made solely on the basis of electrophoretic mobility. Mobilities were measured for a total of 49 peptides. The observed mobility of each peptide was within ten per cent of the calculated mobility in all but three cases. For these three, additional evidence was obtained to confirm the assignments. In no case did the electrophoretic mobility of a peptide suggest an assignment different from that indicated by other methods.

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The Covalent Structure of a Human γG-Immunoglobulin. X. Intrachain Disulfide Bonds*

W. Einar Gall and Gerald M. Edelman

ABSTRACT: The arrangement of the 12 intrachain disulfide bonds in the human γG-immunoglobulin Eu has been determined. The first intrachain bond in each light (κ) chain is formed by half-cystinyl residues at positions 23 (designated I, numbering from the NH_2 terminus) and 88 (II), and the second is formed by half-cystines at positions 134 (III) and 194 (IV). The four intrachain bonds in each heavy (γ1) chain are formed by half-cystines at positions 22 (I) and 96 (II), 144 (III) and 200 (IV), 261 (VIII) and 321 (IX), and 367 (X) and 425 (XI). Each disulfide loop in the C_L and C_H regions of the molecule contains from 57 to 61 residues; the V_L loop contains 66 residues and the V_H loop contains 75 residues. These data indicate that the intrachain bonds form loops which have a linear and periodic arrangement and provide additional evidence for the hypothesis that immunoglobulins evolved by gene duplication.

A previous paper in this series (Gall *et al.*, 1968) described the location and arrangement of the interchain disulfide bonds in the human γG1-immunoglobulin Eu. A linear arrangement of all of the intrachain disulfide bonds of the molecule was first proposed by Waxdal *et al.* (1967). We now present proof of this arrangement.

Peptides containing intrachain disulfide bonds have been isolated from Bence–Jones proteins and light chains of other immunoglobulins by Milstein and coworkers (for a review, see Milstein, 1966b), and the location of these peptides in the light chains has been suggested by comparison with amino acid sequences obtained in several laboratories. Peptides containing some of the intrachain disulfide bonds have been isolated from the four subclasses of γ chains (Frangione and Milstein, 1967; Pink and Milstein, 1967; Frangione *et al.*, 1968, 1969), and these authors also suggested a linear arrangement for these bonds. The results of the present studies, together with those on the interchain bonds (Gall *et al.*, 1968) and the determination of the complete amino acid sequence of protein Eu (Cunningham *et al.*, 1968; Gottlieb *et al.*, 1970; Cunningham *et al.*, 1970; Rutishauser *et al.*, 1970), establish the exact location of all of the disulfide bonds in the molecule.

Materials and Methods

Purification of the immunoglobulin Eu and preparation of the CNBr fragments have been described (Edelman *et al.*,

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1968; Waxdal *et al.*, 1968b). Fab(t)[1] and Fc(t) fragments were prepared by limited digestion with trypsin according to Edelman *et al.* (1968), except that the protein was not partially reduced and alkylated before digestion.

Alkylation of Eu. A solution of the protein (20 mg/ml) in 6 M guanidine hydrochloride, 0.5 M in Tris and 0.002 M in EDTA, apparent pH 8.1, was placed in a water bath at 50°. Iodoacetamide-1-^{14}C was added in a 200-fold molar excess over each half-cystinyl residue. After 4 hr, the protein was separated from reagents by gel filtration on Sephadex G-25 in 50% acetic acid. In a parallel experiment, the protein was reduced for 2 hr with a 100-fold molar excess of dithiothreitol and then alkylated as described above.

Amino acid analyses were performed as described by Edelman *et al.* (1968). The total half-cystine content of Eu, its heavy and light chains, and the Fab(t) and Fc(t) fragments were determined by amino acid analysis after performic acid oxidation (Moore, 1963). Glutathione oxidized with performic acid was used as a standard.

Peptic Digestion. Material to be digested was dissolved (10 mg/ml) in 5% (v/v) formic acid and incubated at 37°. Pepsin (Worthington Biochemical Corp., Freehold, N. J., twice crystallized, lot PM 709) was added in three equal aliquots of 1% by weight at zero time, 3 hr, and 9 hr. After 18 hr, the digest was lyophilized.

Tryptic Digestion. Material to be digested was dissolved (10 mg/ml) in 0.1 M ammonium acetate (pH 7.0) and incubated at 37°. Trypsin (Calbiochem, Los Angeles, Calif., lot 73325, treated with L-(1-tosylamido-2-phenylethyl chloromethyl ketone)) was added (2% by weight), and the digest was lyophilized after 4 hr.

