The Covalent Structure of a Human γG-Immunoglobulin. IV. The Interchain Disulfide Bonds*

W. Einar Gall, Bruce A. Cunningham,† Myron J. Waxdal, William H. Konigsberg, and Gerald M. Edelman

ABSTRACT: The human γG-immunoglobulin, Eu, contains four interchain disulfide bonds. Each light chain is joined to a heavy chain by a single disulfide bond between the COOH-terminal half-cystine residue in the light chain and a half-cystine residue in the region of the heavy chain corresponding to fragment H_4. The two heavy chains are joined by two disulfide bonds in this region. The amino acid sequence of 41 residues of this portion of the heavy chain has been determined; all three half-cystine residues that participate in interchain bonding lie within a linear sequence of ten residues. This region of the heavy chain also contains a point at which trypsin attacks the whole molecule to produce the enzymatic fragments Fab(t) and Fc(t).

γG-Immunoglobulins are composed of four polypeptide chains linked by disulfide bonds. Each light chain is joined to a heavy chain by a single disulfide bond (Fleischman *et al.*, 1963; Pink and Milstein, 1967a; Edelman *et al.*, 1968; Waxdal *et al.*, 1968b), and the two heavy chains are joined by one or more disulfide bonds (Palmer and Nisonoff, 1964). Previous studies (Waxdal *et al.*, 1968b) indicate that the light-chain–heavy-chain disulfide bonds of the human myeloma protein Eu link CNBr fragments L_3 and H_4 and that the heavy-chain–heavy-chain bonds link the two H_4 fragments. All of the interchain disulfide bonds of Eu have been localized in a single fraction obtained by gel filtration of CNBr-treated Eu (Waxdal *et al.*, 1968b).

In this communication we present evidence that Eu contains two disulfide bonds between the heavy chains and a single bond linking the heavy chain to the light chain. The amino acid sequence in the vicinity of these interchain disulfide bonds has been determined. Similar results (Frangione and Milstein, 1967; Pink and Milstein, 1967b; Steiner and Porter, 1967) on the interchain bonds have recently been reported for several other human γG-immunoglobulins.

Materials and Methods

The purification of Eu, the separation of chains, and the preparation of tryptic fragments from partially reduced and alkylated Eu have been previously described (Edelman *et al.*, 1968). The conditions for the production and separation of CNBr fragments and for tryptic and chymotryptic digestion (Waxdal *et al.*, 1968a,b) have also been previously described. Amino acid analysis was performed as described by Edelman *et al.* (1968).

Partial Reduction and Alkylation. Eu was dissolved in 0.15 M Tris buffer (pH 8.0) which was 0.15 M in NaCl and 0.002 M in EDTA; the final protein concentration varied from 22 to 40 mg/ml. Dithiothreitol was added, and the solution was allowed to stand at room temperature. After various times, iodoacetamide (twice recrystallized from water) was added, and the solution was allowed to stand in the dark. Amounts of dithiothreitol and iodoacetamide were varied from experiment to experiment, as described in the Results section. Iodoacetamide-1-^{14}C (New England Nuclear, Boston, Mass., lot 252-43-1-2, sp act. 1.23 μCi/μmole) was used in some experiments. The optimal concentrations and times for partial reduction and alkylation were: 0.005 M dithiothreitol, 40 min; 0.010 M iodoacetamide, 20 min.

Complete Reduction. Disulfide-linked peptides were reduced and alkylated in guanidine as described previously (Waxdal *et al.*, 1968b), using radioactive iodoacetamide. For complete reduction and aminoethylation prior to tryptic digestion, Fab(t)[1] (10 mg/ml) was dissolved in 8 M urea, 1.5 M in Tris, 0.003 M in EDTA, and 0.1 M in 2-mercaptoethanol (pH 8.5). After 4 hr the solution was made 0.8 M in ethylenimine. The reagents were removed by gel filtration on Sephadex G-50, 20 min after addition of the ethylenimine.

Tryptic Digest of Fraction I. Fraction I was prepared from CNBr-treated Eu as previously described (Waxdal

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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.

TABLE I: Incorporation of Iodoacetamide-1-^{14}C into Eu under Different Conditions of Reduction and Alkylation.[a]

Expt	Dithiothreitol Concn (M excess over protein)	Reduction Time (min)	Iodoacetamide Concn (M excess over protein)	Alkylation Time (min)	Moles of Radioact./ Mole of Protein
1	None		0.060 (400 times)	20	0.5 ± 0.01
2	0.005 (33 times)	20	0.030 (200 times)	20	7.7 ± 0.1
		40	0.030	20	7.3 ± 0.2
		60	0.030	20	8.5 ± 0.2
3	0.015 (100 times)	20	0.060 (400 times)	20	7.8 ± 0.9
		60	0.060	20	8.3 ± 0.8
		80	0.060	40	8.9 ± 0.1
		120	0.060	20	8.9 ± 0.3

[a] The protein concentration was 22 mg/ml in 0.15 M Tris, 0.15 M NaCl, and 0.002 M in EDTA (pH 8.0). After alkylation, reagents were removed by gel filtration on Sephadex G-25, and aliquots of the protein fraction were streaked on cellulose acetate strips for counting (see Materials and Methods). Protein concentrations were determined with a modified Folin–Ciocalteu reagent (Lowry *et al.*, 1951). Results are reported as the mean plus and minus the average deviation of duplicate or triplicate samples.

et al., 1968b). Trypsin (12 mg) treated with L-1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK-trypsin, Calbiochem., Los Angeles, Calif., lot 65345) was added to a solution of 615 mg of fraction I in 60 ml of distilled water, pH 4, in a jacketed cell maintained at 37°. The solution was brought to pH 7.2 by the dropwise addition of 2 M NH_4OH, and the pH was maintained at 7.2 with a Radiometer TTT1b pH-Stat. After 4 hr, the turbid solution was brought to pH 3 by the dropwise addition of glacial acetic acid. The resulting clear solution was lyophilized.

