The Covalent Structure of a Human γG-Immunoglobulin. II. Isolation and Characterization of the Cyanogen Bromide Fragments*

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ABSTRACT: A human γG-immunoglobulin (Eu) has been cleaved into ten different fragments by treatment with cyanogen bromide. These fragments have also been obtained by CNBr treatment of the light and heavy chains. The Fab(t) and Fc(t) fragments, produced by limited tryptic proteolysis of Eu, also yielded the expected CNBr fragments with one exception. The region of the heavy chain corresponding to CNBr fragment H_4 was found to be cleaved by trypsin into two fragments, H_{4A} (found in Fab(t)) and H_{4B} (found in Fc(t)). The CNBr fragments were characterized in terms of molecular weight, amino acid composition, and terminal amino acid residues, and it was found that they accounted for the entire γG-immunoglobulin molecule. A partial arrangement of the cyanogen bromide fragments was made on the basis of their terminal amino acids and their location in the chains and tryptic fragments.

Human γG-immunoglobulin has a molecular weight of approximately 154,000, and in order to determine its covalent structure, it is highly desirable to disassemble the molecule into smaller, more workable fragments. The previous paper in this series (Edelman *et al.*, 1968) described the isolation of light and heavy chains after reduction and alkylation of the interchain disulfide bonds and the production of enzymatic fragments by tryptic digestion. These components may be broken down further by specific chemical cleavage with reagents such as CNBr. When a peptide containing an internal methionine is treated with CNBr under acidic conditions, methionine is converted into homoserine lactone, and the peptide is cleaved at this point (Gross and Witkop, 1962).

In this communication, we report the use of CNBr to cleave a human γG myeloma protein (Eu) into ten different fragments. With the exception of two fragments from the light chain, which we have been unable to separate from each other, each of the CNBr fragments has been isolated and characterized. The CNBr fragments were also obtained from light and heavy chains and from Fab(t),[1] Fc(t), and Fd(t) fragments. An unequivocal assignment of each CNBr fragment to these portions of the γG-immunoglobulin molecule was made. The isolation and characterization of the CNBr fragments have proven to be a key step in determining the amino acid sequence of γG-immunoglobulin.

Materials and Methods

The purification of Eu, the isolation of light and heavy chains, and the production of Fab(t) and Fc(t) fragments by tryptic digestion have been previously described (Edelman *et al.*, 1968). Fd(t) was prepared from Fab(t) by gel filtration on Sephadex G-100 in 0.5 M propionic acid (Fougereau and Edelman, 1965). The Fd(t) fragment could not be obtained completely free of light chains. Tryptic digestion of this fragment yielded known peptides of the light chain in 12% yield.

CNBr Cleavage. In preliminary studies, it was found that treatment of the protein with CNBr in dilute HCl or trifluoroacetic acid produced extensively aggregated fragments. Consequently, 70% formic acid was used as the solvent during the CNBr treatment. The protein was dissolved to a final concentration of 50 mg/ml in 70% formic acid. A 1.5-fold (w/w) amount of CNBr was added and the reaction was allowed to proceed at room temperature for 4 hr. The reaction was terminated by the addition of ten volumes of distilled water, followed immediately by lyophilization. Amino acid analyses showed that all methionine residues were lost after this treatment.

Gel Filtration. For large-scale fractionation of the CNBr fragments, Sephadex G-100 was equilibrated with 1 M propionic acid, poured into 5.5 × 200 cm columns,

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Fab(t), Fc(t), Fd(t), tryptic fragments corresponding to Fab, Fc, and Fd (World Health Organization, 1964); dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; Hsr, homoserine; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine.

TABLE I: Properties of CNBr Fragments from Eu.

Fragment	Yield (%)[a]	Mol Wt ($\times 10^{-3}$)	NH_2 Terminus	COOH Terminus	Obtained from				
					Light	Heavy	Fd	Fc	Fab
L_1–S–S–L_2	70	10.5[b]	Asx, Tyr	Hsr	+, +				+, +
L_3	67	13.0[b]	Phe	CMCys	+				+
H_1	56	5.1[b]	(Blocked)	Hsr		+	+		+
H_2	70	0.5[b]	Gly	Hsr		+	+		+
H_3	100	3.0[b]	Phe	Hsr		+	+		+
H_4	88	18.5	Glx	Hsr		+			
H_{4A}		15.5					+		+
H_{4B}		3.2[b]	Thr	Hsr				+	
H_5	75	14.8[c]	Ile	Hsr		+		+	
H_6	60	8.6	Thr	Hsr		+		+	
H_7	100	2.0[b]	His	Gly		+		+	

[a] From Eu. [b] Calculated from amino acid composition. [c] Includes carbohydrate.

and eluted with the same solvent. Loads of 0.5–1 g were fractionated on these columns. Smaller amounts of material were passed through 2.5 × 200 cm columns. Absorbance measurements at 280 and 235 mμ were used to detect fragments in the effluent.

