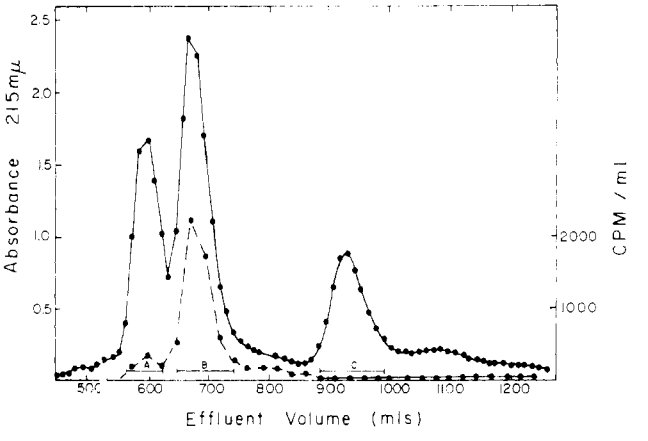

FIGURE 4: The elution profile of the separation of CNBr fragments C-3, C-1-c, and C-1-b from pool III of CB2 after total reduction and alkylation on a column of Sephadex G-50 (3.4 × 180 cm) equilibrated with 0.05 M NH_4HCO_3. Details of the procedure are found in the text. The absorbance is shown by the solid line; the radioactivity, by the dashed line. Fraction size was 10 ml.

TABLE II: Amino Acid Compositions of CNBr Fragments.[a]

	C-1-a_1	C-1-a_2	C-1-b		C-1-c		C-3			C-4		C-5	
Lys	2.4	3.1	2.6	2[a]	7.7	6	9.5	8.2	9	6.0	7	1.0	1
His		0.69	0.85	1	+	1	1.0	1.1	1	0.85	1	2.5	3
Arg	2.3	3.7	0.30		0.72	1	4.2	3.5	4	2.0	2	1.1	1
CMCys		1.9	1.1	1	3.3	4		2.5	2	2.3	2		
Asp	3.8	4.7	1.2	1	4.8	5	8.5	8.8	9	10.3	9	1.1	1
Thr	2.8	5.8	4.2	5	6.0	7	6.9	7.2	8	5.8	6	1.1	1
Ser	3.4	5.4	4.3	5	3.9	5	4.8	5.6	5	9.0	9	2.0	2
Glu	2.6	2.6	2.2	2	4.3	4	11.3	10.9	11	6.5	7	2.1	2
Pro	1.5	3.5	3.1	3	14.0	14	7.9	8.0	8	7.4	8	0.84	1
Gly	4.2	4.3	4.7	5	2.1	2	3.2	3.3	3	2.4	3	1.2	1
Ala	2.2	6.1	2.2	2	3.0	3	4.4	4.3	4	4.1	4	1.9	2
$^1/_2$-Cys							1.7						
Val	2.2	5.7	4.6	5	5.6	6	10.8	11.4	11	9.3	9	0.86	1
Ile	2.3	1.2			2.1	2	3.9	3.5	4	3.2	3	0.99	1
Leu	2.8	3.6	5.1	6	2.3	2	4.7	4.5	5	5.1	5	0.99	1
Tyr	1.9	3.0	1.7	2		0	3.4	3.2	3	4.3	4		
Phe	1.2	1.7	1.9	2	1.9	2	3.5	3.9	3	2.6	3		
Hsr	1.0	1.0	1.0	1	1.0	1	0.83	0.81	1	0.90	1		
Trp[b]	+	+	+	1	0	0	+	+	2	+	+	0	0
CHO[c]	0	0	0	0	±	±	++	++	++	0	0	0	0
Sum				44		65			93		83		18
Pool of origin	CB4	CB3-III-C	CB2-II-C		CB2-II-B		CB2-II	CB2-III-A		CB3-III-A		CB5-1	
N terminal				Thr[d]		Val[e]			Ile[e]		Pro[e]		His[f]
Yield (%)[g]	20	10		19		18			43		20		42