Gel Filtration and Ion-Exchange Chromatography. Gel filtration was carried out at room temperature using either Sephadex G-50 or G-25 fine. Peptides were chromatographed on sulfonated polystyrene resins, and the effluent was monitored with ninhydrin as previously described (Waxdal *et al.*, 1968a). In some cases the column temperature was maintained at 38°. The Arg-tryptic peptides (see Blocking of ϵ-NH_2 Groups) from CNBr fragment H_4 were separated by chromatography on DEAE-cellulose (Cellex-D, 0.87 mequiv/g, Bio-Rad Laboratories, Richmond, Calif.). The peptide mixture (2 μmoles) was loaded on a 1 × 40 cm column of the ion exchanger which had been equilibrated with 0.05 M $NaHCO_3$ at pH 7.6. Elution was performed with a linear gradient from 200 ml of the initial buffer to 200 ml of the same buffer which was 1.0 M in NaCl.

Analysis of Effluent Fractions. The ultraviolet absorbance at 280 and 230 mμ of the effluent fractions from Sephadex columns was measured with a Zeiss PMQII spectrophotometer. In some experiments ninhydrin analysis of the effluent fractions was carried out manually (Moore and Stein, 1954) or automatically using an Auto-Analyzer (Technicon, Ardsley, N. Y.) equipped with an automatic sampler attachment and fraction collector (Cunningham *et al.*, 1968). Cystine-containing fractions were identified by spotting 50- or 100-μl aliquots on paper and staining with nitroprusside (Toennies and Kolb, 1951).

Liquid Scintillation Counting. Samples to be counted were added to 20 ml of a scintillation fluid containing 500 ml of *p*-dioxane, 70 ml of Liquifluor (Pilot Chemicals, Watertown, Mass.), 50 g of naphthalene, and enough toluene to make a final volume of 1 l. Some samples were streaked on a 5 × 20 mm cellulose acetate strip, dried under an infrared lamp, and immersed in the same scintillation fluid for counting. A Packard (Donners Grove, Ill.) Model 574 scintillation counter was used.

Electrophoresis. High-voltage paper electrophoresis was carried out at pH 4.7 (Schwartz and Edelman, 1963) and 6.5 (Brown and Hartley, 1966) in pyridine acetate buffer.

Molecular weights were determined in 6.3 M guanidine-HCl, 0.1 M in Tris (pH 8.4), using the short-column method of Yphantis (1960); in some experiments, the solvent was also 0.1 M in 2-mercaptoethanol. Insulin (bovine pancreas, recrystallized, Mann Research Laboratories, New York, N. Y., lot no. N2054) was used as a standard. The partial specific volume of peptide H–H-1 was estimated from its amino acid composition and corrected for the presence of guanidine-HCl (Tanford *et al.*, 1967). A partial specific volume of 0.73 cc/g was used for insulin. Because of the uncertainty in the partial specific volume and the high density of the guanidine-HCl–Tris solvent, the error in the molecular weight was approximately 10%.

Blocking of ϵ-NH_2 Groups of Lysyl Residues. The CNBr fragment H_4 (Waxdal *et al.*, 1968b) was treated with maleic anhydride (Butler *et al.*, 1967). Subsequent dansylation (Gray, 1967) of an aliquot followed by acid hydrolysis and chromatography on polyamide layers (Woods and Wang, 1967) showed only a trace of ϵ-dansyllysine. The maleoylated H_4 was digested with trypsin as described above (Waxdal *et al.*, 1968a). The products of this procedure have been designated Arg-tryptic peptides to distinguish them from the usual tryptic peptides.

Isolation of Peptides H_4A1 and H_4A1P1. The Arg-tryptic peptide H_4A1 was isolated from a tryptic digest of

TABLE II: Content of *S*-Carboxamidomethylcysteine in Partially Reduced and Alkyated Eu and Isolated Chains and Fragments.

	Moles of Radioactivity/ Mole
Eu, alkylated	0.5
Eu, partially reduced and alkylated	8.0
Eu light chain	0.9
Eu heavy chain	3.2
2 (heavy + light) calculated	8.2
Eu Fab(t)	2.0
Eu Fc(t)	3.7[a]
2 Fab(t) + Fc(t) calculated	7.7

[a] Determined as *S*-carboxymethylcysteine after acid hydrolysis.

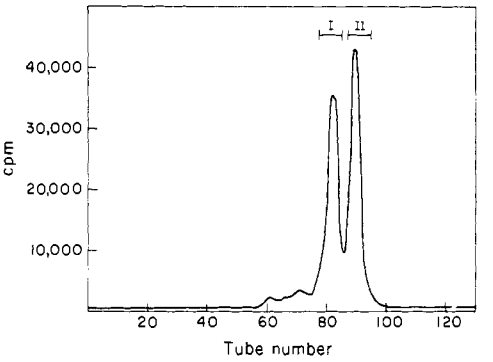

FIGURE 1: Gel filtration of a tryptic digest of Fab(t). The Fab(t) was prepared from partially reduced Eu which was alkylated with iodoacetamide-1-^{14}C and then was fully reduced and aminoethylated before digestion. Column: Sephadex G-25 fine in 2% acetic acid, 1.0 × 150 cm. Volume per tube, 1.0 ml; cpm = counts per minute per tube.

maleoylated H_4 which had been alklyated with iodoacetamide-1-^{14}C. The digest was fractionated by ion-exchange chromatography on DEAE-cellulose, and H_4A1 was isolated from the major radioactive fraction by gel filtration on Sephadex G-50 in 0.02 M NH_4OH.

Peptide H_4A1 was dissolved in 2% formic acid to a final concentration of 2 mg of peptide/ml and digested with pepsin (final concentration 0.04 mg/ml) at 25° for 1 hr. The pH of the solution was raised to 7 with 5 M NH_4OH and the solution was taken to dryness on a flash evaporator. Gel filtration of the digest on Sephadex G-50 in 0.02 M NH_4OH yielded H_4A1P1.

