The Covalent Structure of a Human γG-Immunoglobulin. III. Arrangement of the Cyanogen Bromide Fragments*

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ABSTRACT: The arrangement of ten pairs of fragments produced by the CNBr cleavage of a human γG-immunoglobulin (Eu) has been determined. The three light-chain fragments were ordered on the basis of their NH_2- and COOH-terminal amino acid residues. This order was consistent with the amino acid sequence of tryptic peptides which overlapped the sites of CNBr cleavage.

The seven heavy-chain fragments were placed in order by analysis of tryptic overlap peptides isolated from the Fd(t) and Fc(t) portions of the heavy chains. These assignments provide proof for current models of human γG-immunoglobulins and allow formulation of a more detailed model.

We have reported (Waxdal *et al.*, 1968) the isolation, composition, and location of ten unique CNBr fragments from a human γG-immunoglobulin. In the present paper we describe experiments which have enabled us to deduce the arrangement of regions corresponding to the CNBr fragments in the intact light and heavy chains. The light chains produced only three fragments after CNBr treatment, and the order of these fragments was unambiguously fixed once the NH_2- and COOH-terminal CNBr fragments were identified. These assignments were consistent with the sequences of methionine-containing peptides (overlap peptides) from a tryptic digest of the light chain. The arrangement of the CNBr fragments of the heavy chain was deduced by isolation and partial sequence determination of overlap peptides from tryptic digests of the Fd(t)[1] and Fc(t) portions of the heavy chain.

Materials and Methods

The purification of Eu, subsequent cleavage by CNBr, and isolation of the fragments have previously been described in detail (Edelman *et al.*, 1968; Waxdal *et al.*, 1968).

Tryptic Digestion. Trypsin treated with L-1-tosylamino-2-phenylethyl chloromethyl ketone was obtained from Calbiochem (Los Angeles, Calif.). The CNBr fragments, light chain, and the Fd(t) and Fc(t) portions of the heavy chain were digested with trypsin for 4 hr (protein concentration, 1%; trypsin concentration, 0.01%). The temperature was maintained at 37° and the pH at 7.8 by the use of a pH-Stat. After digestion the pH was lowered to 3.1 and the solution was lyophilized.

Ion-Exchange Chromatography. Peptides were fractionated by ion-exchange chromatography on polystyrene resins substituted with sulfonic or trimethylammonium groups (Bio-Rad Laboratories, Richmond, Calif.) using pyridine acetate buffers (*cf.* Schroeder *et al.*, 1962). The effluent stream was split and a portion continuously monitored before and after hydrolysis in NaOH using a modification of the method described by Catravas (1964).

AG-50X4. The resin (30–35-μ particles) was packed into a 185 × 0.9 cm column and was eluted at 60° at a flow rate of 30 ml/hr maintained with a pump (mini-Pump, Milton Roy, Philadelphia, Pa.). The back pressure under these conditions rose from 80 to 150 psi during the elution. Two linear gradients were used with this resin; in both cases 350 ml of each buffer was placed in separate flasks serially connected by siphons. Flask A was connected to the eluent pump. Gradient I had (1) flask A, 0.2 M pyridine adjusted to pH 3.1 with acetic acid; and (2) flask B, 2.0 M pyridine adjusted to pH 5.5 with acetic acid. Gradient II had (1) flask A, 0.05 M pyridine adjusted to pH 3.1 with acetic acid; (2) flask B, 0.20 M pyridine adjusted to pH 3.1 with acetic acid; and (3) flask C, 2.00 M pyridine adjusted to pH 5.5 with acetic acid.

In some experiments the columns were washed overnight with the pH 5.5 buffer after the gradient was completed in order to elute tightly bound peptides. The resin was regenerated in the column by washing with the initial buffer until the pH and ionic strength of the effluent matched that of the incoming buffer.

AG-50X12. The resin (2–19-μ particles) was packed into a 60 × 0.9 cm column. The conditions of chroma-

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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; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine.