[a] The compositions of C-1-a_1 and C-1-a_2 are based on homoserine equal to 1.0. Whole number columns are the average of many analyses and are supported by sequence data and homology with other sequences. The other columns give single analyses of purified fragments typical of those used for sequence determination. Fragments at this stage of purification gave no significant amounts of amino acids upon N-terminal residue determination other than the ones listed. The tryptic peptides isolated from these fragments accounted for the compositions shown and no other extraneous peptides were isolated in significant amounts. CNBr fragments C-1-b, C-1-c, and C-5 have been completely sequenced. [b] The presence of tryptophan was established by the Ehrlich stain of Easley (1965). Whole numbers reflect actual sequence data. [c] The presence of carbohydrate in a fragment was determined by the appearance of galactosamine or glucosamine on amino acid analysis. [d] Determined by sequence data. See Birshtein *et al.* (1971). [e] Determined by the method of Stark and Smyth (1963). [f] Determined by thin-layer chromatography of the PTH derivative (Turner and Cebra, 1971). [g] Average final yields, based on amount of heavy chain digested with CNBr, are given for fragments prepared using the same fractionation steps shown in Figure 8.

that it contains about 93 residues and is the predominant carbohydrate-containing CNBr fragment.

Complete reduction and alkylation of the remaining disulfides of the major radioactive fraction (pool III), followed by its passage through a column of Sephadex G-50 in 0.05 M NH_4HCO_3, resolved two components, C-1-c and C-1-b (pools B and C, respectively), as depicted in Figure 4. Their analyses are listed in Table II. Fragment C-1-c, distinguished by its high proline content and by the presence of labile half-cystines, is the hinge region fragment. Fragment C-1-b contains about 44 residues and was recovered from CB2-pool III in a yield approximately equimolar to C-1-c.

Pool A from CB2-pool III (analysis in Table I) contained fragment C-3, the bulk of which was isolated from the adjoining CB2-pool II, as described above.

The major fraction from CB2, pool I of Figure 3, contained about 25% of the total radiolabel in CB2 and included components of C-1-a. It will not be treated further here.

Isolation of C-4 and C-1-a_2 from Fraction CB3. Fraction CB3 was initially resolved into several components by gel filtration through a column of Sephadex G-50 equilibrated with 8 M urea, 0.1 M in formic acid (Figure 5). The major fraction (pool III) was freed of radioactivity by passage, after complete reduction and alkylation, through the same column (Figure 6). This cold peptide, the component in pool A (Figure 6), was designated C-4; and its composition, amounting to about 83 residues, can be found in Table II.

Pool C from this column (Figure 6) contained a single peptide, C-1-a_2, whose analysis is found in Table II. This component, labeled with carboxymethyl-^{14}C groups, was obviously present in yields far less than that of the other fragments derived from the IgG(2) digest. However, in other serum pools, such as the one from guinea pigs immunized with dinitrophenylhemocyanin, C-1-a_2 was recovered in considerably higher yield.

As would be expected from the obvious overlap between fractions CB2 and CB3 (Figure 2), CB3 also contains some fragment C-3, which was eluted in the fractions comprising pool II of the G-50 column whose elution pattern is given in Figure 5.

Recovery of C-1-a_1 from Fraction CB4. Passage of CB4 through a column of Sephadex G-50 in 8 M urea 0.1 M in