Preparation of Diagonal Maps. Diagonal electrophoresis (Brown and Hartley, 1966) was carried out on Whatman No. 3MM paper in pyridinium acetate buffer (pyridine–acetic acid–water, 25:25:950, v/v) at pH 4.7 (Schwartz and Edelman, 1963). Electrophoresis in the first dimension was performed for 80 min with a potential gradient of 50 V/cm. After drying, marker strips were cut from the paper. The remaining strip was oxidized over performic acid (40 ml of 97% formic acid and 2 ml of 30% hydrogen peroxide) in a desiccator for 2.5 hr and then dried in a hood. The marker strips were stained for half-cystine with fluorescein mercuric acetate (Karush *et al.*, 1964). The appropriate segment of the oxidized strip was sewn onto a 27 × 92 cm sheet of paper, and electrophoresis was carried out in the second dimension for 3 to 3.5 hr with a potential gradient of 20 V/cm. A mixture of ε-dinitrophenyllysine, aspartic acid, and lysine was used to provide marker substances with neutral, acidic, and basic mobilities. The maps were sprayed with dilute ninhydrin (Bennett, 1967), and the color was developed at room temperature. Peptides appearing off the diagonal in the same vertical zone were presumably linked by a disulfide bond before oxidation. Portions of the paper containing these peptides were cut out, and the peptides were eluted from the paper with 50% (v/v) pyridine. The eluates were dried by rotary evaporation and hydrolyzed for 18 hr at 110° in 6 N HCl prior to amino acid analysis. For clarity, the results are presented as a drawing of the diagonal map. A photograph of an actual map is also included (see Figure 9). The amino acid sequences of the peptides which moved off the diagonal, deduced from their amino acid composition and the amino acid sequence of Eu (Cunningham *et al.*, 1968, 1970; Gottlieb *et al.*, 1970; Rutishauser *et al.*, 1970) are also given.

Other Methods. Gel filtration was carried out as described previously (Gall *et al.*, 1968). Analyses for cystine-containing peptides in the column effluents were made using the fluorescence quenching method of Karush *et al.* (1964). Scintillation counting was done as described by Gall *et al.* (1968).

Results

The γG1-immunoglobulin Eu contains 32 residues of half-cystine/mole (Table I); 11 of these are in each heavy chain and 5 are in each light chain. Each Fab(t) fragment contains 10 half-cystinyl residues and the Fc(t) fragment contains 12. Alkylation of protein Eu with iodoacetamide-1-^{14}C in a denaturing solvent was used to test for the presence of free sulfhydryl groups. Less than 0.3 mole of iodoacetamide was incorporated per mole of protein as measured both by specific activity and amino acid analysis. When alkylation was preceded by treatment with dithiothreitol, 32 moles of iodoacetamide was incorporated per mole of protein. We therefore concluded that there are no free sulfhydryl groups in the protein Eu, and assumed that all of the half-cystinyl residues participate in disulfide bonds.

TABLE I: Total Half-Cystine Content of the Protein Eu, Its Polypeptide Chains, and Tryptic Fragments.[a]

Sample	Residues Half-Cystine/Mole
Eu	32.4
H chain[b]	11.5
L chain[b]	5.2
Fab(t)[c]	10.1
Fc(t)[c]	12.0

[a] Determined as cysteic acid after performic acid oxidation. Results are normalized to the known molar contents of amino acids stable to performic acid oxidation. [b] Determined on chains prepared without alkylation (Edelman *et al.*, 1968). [c] Determined on fragments prepared without reduction or alkylation.

All of the half-cystinyl residues have been located in the amino acid sequence of the light and heavy chains (Cunningham *et al.*, 1968, 1970; Gottlieb *et al.*, 1970; Rutishauser *et al.*, 1970). These residues[2] are numbered in order from I to V in the light chain and from I to XI in the heavy chain. The

[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); $CySO_3H$, cysteic acid.

[2] We have adopted the convention that half-cystinyl residues are designated by Roman numerals beginning with the residue nearest the NH_2 terminus of the polypeptide chain. Residue numbers are subscripted. Intrachain disulfide bonds are designated using parentheses. For example, the first intrachain disulfide bond of the light chain is $\kappa(I_{23}\text{–}II_{88})$. Interchain bonds are designated without using parentheses. For example, $\kappa V_{214}\text{–}\gamma 1 V_{220}$ is the disulfide bond linking the light and heavy chains.

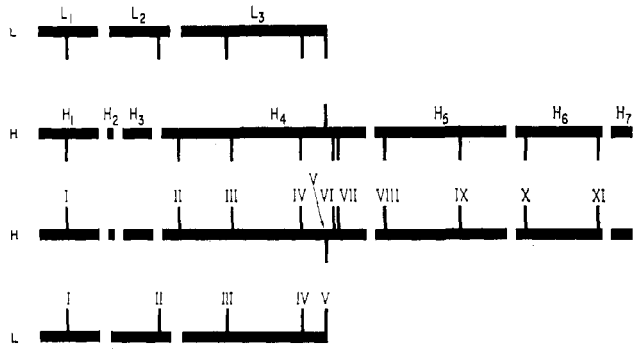

FIGURE 1: The overall structure of protein Eu based on studies of the CNBr fragments (Waxdal *et al.*, 1968a,b). The length of the bar representing each fragment is proportional to the molecular weight of that fragment. The position of the vertical mark representing each half-cystinyl residue corresponds to the position of that residue in the amino acid sequence (Cunningham *et al.*, 1968, 1970; Gottlieb *et al.*, 1970; Rutishauser *et al.*, 1970).

location of each half-cystinyl residue in the CNBr fragments of the molecule is shown in Figure 1. The amino acid sequence around each of these residues is different, and therefore small peptides containing each of the half-cystinyl residues could be unequivocally identified from amino acid compositions.

We have previously shown (Gall *et al.*, 1968) that the two disulfide bonds[2] linking light and heavy chains are κV_{214}–$\gamma 1V_{220}$, and that the disulfide bonds between the heavy chains are $\gamma 1VI_{226}$–$\gamma 1VI_{226}$ and $\gamma 1VII_{229}$–$\gamma 1VII_{229}$. The remaining 24 half-cystinyl residues form intrachain disulfide bonds. The determination of the arrangement of these bonds in the Fab region of the molecule is presented first, followed by the evidence for the arrangement of the intrachain bonds in the Fc region. Because of a relative lack of specificity in the peptic digestions, several different peptides containing the same half-cystinyl residue were in some cases isolated from maps obtained by diagonal electrophoresis (Brown and Hartley, 1966). Only those peptides required to prove the linkage of the half-cystinyl residues will be discussed in detail.