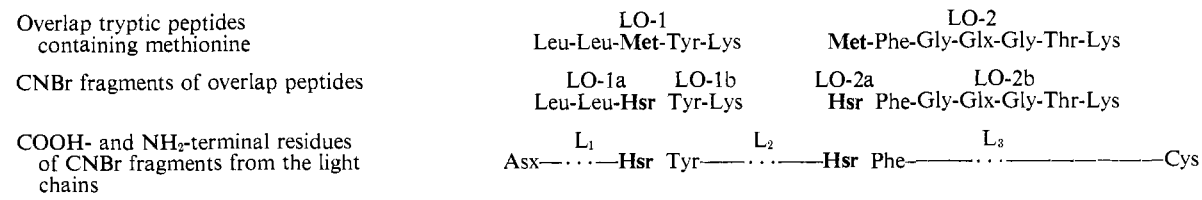

FIGURE 1: Ordering of the CNBr fragments (L_1, L_2, and L_3) of the light chain by comparison of their terminal amino acid residues with the amino acid sequence of tryptic overlap peptides LO-1 and LO-2.

tography were the same as those for AG-50X4 except that the back pressure ranged from 200 to 400 psi.

AG-1X2. The resin (−400 mesh) was packed into a 50 × 0.9 cm column. Elution was performed at a flow rate of 90 ml/hr with back pressure less than 25 psi. The columns were eluted at room temperature with a linear gradient from 0.1 M pyridine adjusted to pH 6.5 with acetic acid to 2.0 M acetic acid. An equal amount (350 ml) of each buffer was used to make gradient III. At the end of the gradient the resin was washed with 25% acetic acid and reequilibrated with the initial buffer.

Identification of Methionine-Containing Peptides. Portions of the pooled ninhydrin-positive material obtained by ion-exchange chromatography were spotted onto Whatman No. 3MM paper and stained with platinic chloride (Easley, 1965). Material which gave a positive stain was subjected to amino acid analysis using a Beckman 120C amino acid analyzer with minor modifications (Edelman *et al.*, 1968).

CNBr Cleavage of Peptides. The overlap peptides were dissolved in 70% formic acid (0.5 μmole of peptide/ml) and CNBr (5 mg/ml) was added. After 4 hr at room temperature the reaction was stopped by the addition of ten volumes of H_2O, and the preparation was lyophilized.

Amino Acid Sequence. Edman degradations were performed by a modification of the procedure of Konigsberg and Hill (1962). The new NH_2-terminal residue exposed after each cycle of the Edman degradation was identified by dansylation (Gray, 1967) followed by acid hydrolysis and thin-layer chromatography of the dansyl-amino acid on polyamide resin plates (Woods and Wang, 1967). An extensive description of this technique is given in a subsequent paper in this series (Cunningham *et al.*, 1968). The NH_2-terminal amino acid residues of the CNBr fragments and of light and heavy chains were also determined by this technique.

TABLE I: NH_2- and COOH-Terminal Amino Acid Residues of the CNBr Fragments and Chains of Eu.[a]

Fragment	NH_2-Terminal Amino Acid	COOH-Terminal Amino Acid
Light Chain	Asx	CMCys
L_1	Asx	Hsr
L_2	Tyr	Hsr
L_3	Phe	CMCys
Heavy Chain	Blocked	Gly
H_1	Blocked	Hsr
H_2	Gly	Hsr
H_3	Phe	Hsr
H_4	Glx	Hsr
H_5	Ile	Hsr
H_6	Thr	Hsr
H_7	His	Gly

[a] From Waxdal *et al.* (1968) and Edelman *et al.* (1968).

Results

The terminal amino acid residues of the CNBr fragments and the light and heavy chains are presented in Table I. The compositions of the isolated tryptic peptides from the heavy and light chains which contained methionine (overlap peptides) are presented in Table II.