End Groups and Amino Acid Sequence. The NH_2-terminal residues of peptides were determined using the dansyl method (Gray, 1967). Amino acid sequences were determined by means of the dansyl-Edman or subtractive-Edman techniques. These procedures are described in detail by Cunningham *et al.* (1968). The COOH-terminal residues were determined by digestion with carboxypeptidase A as described by Guidotti *et al.* (1962).

Assignment of Amides. The electrophoretic mobility of peptides H_4Tn-1, L-H-2, and HO-3 at pH 6.5 was used to determine the presence of glutamine or asparagine.

Results

Localization of Interchain Disulfide Bonds. PARTIAL REDUCTION AND ALKYLATION OF EU WITH IODOACETAMIDE-^{14}C. A series of experiments was carried out to determine the extent of incorporation of iodoacetamide into Eu under different reducing agent concentrations, alkylating agent concentrations, and reaction times (Table I). The data indicate that about 8 moles of iodoacetamide are rapidly incorporated into the protein after exposure to the reducing agent. Alkylation without prior reduction resulted in the incorporation of 0.5 mole of iodoacetamide/mole of protein.

DISTRIBUTION OF RADIOACTIVITY IN CHAINS AND ENZYMATIC FRAGMENTS. A solution of Eu (45 mg/ml) was reduced with 0.005 M dithiothreitol for 40 min at room temperature and alkylated with iodoacetamide-1-^{14}C (final concentration 0.010 M) for 20 min. After extensive dialysis against water to remove excess reagent, 8.0 moles of iodoacetamide had been incorporated into the protein. Eight moles of *S*-carboxymethylcysteine per mole of protein was recovered after acid hydrolysis, indicating that all of the label was present as CM-cysteine.

Heavy and light chains were prepared in 95% yield from the reduced and alkylated protein. The specific activities of the chains are shown in Table II. All of the label in the reduced, alkylated protein was recovered in the isolated chains. These data indicate that the light chain contained 1 mole of CM-cysteine and the heavy chain contained 3 moles.

The enzymatic fragments Fab(t) and Fc(t) were prepared as previously described (Edelman *et al.*, 1968); Fab(t) contained 2 moles of CM-cysteine and Fc(t) contained 4 moles (Table II).

LOCALIZATION OF RADIOACTIVITY WITHIN ENZYMATIC FRAGMENTS. The enzymatic fragments were analyzed to determine whether the label was broadly distributed or was attached to specific half-cystine residues. Fab(t) was fully reduced and aminoethylated in urea and digested with trypsin. The fraction of the digest soluble in 2% acetic acid (containing 94% of the radioactivity) was subjected to gel filtration on Sephadex G-25 (Figure 1). Two peaks of radioactivity were found which together accounted for 83% of the radioactivity loaded. Paper electrophoresis at pH 6.5 of fraction I from Sephadex G-25 gave a single, neutral, radioactive fraction. Fraction II yielded a single, acidic, radioactive fraction. These data suggested that greater than 80% of the radioactivity in Fab(t) was present in two tryptic peptides.

Fc(t) was cleaved with cyanogen bromide as previously described (Waxdal *et al.*, 1968b), and the fragments were separated by gel filtration (Figure 2A). Eighty per cent of the radioactivity loaded on the

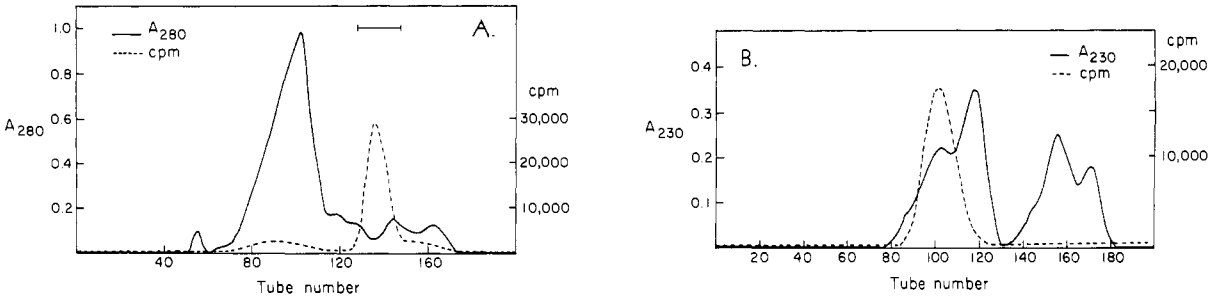

FIGURE 2: Gel filtration studies. (A) Of CNBr-cleaved Fc(t). The Fc(t) was prepared from partially reduced Eu which was alkylated with iodoacetamide-1-^{14}C. Column: Sephadex G-100 in 1 M propionic acid, 1 × 110 cm. Volume per tube, 0.5 ml; A_{280}, absorbance of effluent at 280 mμ; cpm = counts per minute per tube. The bar indicates fractions which were pooled and lyophilized. (B) Of pooled material from tubes 128 to 150 of the chromatogram in part A on Sephadex G-50 in 2% acetic acid. Column: 1.2 × 60 cm. Volume per tube, 0.5 ml; A_{230}, absorbance of effluent at 230 mμ; cpm = counts per minute per tube.

column was recovered, and the indicated fraction contained 75% of the recovered radioactivity. Subsequent purification of this fraction by gel filtration on Sephadex G-50 in 2% acetic acid (Figure 2B) yielded a fragment which was identified as H_{4B} by amino acid composition and end-group analysis (Waxdal *et al.*,1 968b). Fragment H_{4B} contains 2 moles of CM-cysteine, and since there are 2 moles of H_{4B}/mole of Fc(t) (Waxdal *et al.*, 1968a), all of the radioactivity in Fc(t) (Table II) is accounted for in this CNBr fragment.