Disulfide Bonds of the Fab Region. The Fab(t) fragment contains the five half-cystinyl residues of the light chain and

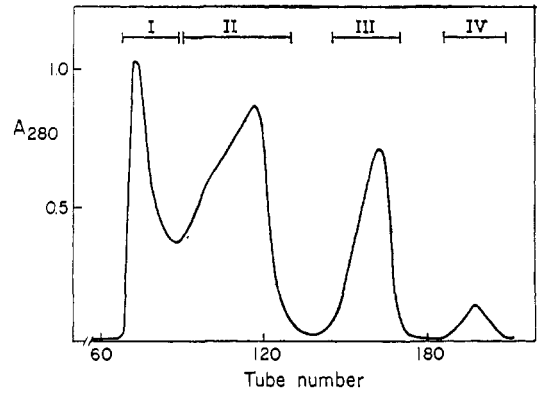

FIGURE 2: Gel filtration of products from CNBr cleavage of Eu Fab(t) fragment (163 mg) prepared without reduction and alkylation. Column: Sephadex G-100 in 1 M propionic acid, 2.5 × 190 cm. Volume per tube, 4.5 ml; A_{280}, absorbance of effluent at 280 mμ. Bars indicate the fractions taken for further analysis.

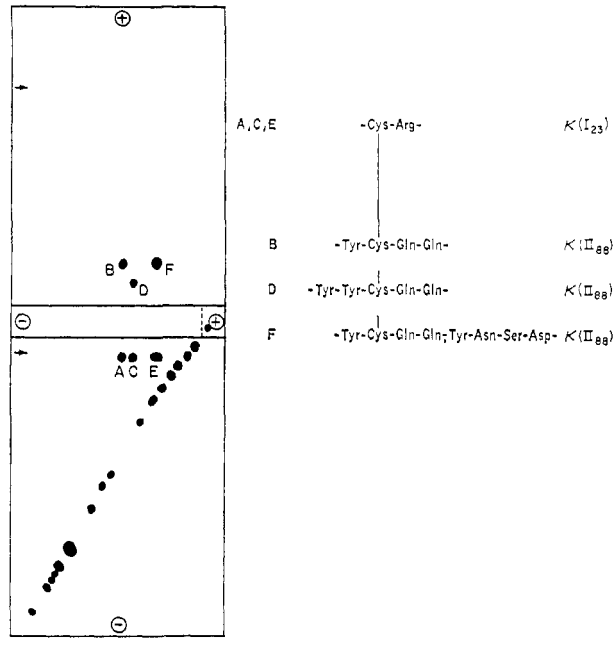

FIGURE 3: Diagonal map of a peptic and tryptic digest of the L_1–L_2 fragment complex. +, anode; −, cathode. Small arrows indicate the position of acidic and neutral marker substances in the second dimension. The amino acid sequence of the peptides which lie off the diagonal, deduced from their composition (Table II) and the known sequence of Eu, is shown at the right of the figure. The half-cystinyl residue contained in each peptide is indicated at the far right.

half-cystinyl residues I–V of the heavy chain (Figure 1). Half-cystines V_{214} of the light chain and V_{220} of the heavy chain form the disulfide bond that links these chains (Gall *et al.*, 1968). In order to obtain evidence for the correct pairing of the remaining half-cystinyl residues, the Fab(t) fragment was cleaved with CNBr, and the products were separated by gel filtration (Figure 2). Two major fractions containing half-cystine were obtained. Fraction II (Figure 2) contained the disulfide-linked CNBr fragment complex composed of fragments H_1, H_{4A}, and L_3 (Waxdal *et al.*, 1968a,b). This fragment

TABLE II: Amino Acid Compositions of Peptides Isolated from a Diagonal Map of a Peptic–Tryptic Digest of Fragment Complex L_1–L_2 (Figure 2).[a]

Peptide	A	B	C	D	E	F
Arg	1.0		1.0		1.0	
$CySO_3H$	0.8	0.8	0.7	0.5	0.9	0.8
Asp						2.0
Ser						0.9
Glu		2.0		2.0		2.0
Tyr		1.0		1.7		1.9
Yield (nmoles)	1.5	2.5	4.5	4.0	4.9	6.7

[a] In this and the following tables of amino acid compositions, amino acids present in amounts corresponding to less than 0.1 residue/mole of peptide are omitted.

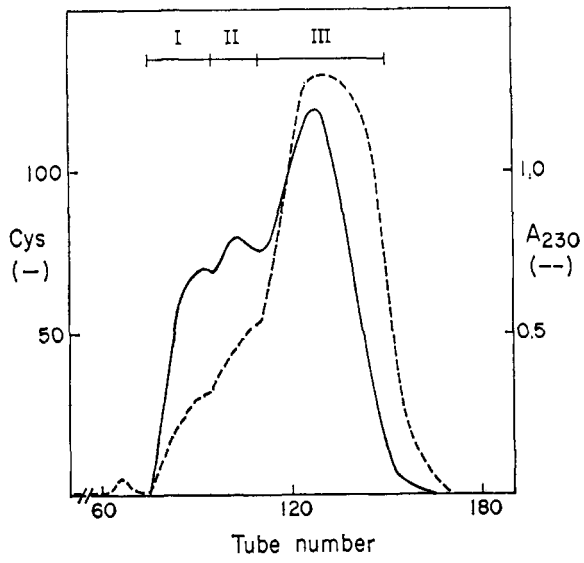

FIGURE 4: Gel filtration of a peptic digest of the H_1–H_{4A}–L_3 fragment complex (85 mg). Column: Sephadex G-50 (fine) in acetic acid–*n*-propyl alcohol–water (2:10:88, v/v), 1.9 × 110 cm. Volume per tube, 1.6 ml; (−) half-cystine content of the effluent, on an arbitrary scale; (---) absorbance of the effluent at 230 mμ. Roman numerals indicate fractions taken for further analysis.