Order of the Light-Chain CNBr Fragments. The order of the CNBr fragments of the light chain (L_1, L_2, and L_3) could be deduced from a knowledge of the termini of the light chain and of the fragments themselves. As shown in the preceding communication (Waxdal *et al.*, 1968), CNBr fragment L_1 contained an NH_2-terminal aspartic acid or asparagine as did the intact light chain (Table I). Because no other major light-chain fragment yielded this end group, L_1 was placed as the NH_2-terminal CNBr fragment. The COOH terminus of both the light chain and L_3 was found to be CM-cysteine (Table I). The presence of a half-cystine residue has previously been reported at the COOH terminus of type K Bence-Jones proteins by a number of authors (Hilschmann and Craig, 1965; Milstein, 1965; Titani *et al.*, 1965). The CM-cysteine residue was produced by reduction and alkylation of the light-chain–heavy-chain disulfide bond during the preparation of these chains from the intact immunoglobulin (Gall *et al.*, 1968). The remaining CNBr fragment from the light chain, L_2, must occupy the position between L_1 and L_3, and the order is L_1–L_2–L_3.

To substantiate this conclusion, the light chain was fully reduced, alkylated, and digested with trypsin. Two tryptic peptides which contained methionine (overlap peptides LO-1 and LO-2) were isolated by chromatography on AG50X4 using gradient II (Cunningham *et al.*, 1968); their amino acid compositions are presented in Table II. The amino acid sequences of these peptides have been determined (Cunningham *et al.*, 1968) and are presented in Figure 1 along with the terminal amino acids of the CNBr fragments of the light chain. The peptide LO-1 overlaps the COOH-terminal portion of

TABLE II: Amino Acid Composition of Methionine-Containing Tryptic Peptides from Eu Light and Heavy Chains.[a]

	LO-1	LO-2	HO-1	HO-2	HO-3	HO-4
Lys	0.9	1.0	1.0			1.0
Arg				1.0	0.8	0.9
Asp			1.0	2.2	0.8	0.4
Thr		1.0		3.8	0.9	2.1
Ser				3.2	1.1	1.6
Glu		1.1	4.2	2.3		2.9
Pro			4.0			1.8
Gly		1.9	4.7			0.4
Ala			1.9	2.0		0.2
Val			0.7	1.3		0.2
Met	1.1	0.9	1.9	0.7	0.9	1.0
Ile			0.7	0.9	1.0	
Leu	2.1		1.0	1.9	1.1	1.2
Tyr	0.9		1.0	0.7		
Phe		1.0	1.1			
Total residues	5	7	24	20	7	13
% yield	20	16	55	10	30	20[b]

[a] Values are reported as residues per mole. Amino acid contamination at less than 0.1 mole/mole of peptide is not reported. The values are from single 20-hr hydrolysates and have not been corrected for destruction of threonine and serine nor for incomplete hydrolysis of valine and isoleucine. [b] After first purification.

CNBr fragment L_1 and the NH_2-terminal portion of L_2. This assignment was made because tyrosine followed methionine in the overlap peptide and was also the NH_2 terminus of the CNBr fragment L_2.

The sequence of the tryptic peptide LO-2, which overlaps L_2 and L_3, is also shown in Figure 1. Methionine was the NH_2 terminus of this overlap peptide, and CNBr cleavage produced free homoserine. The remaining hexapeptide LO-2b showed phenylalanine at the NH_2 terminus as did L_3. This confirms that the order of the light-chain CNBr fragments is L_1–L_2–L_3.

Order of the Heavy-Chain CNBr Fragments. THE Fd(t) PORTION of the heavy chain contains the CNBr fragments H_1, H_2, H_3 and H_{4A} (Waxdal *et al.*, 1968). Reduced and alkylated Fd(t) was digested with trypsin and two methionine-containing tryptic peptides, HO-1 and HO-2, were isolated according to the fractionation scheme shown in Figure 2. The amino acid compositions of these two peptides are presented in Table II. HO-1 contained two methionine residues and therefore must overlap three CNBr fragments. This arrangement is possible only if HO-1 contains a region corresponding to CNBr fragment H_2. As expected, CNBr cleavage of HO-1 produced the fragment H_2 (or HO-1b) in addition to two other peptides, HO-1a and HO-1c (Figure 2). The partial sequence shown in Figure 3 was derived from a knowledge of the NH_2- and COOH-terminal residues of HO-1, HO-1a, HO-1b and HO-1c.