The above data suggest that alkylation of partially reduced Eu takes place at specific half-cystine residues, one of which is located in each light chain, and three of which are in each heavy chain. Two of the three half-cystine residues of the heavy chain have been identified in cyanogen bromide fragment H_{4B}. This is consistent with the previous finding that all of the interchain bonds are located in the CNBr fragments L_3 and H_4 (Waxdal *et al.*, 1968b).

Isolation and Characterization of Tryptic Peptides Containing the Intact Interchain Disulfide Bonds. In order to isolate small fragments of the molecule containing the intact interchain disulfide bonds, 615 mg of fraction I from the CNBr cleavage of Eu (Waxdal *et al.*, 1968b) was digested with trypsin at pH 7.2, and the tryptic digest was fractionated on Sephadex G-50 in *n*-propyl alcohol–acetic acid–water (1:1:3, v/v) (Figure 3). Effluent fractions were pooled as indicated and lyophilized. Fraction A yielded 216 mg of lyophilized material, and fractions B and C each yielded 150 mg. The tryptic peptides containing the intact interchain disulfide bonds were isolated from these three fractions by a combination of gel filtration and ion-exchange chromatography.

To test the possibility that extensive disulfide interchange might occur under the conditions of tryptic digestion, a control digest of fraction I was prepared in the presence of 0.01 M iodoacetamide-1-^{14}C (approximately a fivefold molar excess over the total half-cystine). Separation of the peptides on Sephadex G-50 showed that less than 10% of the half-cystine had been alkylated, as measured by the incorporation of radioactivity. This radioactivity was diffusely distributed in the peptide fractions. If certain disulfide bonds were exceptionally susceptible to interchange, peaks of radioactivity corresponding to highly labeled peptides would have been observed. From these data, we conclude that extensive disulfide interchange does not occur.

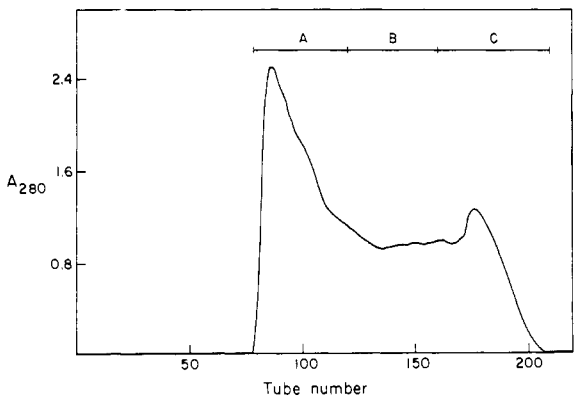

FIGURE 3: Gel filtration of the tryptic digest of fraction I obtained by CNBr cleavage of Eu. Column: Sephadex G-50 fine in *n*-propyl alcohol–acetic acid–water (1:1:3, v/v), 2.5 × 190 cm. Volume per tube, 4.2 ml; A_{280}, absorbance of effluent at 280 mμ.

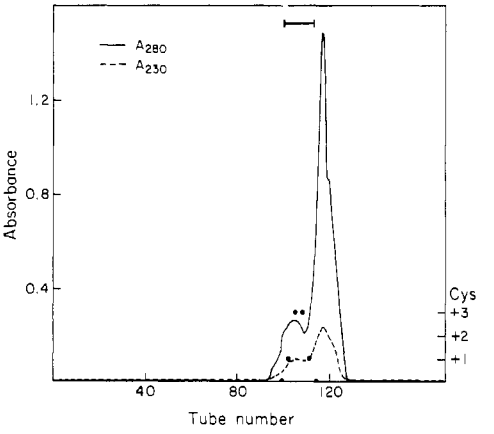

FIGURE 4: Gel filtration of nitroprusside-positive material from fraction C (Figure 3). Column: Sephadex G-25 fine in 5% formic acid, 2.1 × 98 cm. Volume per tube, 2.5 ml. (——) Absorbance of effluent at 280 mμ; (– – –) absorbance of effluent at 230 mμ; Cys (●) estimated intensity of nitroprusside reaction with 50-μl aliquots.