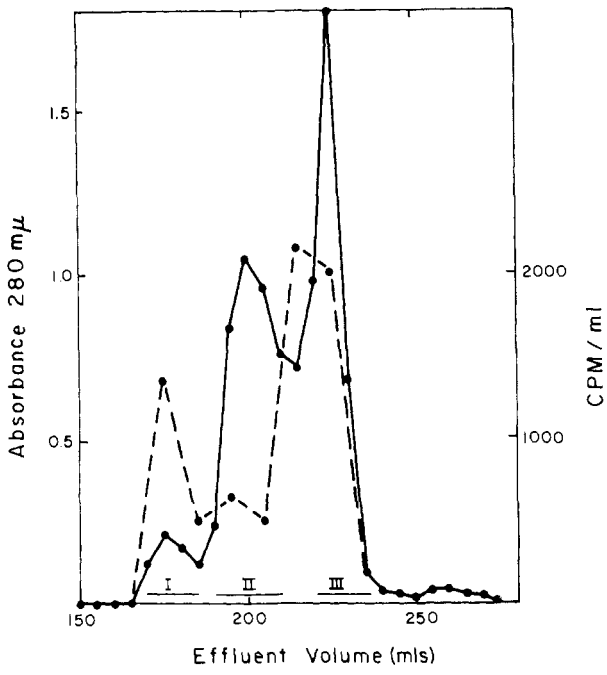

FIGURE 5: The elution profile of the separation of CNBr fragments from CB3 on a column of Sephadex G-50 (2.2 × 170 cm) equilibrated with 8 M urea, 0.1 M in formic acid. Details of the procedure are found in the text. The absorbance is shown by the solid line. The radioactivity by the dashed line. The fraction size was 5 ml.

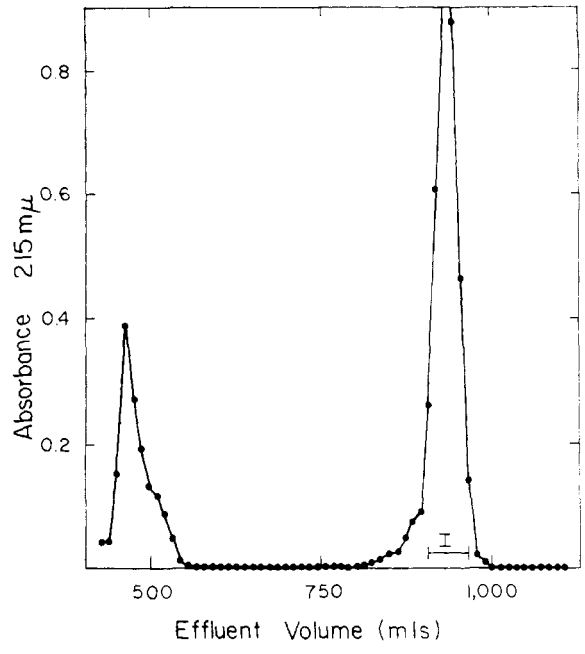

FIGURE 7: The elution profile of the purification of C-5 from pool CB5 on a column of Sephadex G-50 (3.4 × 180 cm) in 0.05 M NH_4OH. Details are found in the text. Fraction size was 10 ml.

formic acid yields C-1-a_1. This component gives the amino acid analysis found in Table II. The peptide contains about 40 residues and has no half-cystines.

Preparation of C-5 from Fraction CB5. Recycling of CB5 through a column of Sephadex G-50 equilibrated with 0.05 M NH_4OH (Figure 7) yielded one major pool (pool I) containing a single peptide, C-5. Its analysis (Table II) shows that it lacks homoserine and consequently places it as the C-terminal octadecapeptide.

Composition of Fraction CB1. After complete reduction and alkylation and subsequent passage through a column of Sephadex G-75, CB1 yielded C-3 and C-1-c as principal components, in addition to an unidentified peptide(s). Since C-3 and C-1-c appear as independent peptides from pools CB2 and CB3, as described above, their presence in CB1 suggests that this portion is composed of aggregates of the fragments already discussed. Consequently, CB1 was not utilized to prepare individual fragments of γ_2 chain.

An outline of the procedures used to isolate the various CNBr fragments is shown in Figure 8.

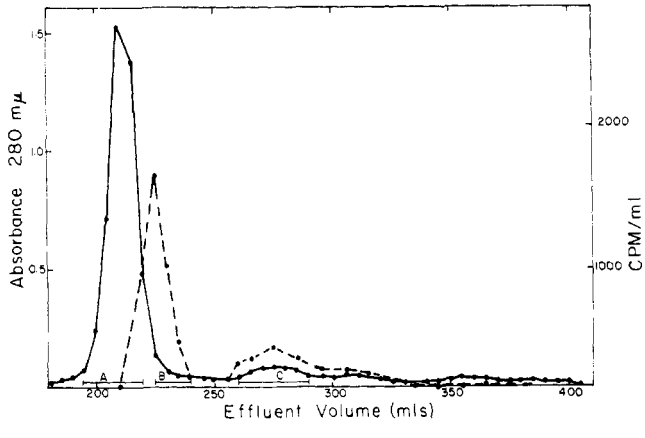

FIGURE 6: The elution profile of the separation of components of pool III from CB3 after total reduction and alkylation on a column of Sephadex G-50 (2.2 × 170 cm) equilibrated with 8 M urea, 0.1 M in formic acid. Details of the procedure are found in the text. A solid line marks the absorbance at 280 mμ; a dashed line, the radioactivity.