Tryptic digestion of the CNBr fragment H_1 yielded a COOH-terminal peptide identical in composition with HO-1a (Figure 3). This established the order of the first two CNBr fragments as H_1–H_2. Phenylalanine was the NH_2-terminal residue of the peptide HO-1c and therefore must be the NH_2 terminus of the third CNBr fragment. Of the two remaining fragments from the Fd(t) portion of the heavy chain (H_3 and H_{4A}) only H_3 possessed an NH_2-terminal phenylalanine (Table I). As expected, tryptic digestion of H_3 yielded an NH_2-terminal peptide with a composition identical with HO-1c. This finding not only establishes the order of the CNBr frag-

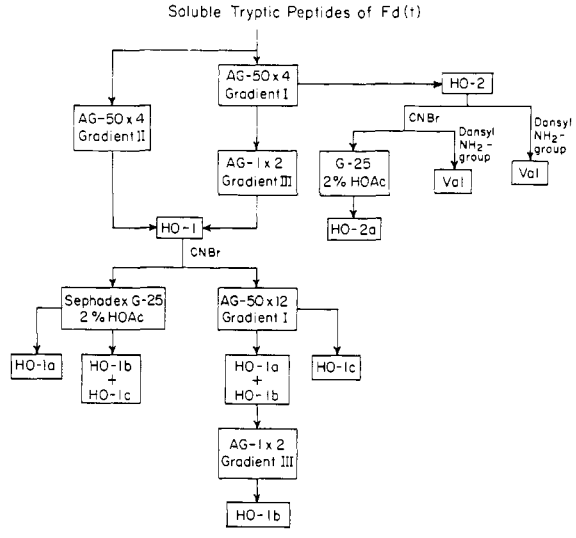

FIGURE 2: Summary of the procedure for the isolation of tryptic overlap peptides HO-1 and HO-2 from the Fd(t) portion of the heavy chain (see Materials and Methods). HOAc, acetic acid.

Overlap tryptic peptide containing methionine	HO-1 Glx(Glx$_2$,Pro,Gly$_2$,Ala,Leu,**Met**,Gly,Pro,Gly,Val,Ile,**Met**,Phe,Asx,Glx,Pro$_2$,Gly,Ala,Tyr)Lys
CNBr fragments of overlap peptides	HO-1a HO-1b HO-1c Glx(Glx$_2$,Pro,Gly$_2$,Ala,Leu)**Hsr**|Gly(Pro,Gly,Val,Ile)**Hsr**|Phe(Asx,Glx,Pro$_2$,Gly,Ala,Tyr)Lys
COOH- and NH_2-terminal tryptic peptides of CNBr fragments from Fd(t)	COOH-terminal tryptic peptide of H_1 H_2 NH_2-terminal tryptic peptide of H_3 Glx(Glx$_2$,Pro,Gly$_2$,Ala,Leu)**Hsr**|Gly(Pro,Gly,Val,Ile)**Hsr**|Phe(Asx,Glx,Pro$_2$,Gly,Ala,Tyr)Lys

FIGURE 3: Ordering of the CNBr fragments (H_1, H_2, and H_3) from the Fd(t) portion of the heavy chain by comparison of their terminal tryptic peptides or amino acid residues with the tryptic overlap peptide HO-1.