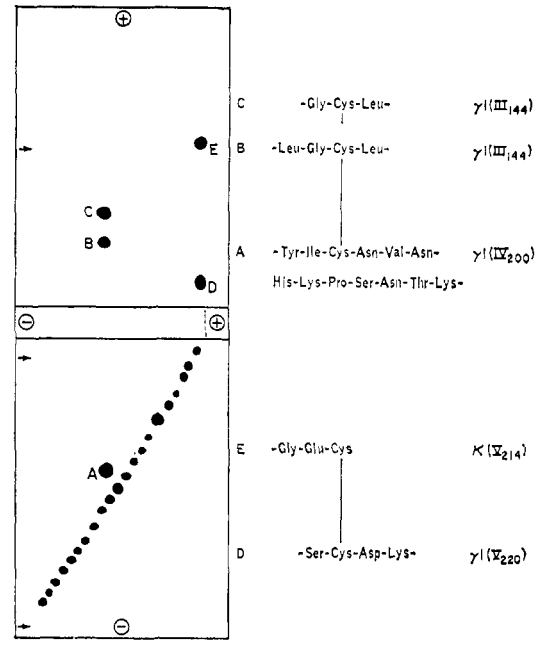

FIGURE 5: Diagonal map of a tryptic digest of fraction II from a peptic digest of the H_1–H_{4A}–L_3 fragment complex (Figure 4). See text and legend of Figure 3 for details.

complex contains three of the four intrachain disulfide bonds in the Fab region. Fraction III (Figure 2) contained the L_1–L_2 fragment complex (Waxdal *et al.*, 1968a,b) in which the remaining disulfide bond is located. Fraction IV (Figure 2) contained fragments H_2 and H_3, which have no half-cystine. Fraction I (Figure 2) was composed of aggregated or uncleaved material.

Fragment L_1 contains half-cystine $\kappa(I_{23})$ and fragment L_2 contains half-cystine $\kappa(II_{88})$. Previous studies (Waxdal *et al.*, 1968a,b) suggested that these two residues formed an intrachain disulfide bond. To provide proof of this linkage, the L_1–L_2 complex (fraction III, Figure 2) was extensively digested with pepsin. The products were further digested with trypsin and a diagonal map of this material was prepared (Figure 3). The amino acid compositions of peptides A–F are given in Table II. Peptides A, C, and E all had the same amino acid composition and contain half-cystine $\kappa(I_{23})$ (Figure 3). The compositions of peptides B, D, and F differ from each other, but all are consistent with the amino acid sequence around $\kappa(II_{88})$ (Figure 3). The yield (Table II) of both peptides in each pair (*i.e.*, A and B, C and D, and E and F) was nearly the same, indicating that both components of the original disulfide-linked peptide had been recovered. These data provide direct proof for the disulfide bond $\kappa(I_{23}$–$II_{88})$ in the Eu light chain.

The H_1–H_{4A}–L_3 fragment complex (fraction II, Figure 2) was used to determine the arrangement of the remaining intrachain disulfide bonds in the Fab region. A peptic digest of this complex was filtered on Sephadex G-50 yielding three fractions (Figure 4). Each fraction was digested with trypsin, and diagonal maps were prepared (Figures 5 and 6). The diagonal map of the digest of fraction I was not significantly different from that of the digest of fraction II and is not shown.

The amino acid compositions of the peptides which migrated away from the diagonal are given in Table III. The composition of peptide A (Figure 5) corresponds to the amino acid sequence around half-cystine $\gamma1(IV_{200})$. The compositions of peptides B and C correspond to the sequence around half-cystine $\gamma1(III_{144})$. Although peptides B and C both contain half-cystine $\gamma1(III_{144})$ and were found in the same vertical zone, the sum of the yields of peptides B and C is equal to the yield of peptide A. These data indicate that there is a disulfide bond $\gamma1(III_{144}$–$IV_{200})$. Peptides D and E (Figure 5) contain half-cystines $\kappa(V_{214})$ and $\gamma1(V_{220})$, in agreement with the pre-

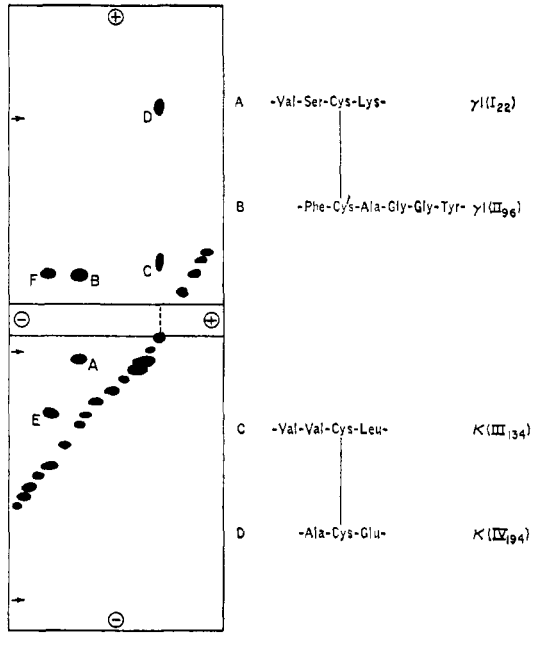

FIGURE 6: Diagonal map of a tryptic digest of fraction III from a peptic digest of the H_1–H_{4A}–L_3 fragment complex (Figure 4). See text and legend of Figure 3 for details.