Complete Reduction and Alkylation. A 1–2% solution of protein in 6 M guanidine hydrochloride, 0.5 M in Tris and 0.002 M in EDTA (pH 8.1), was placed in a screw-cap vial. The tube was flushed with nitrogen, capped, and placed in a 50° water bath for 30 min to denature the protein fully. Dithiothreitol (50 moles/mole of disulfide in the protein) was added; the tube was flushed briefly with nitrogen and maintained at 50° for 4 hr. The solution was then cooled to room temperature and iodoacetamide (100 moles/mole of disulfide) was added with addition of NH_4OH as necessary to maintain constant pH. After 20 min in the dark, the mixture was loaded on a Sephadex G-100 column for the separation of fragments.

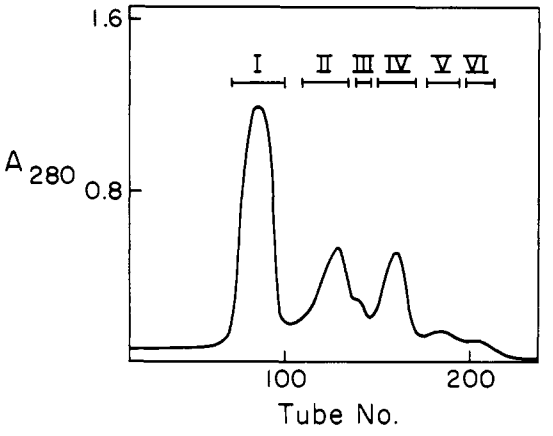


FIGURE 1: Gel filtration of CNBr-treated Eu (500 mg) on Sephadex G-100 (5.5 × 150 cm) in 1 M propionic acid. Volume per tube: 20 ml. A_{280}: absorbance of effluent at 280 mμ. Roman numerals designate the fractions which were pooled for further purification.

Ion-exchange chromatography was carried out on 0.9 × 50 cm columns of SE Sephadex C-25 at 60°. The ion exchanger was equilibrated with initial buffer and degassed prior to pouring into the heated column. The sample was loaded in the initial buffer and elution was effected with a linear gradient from 350 ml of 0.05 M pyridine acetate (pH 3.1) to 350 ml of 1.0 M pyridine acetate (pH 5.6). A constant flow rate of 30 ml/hr was maintained throughout the elution by the use of a pump (miniPump, Milton Roy, Philadelphia, Pa.). After chromatography the SE Sephadex was removed from the column and washed with 4 M pyridine acetate (pH 5.5).

The major portion of the effluent stream was directed to a time-controlled fraction collector set to collect approximately 3-ml fractions. The remainder of the effluent was diverted to an AutoAnalyzer (Technicon, Ardsley, N. Y.) for continuous monitoring, both before and after hydrolysis by NaOH. The manifold of the AutoAnalyzer was a modification of that described by Catravas (1964).

Terminal Amino Acid Residues. NH_2-terminal amino acid residues were determined by the dansyl method of Gray (1967), except that the dansylamino acids were identified by two-dimensional chromatography on polyamide thin layers as described by Woods and Wang (1967). The COOH-terminal amino acids of fragments L_3 and H_7 were determined by sequence analysis of their COOH-terminal tryptic peptides (Cunningham *et al.*, 1968; W. E. Gall and G. M. Edelman, unpublished results). A single homoserine residue was found in each of the other CNBr fragments and was assigned to the COOH terminus (Gross and Witkop, 1962).

Amino acid analyses were performed using the Beckman 120C analyzer, according to the procedures of Spackman *et al.* (1958), modified as described by Edelman *et al.* (1968). Samples for amino acid analysis were hydrolyzed in metal-free 6 N HCl at 110° *in vacuo*. Reported compositions are based on the values obtained after 20-hr hydrolysis. No corrections were made for

the destruction of labile amino acids or for the partial release of amino acids resistant to hydrolysis. Tryptophan analyses are not reported. Half-cystine was determined either as cysteic acid (Moore, 1963) or as CM-cysteine. The reported values for homoserine (Hsr) are the sums of homoserine and homoserine lactone.

Carbohydrate analyses were carried out with anthrone reagent as previously described (Edelman *et al.*, 1968).