Overlap tryptic peptide containing methionine	HO-2 Val(Asx$_2$,Thr$_4$,Ser,Glx,Ala$_2$,Ile,Tyr,**Met**,Glx,Ser$_2$,Leu$_2$)Arg
CNBr fragments of overlap peptide	HO-2a Val(Asx$_2$,Thr$_4$,Ser,Glx,Ala$_2$,Ile,Tyr)**Hsr**
COOH- and NH_2-terminal tryptic peptides of CNBr fragments from Fd(t)	COOH-terminal tryptic peptide of H_3 NH_2-terminal of H_4 Val(Asx$_2$,Thr$_4$,Ser,Glx,Ala$_2$,Ile,Tyr)**Hsr**|Glu(Ser$_2$Leu$_2$)Arg

FIGURE 4: Ordering of CNBr fragments H_3 and H_4 of the heavy chain by comparison with tryptic overlap peptide HO-2.

ments as H_1–H_2–H_3, but completes the ordering of the Fd(t) portion of the heavy chain. H_1 has already been assigned as the NH_2-terminal CNBr fragment of the heavy chain from data on NH_2-terminal residues (Table I, Waxdal *et al.*, 1968). The remaining fragment from the Fd(t) region, H_{4A}, was placed at the COOH-terminal end of the Fd(t) fragment, because H_4 is the site of tryptic hydrolysis of the heavy chain during the production of Fab(t) and Fc(t) (Waxdal *et al.*, 1968). This assignment has been confirmed by analysis of the second overlap peptide from the Fd(t) region, HO-2 (Figure 4). The amino acid composition of HO-2 is presented in Table II. This peptide was treated with CNBr and two smaller peptides, HO-2a and HO-2b, were produced (Figures 2 and 4). Tryptic digestion of H_3 yielded the COOH-terminal peptide identical in composition with HO-2a. The sum of the composition of this peptide and of the NH_2-terminal peptide of H_4 was equal to the composition of HO-2. Consequently, the order of the CNBr fragments in the Fd(t) region of the heavy chain is confirmed as H_1–H_2–H_3–H_{4A}.

THE Fc(t) PORTION of the heavy chain contained the CNBr fragments H_{4B}, H_5, H_6, and H_7. Because H_4 is the site of tryptic hydrolysis to form Fab(t), H_{4B} was assigned to the NH_2-terminal position of Fc(t) (Waxdal *et al.*, 1968). H_7 was placed at the COOH terminus of the heavy chain, because this CNBr fragment contained no homoserine. With the order $H_{4B}(H_5,H_6)H_7$, it was only necessary to confirm the adjacent positions of one pair of CNBr fragments to complete the ordering of the heavy-chain fragments.

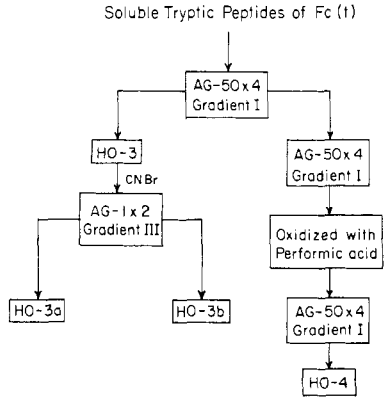

FIGURE 5: Summary of procedures used to isolate tryptic overlap peptides HO-3 and HO-4 from the Fc(t) portion of the heavy chain (see Materials and Methods).

Two of the three possible methionine-containing peptides were isolated from a tryptic digest of the Fc(t) fragment by means of the fractionation procedures summarized in Figure 5. The amino acid composition of overlap peptide HO-3 is presented in Table II. When HO-3 was treated with CNBr, two smaller peptides, HO-3a and HO-3b, were produced (Figures 5 and 6). The COOH-terminal tryptic peptide of H_4 had an amino acid composition identical with HO-3a (Figure 6), and therefore the overlap peptide HO-3 must extend from H_4 to either H_5 or H_6. Both HO-3b and H_5 had isoleucine as their NH_2-terminal residue (Table I, Figure 6). This observation was sufficient to determine the order of the CNBr fragments in Fc(t) as H_{4B}–H_5–H_6–H_7.