TABLE III: Amino Acid Compositions of Peptides Isolated from Diagonal Maps of Enzymatic Digests of the H_1–H_{4A}–L_3 Fragment Complex.

Map Peptide	Fraction II (Figure 5)					Fraction III (Figure 6)			
	A	B	C	D	E	A	B	C[a]	D[a]
Lys	1.9			0.9		0.5			
His	0.8								
$CySO_3H$	0.8	0.9	1.0	0.9	0.9	1.0	0.7	0.8	0.8
Asp	2.9			1.0					
Thr	0.9								
Ser	0.9			0.8		1.1			
Glu					1.0				1.0
Pro	1.0								
Gly		0.8	0.7		0.8		2.0		
Ala							1.0		0.9
Val	0.8					0.9		1.8	
Ile	0.9								
Leu		1.9	1.1					1.0	
Tyr	0.8						1.0		
Phe							0.8		
Yield (nmoles)	84	28	50	61	48	15	12	8	7

[a] Isolated from a diagonal map of fraction III without prior tryptic digestion.

vious conclusion that these residues form an interchain disulfide bond (Gall *et al.*, 1968).

Peptides A and B (Figure 6 and Table III) establish that half-cystines $\gamma 1(I_{22})$ and $\gamma 1(II_{96})$ form the disulfide bond that links CNBr fragment H_1 to H_4 (see Figure 1). The amino acid compositions of peptides C and D (Figure 6) correspond to the amino acid sequence around half-cystines $\kappa(III_{134})$ and $\kappa(IV_{194})$, respectively. These peptides were contaminated with peptides containing half-cystines $\kappa(V_{214})$ and $\gamma 1(V_{220})$ (compare with peptides E and D, Figure 5). This contamination was not observed in peptides corresponding to C and D isolated from a diagonal map of fraction III (Figure 4) which had not been digested with trypsin. These results indicate the existence of the disulfide bond $\kappa(III_{134}$–$IV_{194})$. Peptides F and E (Figure 6) contain half-cystines $\gamma 1(III_{144})$ and $\gamma 1(IV_{200})$.

The above data demonstrate that the arrangement of the intrachain disulfide bonds in the light chain and the Fd region of the Eu heavy chain is linear, *i.e.*, half-cystine I is linked to

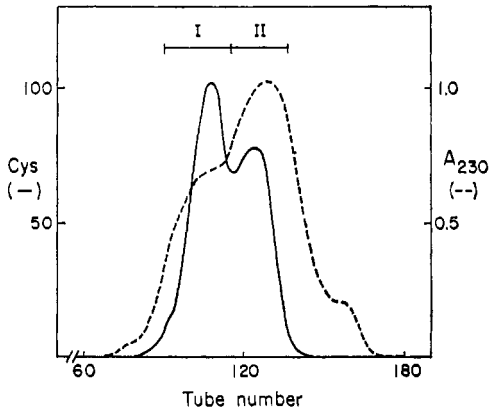

FIGURE 7: Gel filtration of a peptic digest of the Fc(t) fragment (130 mg). Column: Sephadex G-50 (fine) in acetic acid–*n*-propyl alcohol–water (2:10:88, v/v), 1.9 × 110 cm. Volume per tube, 1.6 ml; (—) half-cystine content of the effluent, on an arbitrary scale; (---) absorbance of the effluent at 230 mμ. Roman numerals indicate fractions taken for further analysis.

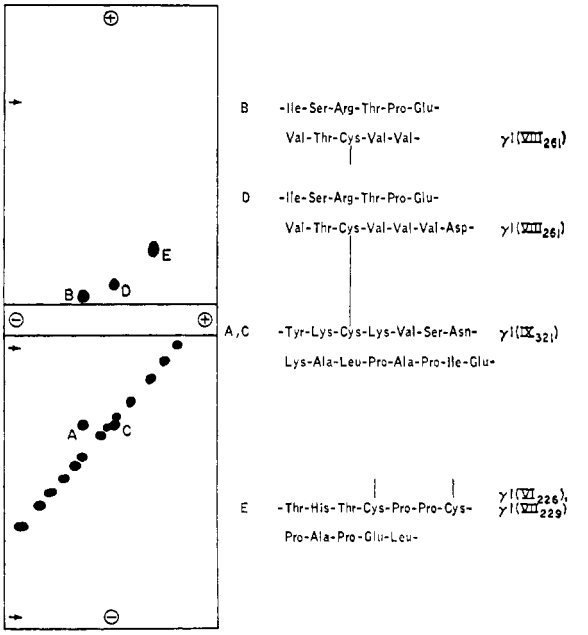

FIGURE 8: Diagonal map of fraction I from a peptic digest of Eu Fc(t) fragment (Figure 7). See text and legend of Figure 3 for details.