Discussion

The treatment of heavy chain from strain 13 guinea pig IgG(2) with CNBr has yielded 5 fragments accounting for the ~303 residues which comprise the C-terminal three-quarters of the chain. This part of γ_2 chain appears homologous with the "constant" part of heavy chain from myeloma IgGs. Subsequent analysis has shown that two of the fragments, C-1-c and C-1-b, are joined by a disulfide bond (Birshtein *et al.*, 1971). Two other fragments, C-3 and C-4, each contain an internal disulfide bond. The fifth fragment, C-5, is the C-terminal octadecapeptide. Radioalkylation of the easily reduced half-cystines places three of them on C-1-c and one on a section N terminal to C-1-b (Turner and Cebra, 1971; Birshtein *et al.*, 1971). Those half-cystines thus marked on C-1-c participate in interheavy chain disulfide bridges while the half-cystine N terminal to C-1-b thus labeled participates in a heavy–light disulfide bridge (Oliveira and Lamm, 1971). Figure 9 shows the tentative alignment of the cyanogen bromide fragments and depicts a number of other structural features described here. Formal alignment was established from a tryptic digest of intact heavy chain, which provided peptides containing methionine (D. C. Benjamin and Q. Z. Hussain, unpublished data). A comparison of the location of methionines in guinea pig heavy chain with those in heavy chains from rabbit (Hill *et al.*, 1967; Fruchter *et al.*, 1970) and a human IgG(1) myeloma protein (Edelman *et al.*,

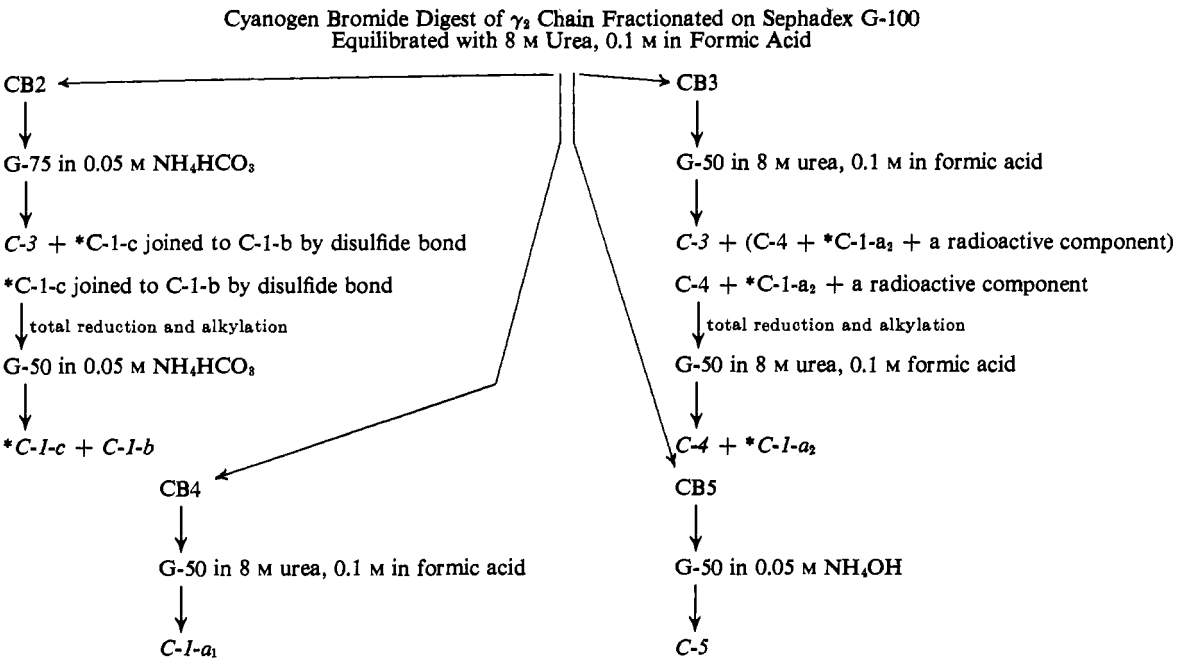

FIGURE 8: An outline of the steps used in isolating the CNBr fragments from γ_2 chain. An asterisk marks the presence of a radioactive CMCys that has resulted from mild reduction and radioalkylation of the parent IgG(2) molecule.