This order is consistent with the partial amino acid sequence of the final overlap peptide, HO-4 (Figure 6). The tryptic peptide was difficult to purify, and it was necessary to oxidize the methionyl residue to the sulfone during the isolation procedure. Because oxidation made subsequent CNBr cleavage impossible, HO-4 was submitted to the dansyl–Edman procedure. The amino acid sequence around the methionyl residue was -Glx-Met-Thr-. The NH_2 terminus of H_6 (Table I) was threonine.

Discussion

The isolation and analysis of methionine-containing tryptic peptides have enabled us to order the CNBr fragments of the γG-immunoglobulin Eu. The order and composition of the fragments and information on the

Overlap tryptic peptide containing methionine	HO-3	HO-4
	Asx-Thr-Leu-**Met**-Ile-Ser-Arg	Thr-Leu-Pro-Pro-Ser-Arg-Glx-Glx-**Met**-Thr-(Ser,Glx,Lys)
CNBr fragments of overlap peptide	HO-3a HO-3b	
	Asx-Thr-Leu-**Hsr**|Ile-Ser-Arg	
COOH- and NH_2-terminal tryptic peptides of CNBr fragments from Fc(t)	COOH-terminal tryptic peptide of H_4 / NH_2-terminal of H_5	COOH-terminal of H_5 / NH_2-terminal of H_6
	Asx-Thr-Leu-**Hsr**|Ile.....	.....**Hsr** Thr....

FIGURE 6: Ordering of the CNBr fragments from the Fc(t) portion of the heavy chain by comparison of their terminal tryptic peptides or amino acid residues with the amino acid sequence of the tryptic overlap peptides HO-3 and HO-4.

interfragment disulfide bonds (Edelman *et al.*, 1968; Waxdal *et al.*, 1968; Gall *et al.*, 1968) may be incorporated in a detailed model of the molecule (Figure 7). The model is composed of two identical halves, each containing ten CNBr fragments and consisting of a pair of heavy and light chains joined by a disulfide bond between the COOH-terminal residue of the light chain and the region of the heavy chain corresponding to CNBr fragment H_{4A}. The half-molecules are joined by two disulfide bonds in the heavy chains located in the region corresponding to fragment H_{4B}. The verification of the number and location of the interchain bonds and the determination of the amino acid sequence of the heavy chain around these bonds are presented in the next paper of this series (Gall *et al.*, 1968). The exact positions of the disulfide bond joining H_1 to H_4 (Figure 7) and of the carbohydrate in fragment H_5 have not been established. The remainder of the disulfide bonds so far accounted for are assigned on the basis of studies of the CNBr fragments (Waxdal *et al.*, 1967, 1968); studies to determine their exact positions are in progress. L_1 and L_2 are joined by a disulfide bond, and there is an intrachain disulfide bond in L_3. Recent studies of the disulfide bonds of another human γG-immunoglobulin (Pink and Milstein, 1967) are consistent with these assignments. The major site where trypsin cleaves the heavy chain to produce the enzymatic fragments Fab(t) and Fc(t) is indicated by the broken arrows (Figure 7).

Both the number and the position of the methionyl residues in Eu light and heavy chains may be compared to the published data on type K Bence–Jones proteins (Hilschmann and Craig, 1965; Milstein, 1965; Titani *et al.*, 1965) and the heavy chain of another human γG-immunoglobulin, Daw (Press *et al.*, 1966a,b).