TABLE IV: Amino Acid Compositions of Peptides from Diagonal Maps of a Peptic Digest of the Fc Fragment.

Map Peptide	Fraction I (Figure 8)					Fraction II (Figure 9)		
	A	B	C	D	E	A	B	C
Lys	2.2		2.7					
His					0.8			
Arg		0.9		0.7				
$CySO_3H$	0.6	0.5	0.7	0.3	1.9	0.9	0.9	0.8
Asp	1.3		1.0	1.0				
Thr		2.3		2.0	1.5			1.0
Ser	1.0	1.2	1.0	1.0		1.9	1.9	
Glu	1.4	1.2	1.0	1.3	1.0			
Pro	2.1	0.7	1.8	0.8	3.7			
Ala	2.0		2.0		1.0			
Val	1.1	2.8	1.0	3.8		1.0	1.0	
Met[a]						1.0	1.0	
Ile	0.9	0.9	1.0	0.8				
Leu	1.1		1.0		1.0			1.4
Tyr	0.4		0.4					
Phe						1.0		
Yield (nmoles)	65	62	40	30	40	12	5	16

[a] As methionine sulfone.

II, and half-cystine III is linked to IV. Diagonal maps prepared from digests of the whole Fab(t) fragment, of the H_1–H_4–L_3 fragment complex (Waxdal *et al.*, 1968a,b), and of the Eu light chain confirmed this arrangement.

Disulfide Bonds of the Fc Region. Fc(t) fragment prepared without prior reduction was digested extensively with pepsin. Gel filtration of this digest yielded two major cystine-containing fractions (Figure 7). Diagonal maps of each fraction are shown in Figures 8 and 9, and the amino acid compositions of peptides which migrated away from the diagonal are given in Table IV.

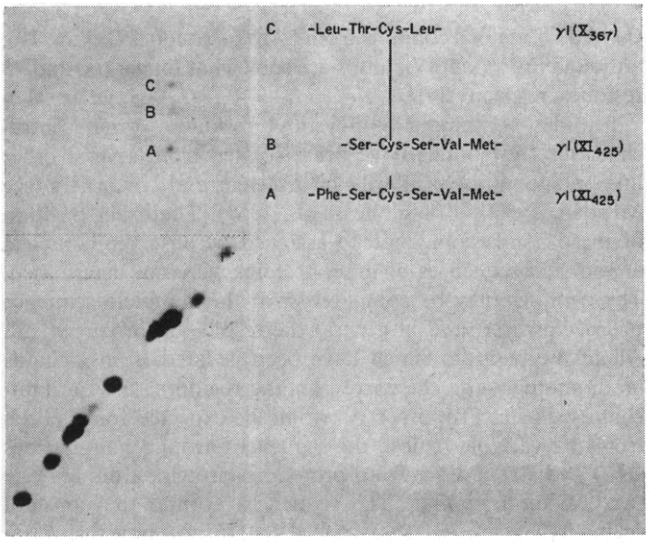

FIGURE 9: Photograph of a diagonal map of fraction II from a peptic digest of Eu Fc(t) fragment (Figure 7). See text and legend of Figure 3 for details.

The amino acid compositions of peptides A and C (Figure 8) were identical and corresponded to the sequence around half-cystine γ1(IX_{321}). Peptides B and D contain half-cystine γ1($VIII_{261}$), indicating that there is a disulfide bond γ1($VIII_{261}$–IX_{321}). Peptide E (Table IV) contains half-cystines γ1(VI_{226}) and γ1(VII_{229}) which form the inter-heavy-chain disulfide bonds (Gall *et al.*, 1968). Because these bonds are symmetrical, oxidation yields only a single peptide.

The diagonal map of fraction II (Figure 9) provides evidence for the disulfide bond γ1(X_{367}–XI_{425}). Although peptides A and B both contain half-cystine γ1(XI_{425}), the sum of their yields is equivalent to the yield of peptide C, which contains half-cystine γ1(X_{367}).

These results indicate that the arrangement of the intrachain bonds in the Fc region of the molecule is linear, *i.e.*, that half-cystine VIII is linked to IX, and X is linked to XI. This is consistent with the observation that cyanogen bromide

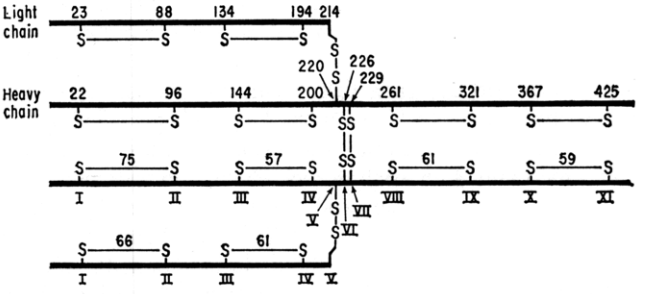

FIGURE 10: The disulfide bonds of the human γG1-myeloma protein Eu. The Arabic numbers above each half-cystine in the top half of the figure indicate the position of that residue in the amino acid sequence of the molecule. The number of residues contained in each disulfide loop is indicated by Arabic numerals in the appropriate positions in the lower half of the figure.

TABLE V: Intrachain Disulfide Bonds in Human Light Chains.