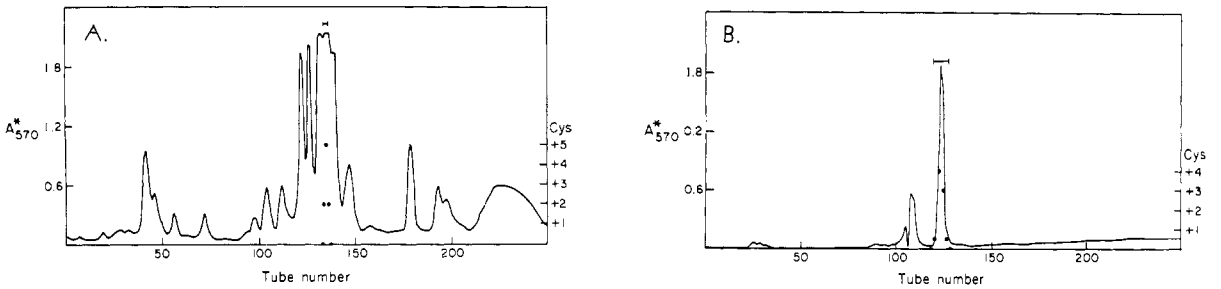

FIGURE 5: Chromatographic studies. (A) Of nitroprusside-positive material from Figure 4. Column: AG50X4, 0.9 × 15 cm a 37°, equilibrated with 0.05 M pyridine acetate (pH 3.1). Volume per tube, 2 ml. Gradient: tubes 1–19 were eluted with initia buffer. A linear gradient from 50 ml of initial buffer to 50 ml of 0.2 M pyridine acetate (pH 3.1) was begun at tube 20. Tubes 55–77 were eluted with 0.2 M pyridine acetate buffer (pH 3.1). A linear gradient from 150 ml of 0.2 M pyridine acetate (pH 3.1) to 150 ml of 1 M pyridine acetate (pH 5.5) was begun at tube 78. Tubes 212–229 were eluted with 1 M pyridine acetate (pH 5.5). A linear gradient from 50 ml of 1 M to 50 ml of 2 M pyridine acetate (pH 5.5) was begun at tube 230. A_{570}*: ninhydrin color value on an arbitrary scale. Cys (●): estimated intensity of nitroprusside reaction with 50-μl aliquots. (B) Of nitroprusside-positive material from part A on AG50X4, 0.9 × 7.5 cm at 37°, equilibrated with 0.2 M pyridine acetate (pH 3.1). Volume per tube, 2 ml. Gradient: tubes 1–69 were eluted with initial buffer. A linear gradient from 170 ml of initial buffer to 170 ml of 1 M pyridine acetate (pH 5.5) was begun at tube 70. Tubes 218 to 250 were eluted with 1 M pyridine acetate (pH 5.5). A_{570}*: ninhydrin color, value on an arbitrary scale. (●) Estimated intensity of nitroprusside reaction of 50-μl aliquots.

THE LIGHT-CHAIN–HEAVY-CHAIN DISULFIDE BOND. Preliminary experiments indicated that the tryptic peptide containing the intact light-chain–heavy-chain disulfide bond contained less than ten residues. For this reason, fraction C (Figure 3) was used as starting material for the isolation of this peptide. It was anticipated (Pink and Milstein, 1967a; Cunningham *et al.*, 1968) that the half-cystine residue contributed to this disulfide bond by the light chain is included in the carboxyl-terminal tripeptide, Gly-Glu-Cys. Therefore peptide fractions having a high ratio of cystine to lysine or arginine and containing glutamic acid and glycine were sought. The nitroprusside stain was used to test column effluent fractions for cystine.

Gel filtration of fraction C on Sephadex G-25 in 5% formic acid gave 65 mg of nitroprusside-positive material which was filtered again on the same column (Figure 4) and yielded 35 mg of a cystine containing a peptide mixture. After a third gel filtration on the same column, this mixture was chromatographed on AG50X4 (Figure 5A). A single nitroprusside-positive fraction was obtained from which peptide L–H-1 (Table III) was isolated by chromatography with a more shallow gradient on AG50X4 (Figure 5B). The yield of L–H-1 after the six steps in the purification was 16%.

Amino-terminal analysis of L–H-1 indicated the presence of two end groups, glycine and serine. High-voltage paper electrophoresis of L–H-1 at pH 4.7 demonstrated the presence of a single, ninhydrin-positive component with a small net negative charge. Following oxidation with performic acid, two negatively charged, ninhydrin-positive components were observed.

Reduction of L–H-1 with dithiothreitol and alkylation with iodoacetamide-1-^{14}C followed by gel filtration on Sephadex G-25 in 2% acetic acid gave two radioactive components, L–H-2 and L–H-3 (Table III), in addition to reagents. The amino acid sequence of L–H-2 was Ser-CMCys-Asp-Lys. This peptide was also isolated from tryptic digests of H_4 and Fd(t) and therefore must be derived from the heavy chain. The sequence of L–H-3 was Gly-Glu-CMCys, the known carboxyl-terminal tryptic peptide of the light chain (Cunningham *et al.*, 1968). These data show that L–H-1 is the tryptic peptide containing the intact light-chain–heavy-chain disulfide bond and that this bond links the COOH-terminal residue of the light chain to a half-cystine residue in that portion of Fd(t) corresponding to CNBr fragment H_{4A} (Waxdal *et al.*, 1968b).

THE HEAVY-CHAIN–HEAVY-CHAIN DISULFIDE BONDS. Preliminary experiments indicated that the two disulfide bonds between the heavy chains were located within a large tryptic peptide having a high proline to cystine ratio. Fractions A and B (Figure 3) were used as starting material for the isolation of this peptide, and peptide fractions were evaluated on the basis of their proline and cystine content. Column effluents were monitored for cystine using the nitroprusside stain.

TABLE III: Amino Acid Composition of Tryptic Peptides Containing the Interchain Disulfide Bonds.

	H–H-1	L–H-1	L–H-2	L–H-3
Lys		1.0	1.1	
His	1.0			
Asp		1.0	1.0	
Thr	1.9			
Ser	1.1	0.9	0.9	
Glu	1.1	1.0		1.1
Pro	4.7			
Gly	2.0	1.0		1.0
Ala	1.0			
Cys	1.8	2.1		
CMCys			1.0	1.0
Val	1.0			
Leu	2.0			
Phe	1.0			
Total residues	19	7	4	3