The partial amino acid sequences of the several type K Bence–Jones proteins show only one methionine residue at position 4 in the chain (Hilschmann and Craig, 1965; Milstein, 1965; Titani *et al.*, 1965). The κ chain of Eu contains two other methionine residues in the variable region which are the major sites of cleavage upon CNBr treatment (Waxdal *et al.*, 1968). The methionyl residues in the heavy chain of Eu did not correspond to those in Daw (Press *et al.*, 1966a), with the exception of the residues linking H_4 and H_5 (see Gall *et al.*, 1968) and H_6 and H_7. The COOH-terminal CNBr fragment of heavy chain, H_7, which contains 18 amino acid residues, is identical with the corresponding fragment from the heavy chain of Daw (Waxdal *et al.*, 1968). Pooled human γG-immunoglobulin has also been found to contain this fragment (Piggot and Press, 1966), and CNBr fragments of the same size, but differing in composition by two residues, have been isolated from the γG-immunoglobulin heavy chains of rabbit (Givol and Porter, 1965; Hill *et al.*, 1966b) and horse (Press *et al.*, 1966a). The NH_2-terminal CNBr fragment of the heavy chain from Eu (H_1) was found to be larger than the corresponding fragment from Daw. In both cases the fragments are linked by a disulfide bond to a large central CNBr fragment (Press *et al.*, 1966a; Waxdal *et al.*, 1968).

Thorpe and Deutsch (1966) have reported the sequence of a peptide similar to HO-4 and have suggested that this peptide contains the marker for γ_{2b}, Gm (a^-) heavy chains. Although Eu has been typed as Gm (a^-), the partial amino acid sequence of HO-4 (Thr-Leu-Pro-Pro-Ser-Arg-Glx-Glx-Met-Thr-, Figure 6) differs from the sequence of the peptide given by Thorpe and Deutsch (Thr-Leu-Pro-Pro-Ser-Arg-Met-Glu-Glu-Thr-Lys). Another human myeloma protein, He, of the same Gm type as Eu, is currently being studied in our laboratory. CNBr treatment of He produces fragments apparently identical with H_5, H_6, and H_7 from Eu (M. J. Waxdal and G. M. Edelman, unpublished results). The location of the variable region of the heavy chain of this Gm type may emerge from a comparison of the CNBr fragments in the Fd region of Eu and He.

The partial amino acid sequence of peptide HO-4 is similar to the sequence of the corresponding region of

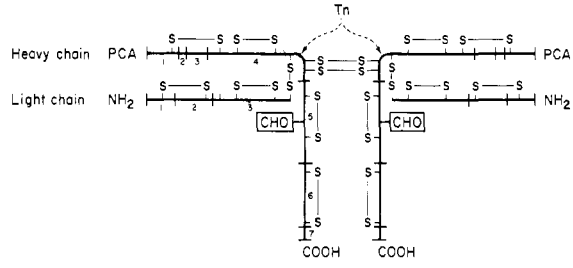

FIGURE 7: Linear model of the light and heavy chains of the human γG-immunoglobulin Eu. The large vertical divisions in each chain denote the positions of the methionyl residues cleaved by the CNBr treatment. The relative lengths of the lines between the vertical bars are proportional to the molecular weights of each of the CNBr fragments. The numbers under the lines identify the fragments. CHO indicates that fragment H_6 contains the carbohydrate group. PCA indicates the blocked NH_2 terminus of the heavy chain, probably pyrrolidonecarboxylic acid. The major site of proteolysis of the heavy chain by trypsin (Tn) to produce Fab(t) and Fc(t) fragments is denoted by arrows. Assignment of the intrachain disulfide bonds is made on the basis of studies of the CNBr fragments and is provisional. The interchain bonds have been located and identified (*cf.* Gall *et al.*, 1968).

pooled rabbit Fc fragments (Hill *et al.*, 1966b). In addition, the peptide HO-3 which overlaps H_4 and H_5 of Eu is identical in sequence with a methionine-containing peptide from pooled rabbit Fc fragments (Hill *et al.*, 1966b) and is found at approximately the same position as in rabbit γG-immunoglobulin (Hill *et al.*, 1966b). These homologies and the close resemblance of the sequences around the heavy-chain–heavy-chain disulfide bonds (Steiner and Porter, 1967; Gall *et al.*, 1968) provide additional evidence for the close evolutionary relationships (Hill *et al.*, 1966a) among immunoglobulins of different species.

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