Molecular weights of the CNBr fragments were determined in the Beckman Model E analytical ultracentrifuge using the methods described by Yphantis (1960, 1964). The measurements were made in a dissociating solvent consisting of 20% acetic acid and 0.5% sucrose in water. The partial specific volume of each CNBr fragment was estimated from its amino acid composition and carbohydrate content.

Starch gel electrophoresis in formate–urea was performed as described by Edelman and Poulik (1961).

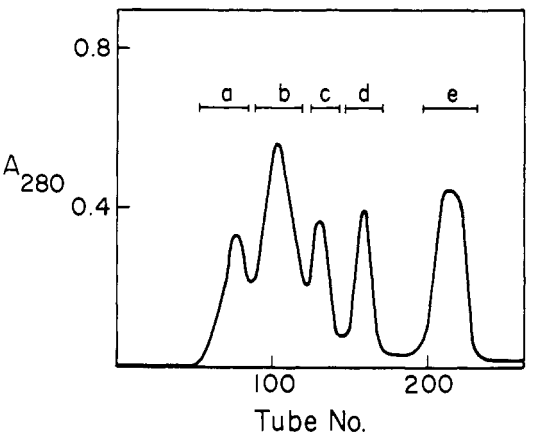


FIGURE 2: Gel filtration of 250 mg of completely reduced and alkylated fraction I (Figure 1) on Sephadex G-100 (5.5 × 150 cm) in 1 M propionic acid. Volume per tube: 20 ml. A_{280}: absorbance of effluent at 280 mμ.

Results

The intact molecule contains 18 methionyl residues, 3 of which are in each light chain and 6 of which are in each heavy chain (Edelman *et al.*, 1968). If the two half-molecules of Eu were identical, eleven unique CNBr fragments would be expected, four from each of the light chains and seven from each of the heavy chains. As will be shown below, only a small amount of cleavage occurred at the first methionyl residue near the NH_2 terminus in the light chains, despite the fact that no remaining methionine could be detected by amino acid analysis. Hence, the light chain was cleaved into only three major CNBr fragments, and a total of ten unique fragments was obtained from the whole molecule.

The fragments which arise from the light chain will be designated L_1, L_2, and L_3 and those from the heavy chain will be designated H_1 to H_7. For convenience in following the description of the fractionation, the reader may refer to Table I for a summary of the yields, molecular weights, and terminal amino acid residues of the fragments, as well as the portions of Eu from which the fragments have been isolated.

Separation of the CNBr Fragments of Eu. After treatment with CNBr, Eu was subjected to gel filtration on Sephadex G-100 in 1 M propionic acid (Figure 1). The material in each of the pooled fractions (I–VI) was subjected to further fractionation and purification. Fraction I contained three CNBr fragments joined by disulfide bonds. Fractions II and III contained a mixture of three components. Fractions IV and V each contained two distinct components, and fraction VI contained a single component of low molecular weight. Each fraction was characterized by gel filtration, starch gel electrophoresis, amino acid analysis, and end-group determination. These fractions were compared to corresponding fractions obtained after CNBr cleavage of light and heavy chains, and Fab(t), Fc(t), and Fd(t) fragments.

Fraction I. The material in fraction I (Figure 1) was completely reduced and alkylated. Gel filtration over Sephadex G-100 in 1 M propionic acid resulted in the pattern shown in Figure 2. Fraction a of Figure 2 was found to contain aggregated material and was not studied further. Fraction b was purified from the material in fractions a and c by another gel filtration over Sephadex G-100 in 1 M propionic acid. The repurified material (H_4) gave a single band on starch gel electrophoresis in formate–urea. Fragment H_4 could also be isolated from CNBr-cleaved heavy chain, but could not be detected in the cleavage products of light chains, Fab(t), Fd(t), or Fc(t). The failure to isolate H_4 from the enzymatically produced fragments suggests that the tryptic cleavage of the heavy chain which gives rise to Fd(t) and Fc(t) occurs at some point in the region corresponding to the CNBr fragment H_4. As will be shown below, all of the other CNBr fragments could be isolated from cleavage products of Fab(t), Fc(t), or Fd(t) fragments.

The material in fraction c (Figure 2) was separated from H_4 by gel filtration over Sephadex G-100. This CNBr fragment (L_3) was also obtained by the CNBr cleavage of Fab(t) (see Figure 5, fraction c) and light chains. Fraction d (Figure 2) contained fragment H_1 which was also isolated from heavy chains, where it was found linked to H_4 by a disulfide bond. It was also found in Fab(t) and Fd(t) attached to a part of H_4 (H_{4A}) by a disulfide bond. Fraction e (Figure 2) contained reagents only. Thus, after reduction and alkylation, material from fraction I (Figure 1) yielded two fragments (H_1 and H_4) from the heavy chain and one fragment (L_3) from the light chain.