	I. Variable Regions[a]	
κ chains		
	18 I 27	83 II 92
I Eu	-Arg-Val-Thr-Ile-Thr-Cys-Arg-Ala-Ser-Gln-	-Phe-Ala-Thr-Tyr-Tyr-Cys-Gln-Gln-Tyr-Asn-
	Ile Ala Gln	Ile Phe Asp Glu
II HBJ 3	-Pro-Ala-Ser-Ile-Ser-Cys-Arg-Ser-Ser-Gln-	-Val-Gly-Val-Tyr-Tyr-Cys-Met-Gln-Ala-Leu-
	Thr	Gln Met Arg
III Rad	-Arg-Ala-Thr-Leu-Ser-Cys-Arg-Ala-Ser-Gln-	-Phe-Ala-Val-Tyr-Tyr-Cys-Gln-Gln-Tyr-Glu-
λ chains		
	17 I 26	82 II 91
I New	-Lys-Val-Thr-Ile-Ser-Cys-Ser-Gly-Gly-Ser-	-Glu-Ala-Asp-Tyr-Tyr-Cys-Ala-Thr-Trp-Asp-
	Arg Ala Ile Ser	His His Gln Ala
	Gly	
	Ala	
II Bo	-Ser-Val-Thr-Ile-Ser-Cys-Thr-Gly-Thr-Ser-	-Glx-Ala-Asx-Tyr-Tyr-Cys-Ser-Ser-Tyr-Val-
	Thr Ile	
III Sh	-Thr-Val-Arg-Ile-Thr-Cys-Gln-Gly-Asp-Ser-	-Glu-Ala-Asp-Tyr-Tyr-Cys-Asn-Ser-Arg-Asp-
IV Kern	-Thr-Ala-Val-Ile-Thr-Cys-Ser-Gly-Asp-Asn-	-Glu-Ala-Asp-Tyr-Phe-Cys-Gln-Thr-Trp-Asp-
	Ser Gly Lys	Tyr Ala
	II. Constant Regions[b]	
κ chains		
	129 III 138	189 IV 198
Eu	-Thr-Ala-Ser-Val-Val-Cys-Leu-Leu-Asn-Asn-	-His-Lys-Val-Tyr-Ala-Cys-Glu-Val-Thr-His-
		Leu
λ chains		
New	-Lys-Ala-Thr-Leu-Val-Cys-Leu-Ile-Ser-Asp-	-His-Arg-Ser-Tyr-Ser-Cys-Gln-Val-Thr-His-

[a] For variable regions, portions of the sequence of one protein from each subgroup, indicated by the Roman numerals, are given. Data for proteins other than Eu are from Dreyer *et al.* (1967), HBJ 3; Milstein (1967), Rad; Langer *et al.* (1968), New; Putnam *et al.* (1967), Bo; Wikler *et al.* (1967), Sh; Ponstingl *et al.* (1968), Kern. Variants which have been observed are shown in the appropriate positions. For reference to these variants, see Edelman and Gall (1969). [b] Residue 191 is leucine in Inv(2+) κ chains and valine in Inv(3+) κ chains (Baglioni *et al.*, 1966).

fragments H_5 and H_6 (Figure 1) can be isolated without prior reduction and alkylation (Waxdal *et al.*, 1968b).

Discussion

The arrangement and exact location of the disulfide bonds of the immunoglobulin Eu are shown on a linear model of the molecule in Figure 10. There are four interchain disulfide bonds, one between each light chain and its corresponding heavy chain and two between the heavy chains (Gall *et al.*, 1968). There are 12 intrachain disulfide bonds; 2 in each light chain and 4 in each heavy chain. Several important features of the arrangement and location of the disulfide bonds are illustrated in Figure 10. (1) The interchain bonds are grouped in a small region of the molecule about the middle of the heavy chain. (2) The arrangement of the intrachain bonds is linear, *i.e.*, half-cystine I is linked to II, III to IV, etc. (3) The corresponding half-cystinyl residues are located in nearly parallel positions in the amino acid sequence of the light and heavy chains. For example, half-cystine $\kappa(I_{23})$ is aligned with half-cystine $\gamma1(I_{22})$. (4) Each disulfide loop in the C_H and C_L regions contains from 57 to 61 residues (Figure 10), although the V_L and V_H loops are somewhat longer (66 and 75 residues, respectively).

Peptides containing half-cystinyl residues forming intrachain disulfide bonds have been isolated from several other immunoglobulins, primarily by Milstein and coworkers (see Milstein, 1966b; Frangione *et al.*, 1969). The exact position of these bonds could not be assigned because the complete amino acid sequences of these proteins were not determined. The peptides may be compared with the complete sequence of Eu, however, and in general the results are in agreement. All of the peptides which have been isolated from κ chains are homologous to the corresponding region of the Eu light-chain sequence (Table V). As would be expected for peptides from the variable region, the sequences around half-cystines $\kappa(I_{23})$ and $\kappa(II_{88})$ differ from protein to protein, although any two are quite similar. The sequences around the constant region half-cystines $\kappa(III_{134})$ and $\kappa(IV_{194})$ do not show this variation. Similar results have been obtained for λ chains (Table V), and peptides containing the constant region disulfide bridges have been isolated from pooled normal human light chains

TABLE VI: Intrachain Disulfide Bonds in Human Heavy Chains.[a]