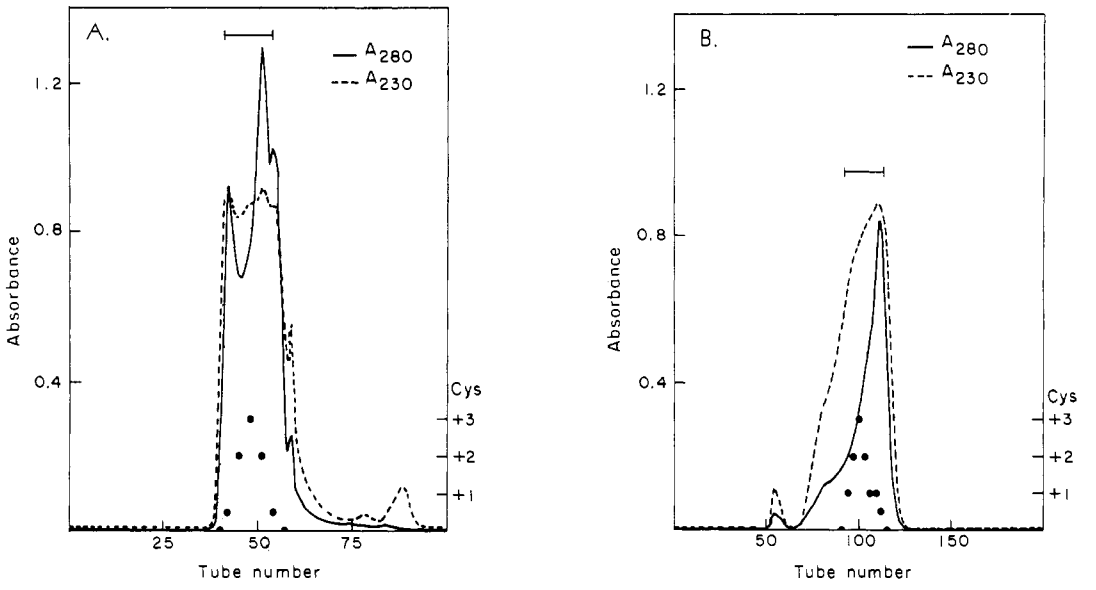

FIGURE 6: Gel filtration studies. (A) Of nitroprusside-positive material from fractions A and B (Figure 3). Column: 1.5 × 150 cm, Sephadex G-25 fine in 2% acetic acid. Volume per tube, 3 ml. (——) Absorbance at 280 mμ; (– – –) absorbance at 230 mμ. (●) Estimated intensity of nitroprusside reaction with 100-μl aliquots. The fraction indicated by the bar was lyophilized and filtered again on the same column. (B) Of nitroprusside-positive material from part A. Column: 1.5 × 150 cm, Sephadex G-50 in 2% acetic acid. Volume per tube, 1.75 ml. (——) Absorbance at 280 mμ; (– – –) absorbance at 230 mμ; (●) estimated intensity of nitroprusside reaction with 50-μl aliquots.

Chromatography of 216 mg of fraction A and 57 mg of fraction B on Sephadex G-50 in *n*-propyl alcohol–acetic acid–water (1:1:3, v/v) yielded 28 mg of a peptide fraction having a high proline to cystine ratio. Gel filtration of this material on Sephadex G-25 in 2% acetic acid (Figure 6A) yielded a single nitroprusside-positive fraction which was again filtered on Sephadex G-25 and then filtered on Sephadex G-50 (Figure 6B). The cystine-containing portion of this peptide mixture was chromatographed on AG50X4 (Figure 7) to yield peptide H–H-1 (Table III). The yield after the five steps of purification was 20%.

Amino-terminal analysis of H–H-1 gave only threonine, and the amino acid composition of H–H-1 (Table III) suggested that it was a single peptide containing two half-cystine residues per histidine residue. Alkylation of H–H-1 with iodoacetamide-1-^{14}C in guanidine in the absence of reducing agent did not lead to incorporation of radioactivity into the peptide. This indicates that H–H-1 contained no sulfhydryl groups and that the half-cystine residues were present in a disulfide linkage.

In order to show that H–H-1 is composed of two identical polypeptide chains linked by two disulfide

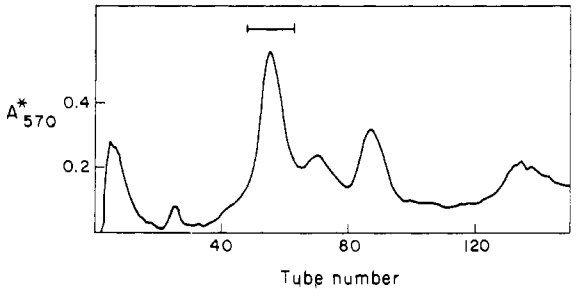

FIGURE 7: Chromatography of nitroprusside-positive material from Figure 6B. Column: AG50X4, 0.9 × 7.5 cm at 37° equilibrated with 0.2 M pyridine acetate (pH 3.1). Volume per tube, 2 ml. Gradient: tubes 1–8 were eluted with initial buffer. A linear gradient from 170 ml of initial buffer to 170 ml of 1 M pyridine acetate (pH 5.5) was started at tube 9. The indicated fraction contained peptide H–H-1. A_{570}*: ninhydrin color value in arbitrary units.

TABLE IV: Molecular Weight of Peptide H–H-1 before and after Reduction.[a]

Sample	Guan	Guan-SH
H–H-1		
Observed	3870 ± 80	2180 ± 140
Predicted	3840	1920
Insulin		
Observed	5800 ± 20	3190 ± 20
Predicted	5734	2970

[a] Guan = 6.3 M guanidine-HCl, 0.1 M in Tris (pH 8.4); Guan-SH = 6.3 M guanidine-HCl, 0.1 M in Tris and 0.1 M in 2-mercaptoethanol (pH 8.4). Observed molecular weights are weight-average molecular weights plus and minus average deviation of two channels with a twofold difference in initial concentration. Because of the uncertainty in the partial specific volume and the high solvent density, results are considered reliable only within 10%. Predicted molecular weights for insulin are based on the known sequence. The minimal molecular weight of H–H-1, based on its amino acid composition, is 1920.

TABLE V: Composition[a] and Yield of Peptides Used to Determine Sequence around the Interchain Disulfide Bonds.

	H_4Tn-1	L-H-2	FcCh-1	FcCh-2	FcCh-3	FcCh-4	HO-3	FcTn-1	H-H-2	H_4A1	H_4-A1P1	H_{4B}
Lys	1.0	1.0				2.2		2.1		4.8	2.0	2.0
His									1.0	1.0	0.9	1.0
Arg							0.8					
CMCys		0.9	1.8		1.8			1.8	2.0	3.8	2.9	1.9
Asp		1.1				1.0	0.8			2.2	1.1	1.0
Thr			0.9		1.0	1.0	0.9	0.8	1.8	3.1	1.8	2.9
Ser		0.9		1.1	1.1		1.1	0.9	1.1	5.7	0.9	1.0
Glu	1.0		1.1		1.1			1.1	1.1	5.7	2.0	1.1
Pro	1.1		4.2	1.0	5.2	3.1		7.7	5.0	7.1	5.1	8.2
Gly			0.2	2.1	2.2			2.2	2.2	4.2		2.1
Ala			1.0		1.1			0.9	1.1	2.8	1.1	1.1
Val	0.9			0.9	0.9			1.1	1.0	6.1	0.9	0.9
Met[b]							0.9					1.0
Ile							1.0					
Leu			2.1		1.8	2.0	1.1	3.2	2.1	3.0	2.0	4.0
Phe				0.8	1.0	0.9		1.9	1.0	1.1		2.0
% yield	40	75	20	15	40	50	30	70		40	30	85
Source	H_4	H_4	Fc(t)	Fc(t)	Fc(t)	Fc(t)	Fc(t)	Fc(t)	*d*	H_4	H_4	Fc(t)
Type of digest[c]	T	T	C	C	C	C	T	T	T	T	P	CNBr