Fractions II and III. Fraction II (Figure 1) could be partially resolved into three components by subjecting 50-mg quantities to gel filtration on 2.5 × 200 cm columns (Figure 3). Peak b contained H_5 and peak c contained H_6. Peak a contained material having an amino acid composition corresponding to an equimolar mixture of H_5 and H_6. This material had a single NH_2-terminal isoleucine residue, which was the same as the NH_2-terminal residue of H_5. Material from fraction a showed a single band on starch gel electrophoresis in formate–urea, both before and after reduction and alkylation, and hence did not appear to be a mixture of H_5 and H_6 joined by disulfide bonds. Therefore, the material in peak a was designated $H_{5,6}$. It is not known

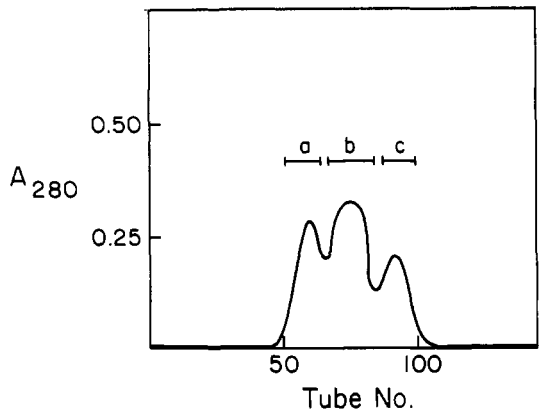


FIGURE 3: Gel filtration of 50 mg of fraction II (Figure 1) on Sephadex G-100 (2.5 × 200 cm) in 1 M propionic acid. Volume per tube: 5 ml. A_{280}: absorbance of effluent at 280 mμ.

whether the presence of this fragment is due to incomplete CNBr cleavage at the methionyl residue or to the replacement of the methionyl residue in some of the heavy chains by another amino acid residue. Fraction III (Figure 1) contained H_6. Fragments H_5, H_6, and $H_{5,6}$ were also found after the CNBr treatment of heavy chains and Fc(t).

Fraction IV. Material from fraction IV (Figure 1), after separation from contaminating fraction III by gel filtration on Sephadex G-100, gave a single band on starch gel electrophoresis in formate–urea. Reduction and alkylation of this material resulted in an apparent decrease in molecular weight as shown by gel filtration and the appearance of two bands in starch–urea gels. Attempts to separate by other means the two components produced after reduction and alkylation of fraction IV were unsuccessful. Nevertheless, the presence of two NH_2-terminal residues in fraction IV and the appearance of two electrophoretically distinct bands in the reduced material have led to the conclusion that fraction IV contains two CNBr fragments linked to each other by a disulfide bridge. This view is supported by the amino acid composition of the reduced and alkylated material which showed the presence of two residues of CM-cysteine. The same fragments were also obtained by CNBr cleavage of light chains and Fab(t) (see Figure 5, fraction c). In these two cases they were not separated from L_3. Because both fragments were derived from the light chains, they have been designated as L_1 and L_2. The amino acid composition of the disulfide-linked component (L_1–S–S–L_2) which comprises fraction IV (Figure 1) is consistent with the amino acid sequence of the NH_2-terminal portion of the light chain (Cunningham *et al.*, 1968).

Fractions V and VI. The components of fractions V and VI (Figure 1) were purified by ion-exchange chromatography on SE Sephadex. The material in fraction V was resolved into two CNBr fragments (H_3 and H_7) by this technique (Figure 4). Both of these fragments were also obtained from CNBr-treated heavy chains. Fragment H_3 was isolated from Fab(t) (see Figure 5, fraction d) and Fd(t). Fragment H_7 was also obtained from Fc(t) (see Figure 6, fraction c).

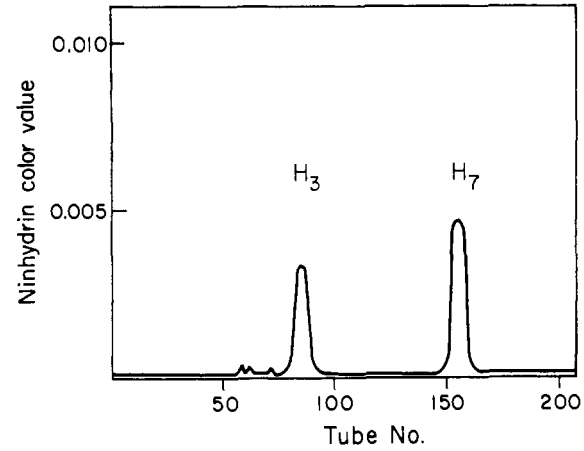


FIGURE 4: Separation of H_3 and H_7 on SE Sephadex C-25 (0.9 × 50 cm) at 60°. Elution was performed with a linear gradient of pyridine acetate (see Materials and Methods). Peptides were detected by diverting 1% of the effluent for continuous ninhydrin monitoring. Ninhydrin color value: absorbance at 570 mμ of ninhydrin reaction.