		I. Variable Regions	
		18 I 27	92 II 100
γ1	Eu	-Val-Lys-Val-Ser-Cys-Lys-Ala-Ser-Gly-Gly-	-Ala-Phe-Tyr-Phe-Cys-Ala-Gly-Gly-Tyr-
	Dee	-Val-Arg-Ile(Ser,Cys,Lys,Ala,Ser,Gly)	-Tyr-Tyr-Cys-Thr-Gly-Arg-Gly-Met-
	Daw	-Leu-Thr-Leu-Thr-Cys-Thr-Phe-Ser-Gly-Phe-	-Ala-Thr-Tyr-Tyr-Cys-Ala-Arg-Ser-Cys-
γ4	Vin	-Leu-Ser-Cys-Ala-Ala-Ser-Gly-Phe-	-Ala-Val-Tyr-Tyr-Cys-Ala-Arg-
		18 27	93 99
μ	Ou	-Leu-Thr-Leu-Thr-Cys-Thr-Phe-Ser-Gly-Phe-	-Ala-Thr-Tyr-Tyr-Cys-Ala-Arg-
		II. Constant Regions	
		140 III 147	198 IV 205
γ1	Eu, Dee, Car	-Ala-Ala-Leu-Gly-Cys-Leu-Val-Lys-	-Tyr-Ile-Cys-Asn-Val-Asn-His-Lys-
γ2	Sa	-Ala-Ala-Leu-Gly-Cys-Leu-	-Tyr(Thr,Cys,Asx,Val,Asx,His,Lys)
γ3	Kup Bru	(Ala,Ala,Leu)Gly-Cys-Leu-	(Tyr,Thr,Cys,Asx,Val,Asx,His,Lys)
γ4	Vin	-Ala-Ala-Leu-Gly-Cys-Leu-	-Tyr-Thr-Cys-Asn-Val-Asp-His-Lys-
		258 VIII 265	319 IX 325
γ1	Eu, Cra	-Glu-Val-Thr-Cys-Val-Val-Val-Asp-	-Tyr-Lys-Cys-Lys-Val-Ser-Asn-
γ2	Sa	(Glu,Val,Thr,Cys,Val)	-Thr-Lys-Cys-Lys-Val-Ser-Asn-
γ3	Zuc	-Glu-Val-Thr-Cys-Val(Val,Val,Asp)	-Tyr-Lys-Cys-Lys-Val-Ser-Asn-
γ4	Vin	(Glu,Val,Thr,Cys,Val)	-Tyr-Lys-Cys-Lys-Val-Ser-Asn-
		364 X 370	423 XI 429
γ1	Eu, Cra	-Ser-Leu-Thr-Cys-Leu-Val-Lys-	-Phe-Ser-Cys-Ser-Val-Met-His-
γ2	Sa	-Thr(Cys,Leu)	-Phe-Ser(Cys,Ser,Val,Met)
γ3	Zuc	-Leu-Thr(Cys,Leu)	-Phe-Ser(Cys,Ser,Val,Met)
γ4	Vin	-Leu-Thr-Cys-Leu-	(Phe,Ser,Cys,Ser,Val,Met)

[a] Data for proteins other than Eu are from Frangione *et al.* (1969), with the exception of Ou (Wikler *et al.*, 1969) and Daw (Press and Piggot, 1967; Press and Hogg, 1969).

(Milstein, 1966a). We may therefore conclude that human light chains have two intrachain disulfide bridges, one in the variable region and one in the constant region. Both of these bridges form loops containing about 60 residues. Because of the diversity of V_L regions, it might be expected that some light chains which contain extra half-cystinyl residues may be found. The amino acid sequence of a λ chain with an extra half-cystinyl residue in the variable region has been reported (Milstein *et al.*, 1968). This cysteinyl residue is partly blocked through formation of a disulfide bond with free cysteine. The characteristic structure of light chains is preserved, however.

Only a few peptides containing half-cystine have been isolated from heavy chains (Table VI), but they show the expected similarity to the sequence of the heavy chain of protein Eu. In contrast to the variation shown by interchain bonds (Frangione and Milstein, 1968; Frangione *et al.*, 1969), the pattern of intrachain bonding in the four subclasses of γ chains seems to be preserved. There are three disulfide loops in the constant region of the heavy chain, and differences in sequences around the half-cystines in this region probably are specific for each subclass. If all γ chains are assumed to have nearly the same length, then each intrachain bond in the C_H region forms a loop of about 60 residues similar to the C_L loop. The V_H loop is somewhat longer, in the Eu heavy chain and may also be longer in other γ chain variable regions. The amino acid sequence of a portion of the variable region of the heavy chain from an immunoglobulin of the γM class has also been reported (Wikler *et al.*, 1969). There is a disulfide bond (Table VI) between Cys_{22} and Cys_{97}, forming a loop of 76 residues similar in length and position to the bond γ1 (I_{22}–II_{96}) of protein Eu.

It should be noted that the V_H region of the protein Daw (Press and Piggot, 1967; Press and Hogg, 1969) contains two more half-cystinyl residues than the V_H region of the Eu heavy chain. These residues, at positions 35 and 101, presumably from a disulfide bond in addition to the bond assumed to link half-cystines at positions 22 and 97 (Press and Hogg, 1969). Although these bonds are staggered, they are shifted by only a few residues and thus connect the same regions of the chain.

The data on the intrachain disulfide bonds of heavy and light chains suggest that the linear and periodic arrangement of these bonds may be a common structural feature of γG-immunoglobulins and perhaps of other classes as well. This arrangement provides additional evidence for the idea

(Hill *et al.*, 1966; Singer and Doolittle, 1966) that immunoglobulins evolved by duplication of a precursor gene sufficient in size to specify a polypeptide of about 110 residues in length.

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