[a] Contamination by other residues at 0.1 mole or less is not reported. [b] Methionine (HO-3) or homoserine plus homoserine lactone (H_{4B}). [c] T, tryptic digest; C, chymotryptic digest; P, peptic digest. [d] Prepared by reduction and alkylation of H-H-1.

bonds rather than a single peptide chain containing an internal disulfide bond, the molecular weight of H-H-1 was compared with the minimal molecular weight calculated from the amino acid composition. The molecular weight of H-H-1 (measured in guanidine in order to dissociate noncovalently bonded aggregates) was found to be twice the minimal molecular weight (Table IV), suggesting that the peptide is composed of 38 residues (Table III). Control experiments with bovine insulin (Table IV) indicated that 6.3 M guanidine-HCl and 0.1 M mercaptoethanol were sufficient to reduce and dissociate insulin into its constituent chains. Under these conditions, the observed molecular weight of H-H-1 decreased by about half. H-H-1 was reduced and carboxamidomethylated in guanidine, and reagents were removed by gel filtration on Sephadex G-25 in 2% acetic acid. The amino acid composition of the reduced and alkylated peptide (H-H-2) was identical with that of H-H-1 except that two residues of CM-cysteine were found instead of two residues of half-cystine. The data indicate that H-H-1 is a symmetrical dimer linked by two disulfide bonds (*cf.* Figure 9).

The amino acid sequence of H-H-2, determined by the dansyl-Edman procedure, was Thr-His-Thr-CMCys-Pro-Pro-CMCys-Pro-Ala-Pro-Glx-Leu-Leu-Gly-Gly-Pro-Ser-Val-Phe. A radioactive peptide was isolated from a chymotryptic digest of heavy chain prepared from partially reduced Eu which had been alkylated with iodoacetamide-1-^{14}C. The partial sequence of this peptide was Thr-CMCys-Pro-Pro-CMCys-Pro-Ala-(Pro,Glx,Leu_2). We therefore conclude that the half-cystine residues of H-H-2 participate in the interchain bonds.

Amino Acid Sequence of the Heavy Chain around the Interchain Disulfide Bonds. The amino acid sequences of L-H-1 and H-H-1 are summarized in Figure 9. In order to extend the sequences, peptides were isolated from tryptic and chymotryptic digests of H_4 and Fc(t) by ion-exchange chromatography and gel filtration. The source, yield, and amino acid composition of the peptides are presented in Table V. The sequence shown in Figure 8 was deduced by analyzing the chymotryptic peptides FcCh-1, FcCh-2, FcCh-3, and FcCh-4, the tryptic peptides, H_4Tn-1, L-H-2, FcTn-1, H-H-2, and HO-3 (Waxdal *et al.*, 1968a), and the peptic peptide H_4A1P1. Additional evidence was obtained by comparison of the sequence of these peptides with CNBr fragment H_{4B} and enzymatic fragment Fc(t). The partial or complete amino acid sequences of these peptides were determined by the dansyl-Edman procedure.

The data shown in Figure 8 establish the amino acid sequence of 38 residues in the CNBr fragment H_4. The sequence contains the three CM-cysteine residues of the heavy chain involved in interchain bonding and includes a region corresponding to CNBr fragment H_{4B} which begins the Fc(t) region of the Eu heavy chain (Waxdal *et al.*, 1968a). The amino acid residues are numbered from the COOH-terminal homoserine