Fraction VI (Figure 1) was freed of contaminating material by chromatography on SE Sephadex. This fragment (H_2) was also obtained by the CNBr cleavage of Fab(t) (see Figure 5, fraction e), Fd(t), and heavy chain.

CNBr Cleavage of Fab(t) and Fc(t). CNBr cleavage of the enzymatically produced fragments and of light and heavy chains was undertaken in order to assign the CNBr fragments to the appropriate positions in the whole molecule. It was first necessary to identify the CNBr fragments from Fab(t) and Fc(t) which together constitute H_4. The gel filtration pattern obtained after CNBr treatment of Fab(t) is shown in Figure 5. Peak a appeared to consist of aggregated material and was not characterized further. Peak b contained a single major component which could be separated by gel filtration into two fragments after reduction and alkylation. One of these fragments was H_1. The other fragment (H_{4A}) had the same NH_2-terminal residue as H_4, but had a different amino acid composition and a lower molecular weight (Table I). It was tentatively concluded that H_{4A} is the NH_2-terminal portion of H_4 and is at the COOH-terminal position of the Fd(t) portion of the heavy chain. Peak c (Figure 5) contained CNBr fragments L_1, L_2, and L_3. When the material in this peak was reduced, alkylated, and subjected to gel filtration under the same conditions, two peaks were obtained. The first contained L_3 and the second contained L_1 and L_2. As discussed above, L_1 and L_2 appear to be linked by a disulfide bond and are not separated by gel filtration after reduction and alkylation. Peak d (Figure 5) contained fragment H_3, and peak e contained fragment H_2. Thus, Fab(t) is made up of CNBr fragments L_1, L_2, L_3, H_1, H_2, and H_3, as well as a portion of H_4, designated H_{4A}.

Gel filtration of CNBr-treated Fc(t) is illustrated in Figure 6. Fraction a contained three components which could be resolved by gel filtration at low concentrations, and a pattern similar to that shown in Figure 3 was obtained. Amino acid analyses and end-group determina-

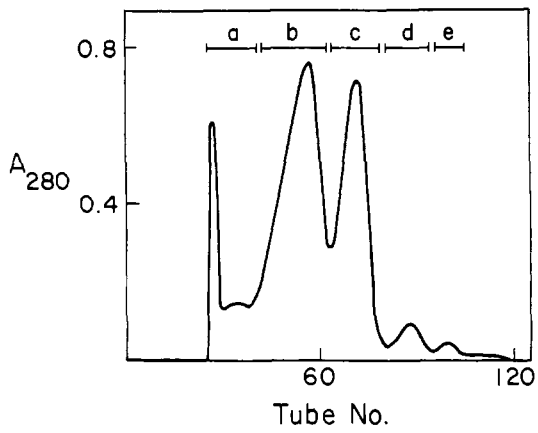


FIGURE 5: Gel filtration of CNBr-treated Fab(t) (100 mg) on Sephadex G-100 (2.5 × 150 cm) in 1 M propionic acid. Volume per tube: 5 ml. A_{280}: absorbance of effluent at 280 mμ. (a–e) Fractions pooled for further purification.

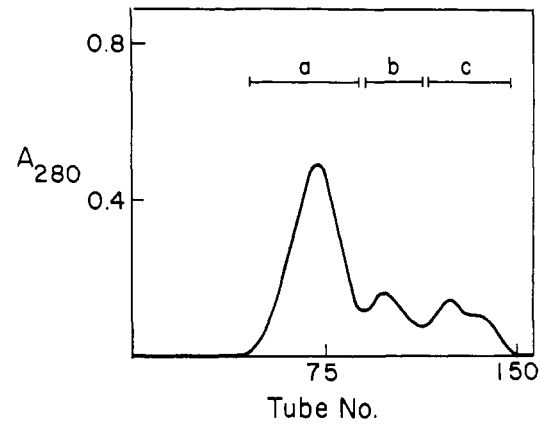


FIGURE 6: Gel filtration of CNBr-treated Fc(t) (55 mg) on Sephadex G-100 (2.5 × 100 cm) in 1 M propionic acid. Volume per tube: 5 ml. A_{280}: absorbance of effluent at 280 mμ. (a–c) Fractions pooled for further purification.