1969) is shown in Figure 9. The positions marked by the C terminus of C-1-c and the C terminus of C-4 are conserved in these three examples.

Fragments composing a section homologous with the "variable" region of myeloma heavy chain have not been as clearly distinguished as those from the constant region. There is reason to suspect that the methionine residues at which CNBr cleaves are not constant within the variable region. Positions N-34 and N-85 may be two such positions. Upon tryptic digestion, pools CB2-I, CB3-III-C, and CB4 have yielded peptides, which, by analogy with rabbit and Eu heavy chain sequences, come from the variable region. CB4 seems to span the region between residues 34 and 85 while CB3-III-C, a minor component from normal immunoglobulin, contains the peptide T37, which has been formally placed immediately N terminal to C-1-b (Birshtein *et al.*, 1971).

Cyanogen bromide cleavage of anti-dinitrophenyl antibodies leads to a digest, which, upon fractionation, shows dramatic changes in the proportions of fragments coming from the variable region. In contrast to the low yield of pool C in Figure 6, which contains C-1-a_2, the corresponding pool derived from anti-dinitrophenyl antibodies is found in high yield and contains the majority of specifically reacted affinity label (A. Ray, unpublished data).

Succeeding papers will deal with the sequence of the CNBr fragments from the C-terminal three-quarters of heavy chain. In addition, the localization of the site of affinity-labeling for anti-dinitrophenyl antibodies will be presented as a complementary step in demarcating the region containing the antibody site.

Based on the description of the major sequence underlying guinea pig IgG(2) heavy chain, additional subclasses of this chain may come to be defined. It is hoped that subgroups of the variable region, if they exist, can be resolved and that comparisons of the regions composing the antibody sites in antibodies directed toward various antigens can be achieved.

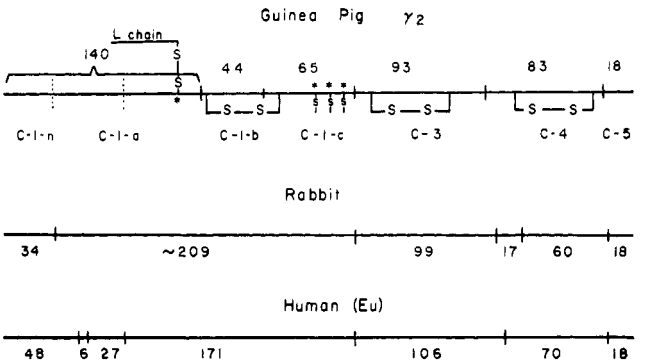

FIGURE 9: Schematic representation of the CNBr fragments of guinea pig γ_2 chain as compared with rabbit and Eu. The location of methionine is marked by a solid vertical line. A dotted vertical line marks the possible location of methionines. Disulfide bridges are indicated by –S–S–. Half-cystines labeled upon mild reduction and radioalkylation are marked with an asterisk.

To this end, the raising of less heterogeneous antibodies in the guinea pig seems essential. The success in eliciting antibodies in rabbits with a distribution of isoelectric points and association constants more restricted than found in most antisera (Eichmann *et al.*, 1970; Pincus *et al.*, 1970; Nisonoff *et al.*, 1967) is therefore encouraging. Since the magnitude of the immune response in these rabbits seems under some form of genetic control (Braun *et al.*, 1969), we are hopeful that the appropriate choice of antigen and route of administration will evoke a similar type of response in inbred guinea pigs.

Acknowledgments

We thank Mrs. Sandra Graziano for excellent technical assistance.

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