tions on the fractionated material showed that fraction a consisted of H_5, H_6, and $H_{5,6}$. Fraction b (Figure 6) consisted of peptides varying in size and yield. Amino acid analysis of these peptides showed that they were rich in proline and contained CM-cysteine and homoserine. The largest of the peptides (H_{4B}) was purified by chromatography on SE Sephadex; it had a composition which, when added to that of H_{4A}, accounted for all of H_4. H_{4B} showed only threonine as the NH_2-terminal residue and contained two residues of CM-cysteine and a single residue of homoserine. It was tentatively concluded that H_{4B} is the COOH-terminal portion of H_4 and is at the NH_2-terminal position in Fc(t). Fraction c contained CNBr fragment H_7, which was separated from contaminating material by chromatography on SE Sephadex. Thus, Fc(t) is made up of CNBr fragments H_5, H_6, and H_7, as well as a portion of H_4, designated H_{4B}.

Characterization of CNBr Fragments. The yield, molecular weight, and NH_2- and COOH-terminal residues of each CNBr fragment are presented in Table I, and the amino acid composition of each of the fragments is presented in Table II. From these data we conclude that the isolated CNBr fragments account for the entire γG-immunoglobulin molecule. The yields of the major CNBr fragments ranged from 56% for H_1 to 100% for H_3 and H_7. The lower recovery of H_1 is thought to be a result of the tendency of this fragment to aggregate in the acidic buffers used to fractionate the fragments.

The molecular weights of the larger fragments were measured in the ultracentrifuge. The molecular weights of H_1, H_2, H_3, and H_7 were calculated from their amino acid compositions normalized to the amino acid present in the smallest amount. The molecular weight of L_1–S–S–L_2 was calculated on the basis of the amino acid composition of peak IV (Figure 1) normalized to seven aspartic acid residues. The calculated molecular weights were consistent with the behavior of the fragments during gel filtration.

The sum of the molecular weights of L_1–S–S–L_2 and L_3 was 23,500. This is the same as the molecular weight of the intact light chain, 23,500 ± 1200 (Edelman *et al.*, 1968). Summation of the molecular weights of all of the CNBr fragments of the heavy chain gave a value of 52,500. The molecular weight of the heavy chain was 51,600 ± 2600 (Edelman *et al.*, 1968).

When the molecular weights of all of the fragments from light and heavy chains are summed and multiplied by two to account for the pairing of chains in the intact molecule, the resulting value is 152,000, compared to a molecular weight of 154,000 ± 8000 determined for the intact molecule (Edelman *et al.*, 1968). The molecular weights of fragments derived from Fab(t) (L_1, L_2, L_3, H_1, H_2, H_3, and H_{4A}) were summed and gave a value of 47,600 which may be compared to the measured value, 46,000 ± 2300 (Edelman *et al.*, 1968). The sum of the molecular weights of H_{4B}, H_5, H_6, and H_7 is 28,600. This is close to half the measured value for the Fc(t) fragment (48,000 ± 2400) (Edelman *et al.*, 1968).

The amino acid compositions of each of the CNBr fragments from Eu are presented in Table II. The compositions of fragments H_4, H_{4A}, and H_5 have been normalized to the molecular weights of the fragments (Table I). The composition of H_6 is normalized to one homoserine residue. The compositions of the other fragments are normalized to the amino acid present in the smallest amount. The amino acid compositions of the light and heavy chains (Edelman *et al.*, 1968) are also presented for comparison with the sum of the compositions of their constituent CNBr fragments. These comparisons show good agreement within experimental error.

Most of the CNBr fragments had NH_2-terminal residues which differed from each other, and thus the fragments could be identified by this residue. H_1 was the only CNBr fragment in which an NH_2-terminal residue could not be detected. L_1–S–S–L_2 showed NH_2-terminal aspartic acid (or asparagine) and tyrosine, both before and after reduction and alkylation. Because the NH_2-terminal amino acid residues were determined as the *N*-dansyl derivatives after acid hydrolysis, no distinction could be made between aspartic acid and asparagine. Since the NH_2-terminal residue of the light chain was aspartic acid (or asparagine), the CNBr fragment which showed this NH_2-terminal residue was arbitrarily

TABLE II: The Amino Acid Composition of the CNBr Fragments of Eu.[a]

	H_1	H_2	H_3	H_4	H_5	H_6	H_7	Sum of H_1–H_7	Heavy Chain[d]	L_1–S–S–L_2	L_3	Sum of L_1–L_3	Light Chain[d]
Lys	4.0		1.2	9.9	8.6	5.2	1.0	29.9	31.1	5.5	10.6	16.1	14.7
His				2.5	1.8	0.5	3.0	7.8	8.8		1.7	1.7	3.0
Arg	1.9		1.1	3.0	2.8	1.2		10.0	10.5	2.9	1.8	4.7	5.2
Asp	1.0		3.0	9.9	11.4	9.5	1.0	35.8	31.7	7.1	10.3	17.4	16.4
Thr	2.0		4.1	13.7	7.1	5.0	0.9	32.8	33.3	9.3	9.3	18.6	17.2
Ser	6.6		1.3	21.4	8.0	8.6	2.9	48.8	56.0	14.6	15.1	29.7	29.1
Glu	7.0		3.2	14.7	13.6	7.9	2.0	48.4	44.3	10.3	16.6	26.9	24.6
Pro	2.6	1.1	2.2	14.6	9.0	3.4	1.0	33.9	38.6	5.7	5.6	11.3	11.8
Gly	5.8	2.0	1.9	15.3	4.9	4.5	1.1	35.5	32.8	6.5	7.6	14.1	14.2
Ala	3.9		3.4	12.0	2.9	1.5	1.1	24.8	22.1	6.3	7.2	13.5	13.1
Cys[b]	0.9			6.1	2.0	1.8		10.8	11.8	1.6	2.8	4.4	5.2
Val	6.2	0.9	1.3	14.8	12.0	7.6		42.8	46.5	3.5	10.7	14.2	16.9
Met[c]	1.0	1.0	1.0	0.9	0.9	1.0		5.8	5.9	2.8		2.8	2.9
Ile	1.5	0.9	1.1	2.5	2.3	1.1		9.4	9.5	4.6	1.4	6.0	6.0
Leu	2.6			13.1	6.6	5.7	3.0	31.0	30.2	6.9	7.7	14.6	15.0
Tyr	0.5		1.5	4.8	4.8	2.9	1.0	15.5	18.7	4.4	3.7	8.1	8.7
Phe	1.3		2.0	5.6	2.8	4.0		15.7	16.1	3.1	5.1	8.2	7.9
Total residues	48.8	5.9	28.3	164.8	101.5	71.4	18.0	438.7	447.9	95.1	117.2	212.3	211.9

[a] Values reported are expressed as residues per mole and are not corrected for incomplete hydrolysis of valine and isoleucine and destruction of threonine and serine. [b] Determined as CM-cysteine or cysteic acid. [c] Methionine sulfone or the sum of homoserine and homoserine lactone. [d] From Edelman *et al.* (1968).

designated L_1. The fragment with NH_2-terminal tyrosine was designated L_2. A trace of dansylthreonine was detected in end-group analysis of L_1–S–S–L_2. The presence of this end group is thought to result from partial CNBr cleavage of the methionyl residue at position four (Cunningham *et al.*, 1968).

The NH_2-terminus of the heavy chain failed to react with dansyl chloride, dinitrofluorobenzene, or cyanate. Because H_1 is the only CNBr fragment which failed to give an NH_2-terminal residue, it was assigned to the NH_2-terminal position in the heavy chain. Press and coworkers (Press *et al.*, 1966; Piggot and Press, 1967) have described a CNBr fragment with properties similar to H_1 from another human pathologic γG-immunoglobulin, Daw, and have also assigned it to the NH_2-terminal end of the heavy chain.

All of the CNBr fragments from the intact molecules or the isolated chains contained homoserine except for L_3 and H_7 (Table I). Consequently these CNBr fragments were assigned to the COOH-terminal position in their respective chains.

Eu contained approximately 4000 g of carbohydrate/mole of protein (Edelman *et al.*, 1968). A single glycopeptide has been isolated from the complete tryptic hydrolysate of the Fc(t) portion of the heavy chain (M. J. Waxdal and G. M. Edelman, unpublished results). If there were one such peptide per heavy chain the anthrone-positive carbohydrate in this glycopeptide would account for all the carbohydrate in the entire molecule. Carbohydrate assays on all the CNBr fragments indicated that H_5 was the only fragment which contained carbohydrate.

Discussion

The CNBr treatment of Eu, light chains, heavy chains, Fab(t), Fd(t), and Fc(t) produced the expected number of fragments with one exception. Only a small amount of cleavage occurred at the methionine in position 4 of the light chain (Cunningham *et al.*, 1968). The major fragment, L_1, from this portion of the light chain had the same amino terminus as the intact chain, and its composition suggested that the amino-terminal tetrapeptide was not released following CNBr treatment.

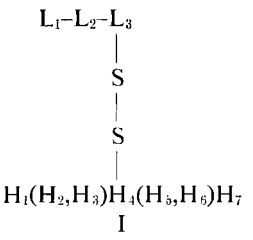
The yields of each CNBr fragment from the heavy chain ranged from approximately 60% for H_1 to 100% for H_3 and H_7. H_1 has been detected in material eluted at the void volume during gel filtration and the low yield for H_1 is probably accounted for by its tendency to aggregate. The light-chain CNBr fragments, L_1–S–S–L_2, were isolated in 70% yield and L_3 was recovered in 67% yield. Under the conditions of reaction with CNBr used in these experiments, all of the methionyl residues in the immunoglobulin, light and heavy chains, and proteolytic fragments were destroyed. Preliminary evidence suggests that some tryptophan and histidine may be destroyed by CNBr treatment of the immunoglobulin, but no other amino acid residue was observed to be altered. Because each of the CNBr fragments from the entire protein molecule was recovered in good yield, we can conclude that these CNBr fragments account for the entire γG-immunoglobulin molecule. This conclusion is further supported by the molecular weights and amino acid compositions of the CNBr fragments.

Press and coworkers (1966) have described the CNBr fragments of another human γG-immunoglobulin, Daw. CNBr fragment H_7 is identical in sequence (M. J. Waxdal and W. E. Gall, unpublished results) with the COOH-terminal octadecapeptide of protein Daw and pooled heavy chains (Piggot and Press, 1966). None of the other CNBr fragments of the two proteins appear to be identical.

The recovery of ten unique CNBr fragments indicates that the molecule is composed of two identical light chains and two identical heavy chains. Further support for this conclusion is provided by amino acid sequence analysis of the light (Cunningham *et al.*, 1968) and heavy (M. J. Waxdal, G. M. Edelman, and W. H. Konigsberg, unpublished results) chains of Eu.

From the data on the CNBr fragments, the interchain disulfide bonds of Eu can be localized (see Gall *et al.*, 1968). During the isolation and characterization of the CNBr fragments, the light-chain fragment L_3 was found to be linked to the heavy-chain fragment H_4 by a disulfide bond which corresponds to the light-chain–heavy-chain disulfide bond in the intact molecule. The position of the bonds between the heavy chains was not clear at this stage of our work. CNBr fragments H_2, H_3, and H_7 did not contain half-cystine. Fragments H_5 and H_6 each contained two half-cystine residues which appeared to be joined in intrafragment disulfide bonds. The remaining heavy-chain fragments, H_1 and H_4 (or H_{4A}), were joined by a disulfide bond regardless of whether they were obtained from the intact molecule, heavy chain, Fab(t), or Fd(t). The disulfide bond linking H_1 and H_4 could not be the heavy-chain–heavy-chain bond, because all interchain disulfide bonds were reduced and alkylated prior to separation of the chains and prior to digestion with trypsin to form the enzymatic fragments (Edelman *et al.*, 1968). Studies on the enzymatic cleavage of unreduced rabbit γG-immunoglobulin indicate that the light-chain–heavy-chain disulfide bond is in the Fab portion of the molecule and that all of the heavy-chain–heavy-chain disulfide bonds are in the Fc portion (Marler *et al.*, 1964). The CNBr fragment H_{4B}, obtained from the Fc(t) region of the heavy chain of Eu, contained two residues of CM-cysteine which arose from the reduction and alkylation of the interchain disulfide bonds. Thus, the region corresponding to H_{4B} probably is the site of two disulfide bonds linking the heavy chains in the intact molecule. A later communication in this series (Gall *et al.*, 1968) provides evidence that this is the case.

Fragment H_4 may be assigned to the center of the heavy chain because the region corresponding to this fragment is cleaved by the partial tryptic digestion of the heavy chain in the production of Fab(t) and Fc(t). Localization of the fragments contained in the Fd(t) and Fc(t) region of the heavy chain and knowledge of their NH_2- and COOH-terminal amino acid residues suggest the partial ordering $H_1(H_2,H_3)H_4(H_5,H_6)H_7$. The Fab(t) fragment contained L_1, L_2, and L_3 which are arranged in the light chain in the order L_1–L_2–L_3. A half-molecule of Eu is presented in I. Fragment L_3 is linked to fragment H_{4A} of Fab(t), and fragment H_{4B} of



I

Fc(t) contains heavy-chain–heavy-chain disulfide bonds. This substantiates current models (Porter, 1962; Edelman and Gally, 1964; Fougereau and Edelman, 1965) of the gross structure of human γG-immunoglobulin. The complete order of the CNBr fragments has been established by isolation and characterization of methionine-containing tryptic peptides from the light and heavy chains (Waxdal *et al.*, 1968).

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