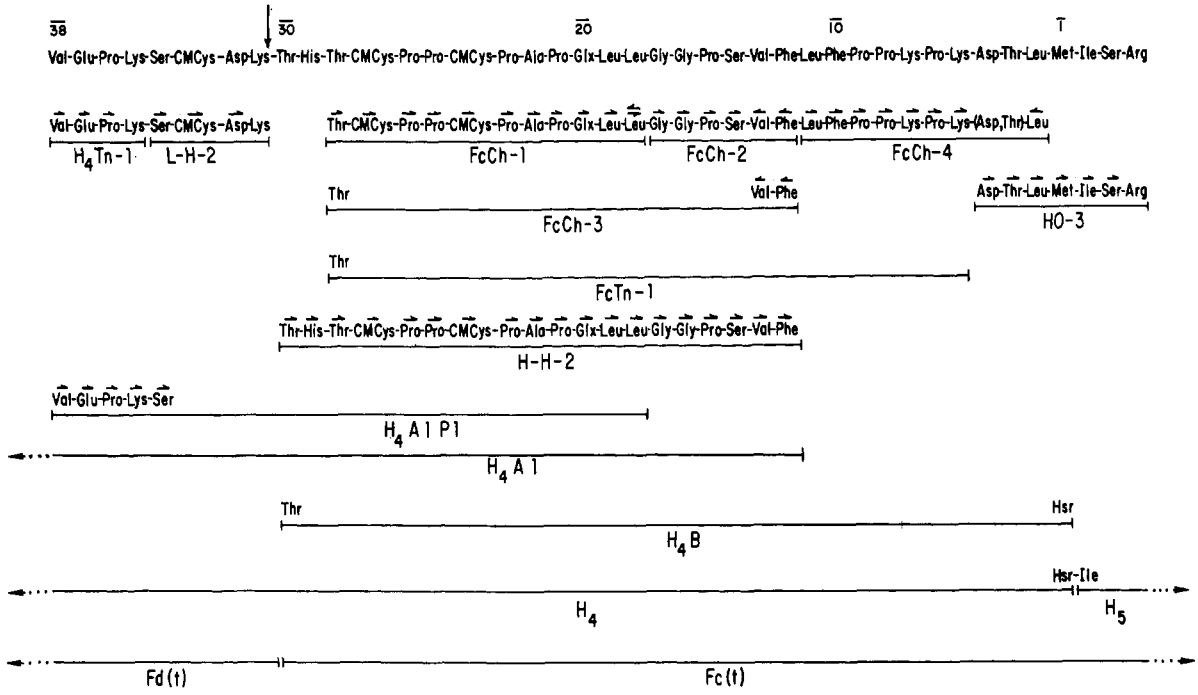

FIGURE 8: Peptides used to establish the sequence of Eu heavy chain in the region of the interchain disulfide bonds. The residues are numbered from the carboxyl-terminal homoserine residue of CNBr fragment H_4. (→) Sequence established by dansyl-Edman degradation. (←) Sequence established by treatment with carboxypeptidase A. The arrow at lysine $\overline{31}$ indicates a point of proteolysis by trypsin to yield Fab (t) and Fc (t).

residues of H_4. To avoid confusion with other numbering systems, a bar has been placed over the residue number.

CNBr fragment H_4 is made-up of H_{4A} and H_{4B} (Waxdal *et al.*, 1968b) and H_{4B} represents residues $\overline{1}$–$\overline{30}$ of H_4 (Figure 8). The sequence contained in H_{4B} is extended to residue $\overline{38}$ by peptides H_4A1P1, H_4Tn-1, and L–H-2. FcCh-1, FcCh-2, and FcCh-4 account for all the residues in H_{4B} except the COOH-terminal homoserine and the NH_2-terminal two residues, threonine and histidine. The sequence of peptide H–H-2 orders peptides FcCh-1 and FcCh-2 and completes the amino-terminal sequence of H_{4B}. Peptide FcCh-4 was placed in order by analyzing peptide FcTn-1 which had NH_2-terminal threonine and a composition consistent with the presence of residues $\overline{5}$–$\overline{28}$ (Table V). The sequence of H_{4B} is completed by peptide HO-3 which overlaps CNBr fragments H_4 and H_5 (Waxdal *et al.*, 1968a).

The peptic peptide H_4A1P1 was derived from the Arg-tryptic peptide H_4A1 (Table V) and overlaps fragments Fd(t) and Fc(t). The sequence of the first four residues of H_4A1P1 enables us to position L–H-2, which contains the CM-cysteine arising from the light-chain–heavy-chain disulfide bond. The sequence of HO-3 and of the amino-terminal portion of H_4A1P1 completes the sequence of 41 residues of the heavy chain in the vicinity of the interchain disulfide bonds.

Discussion

The present studies indicate that Eu contains four interchain disulfide bonds, all of which are found in CNBr fraction I (Waxdal *et al.*, 1968b). Each light chain is joined to a heavy chain by a single disulfide bond between the carboxyl-terminal half-cystine residue of the light chain and a half-cystine residue in the region of the heavy chain corresponding to CNBr fragment H_4 (Waxdal *et al.*, 1968b). The two heavy chains are joined by two disulfide bonds which are also in the H_4 region. All three half-cystine residues of the heavy chain that participate in the interchain bonding are in a linear stretch of ten residues.

The experiments on the alkylation of the easily reduced disulfide bonds with iodoacetamide-1-^{14}C provide the major evidence for the presence of four interchain bonds in the molecule. The light chain was prepared in high yield from partially reduced and alkylated Eu and contained only one residue of radioactive CM-cysteine. It is therefore attached to the heavy chain by only one disulfide bond. The heavy chain contained three residues of CM-cysteine per mole, one of which originated from the light-chain–heavy-chain bond. The remaining two half-cysteines must participate in linking the heavy chains.

As shown by the radioactive-labeling experiments, the light-chain–heavy-chain disulfide bond is contained within Fab(t). The heavy-chain–heavy-chain disulfide bonds are within the portion of Fc(t) corresponding to CNBr fragment H_{4B} and thus they are distal to the light-chain–heavy-chain disulfide bond.

In order to place the interchain bonds more precisely in the molecule, tryptic peptides containing intact disulfide bridges were isolated. Extensive disulfide interchange did not occur, as indicated by control experiments which included radioactive iodoacetamide in the

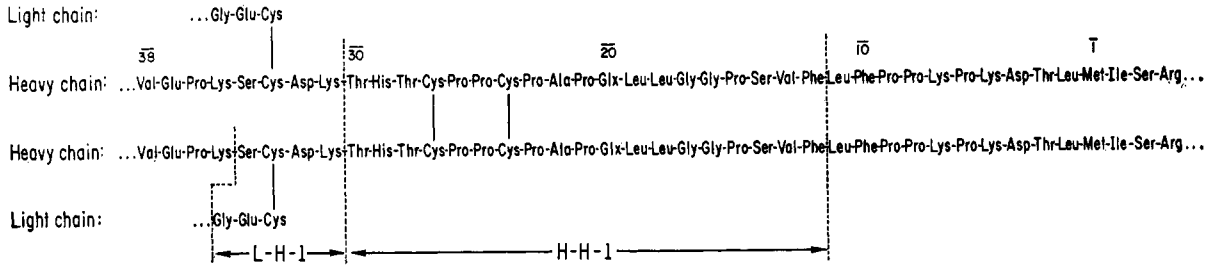

FIGURE 9: Sequence of a human γG-immunoglobulin (Eu) in the region of the interchain disulfide bonds. L–H-1 and H–H-1 indicate the disulfide-linked peptides isolated as described in Results. Evidence for the remainder of the sequence presented is shown in Figure 8.

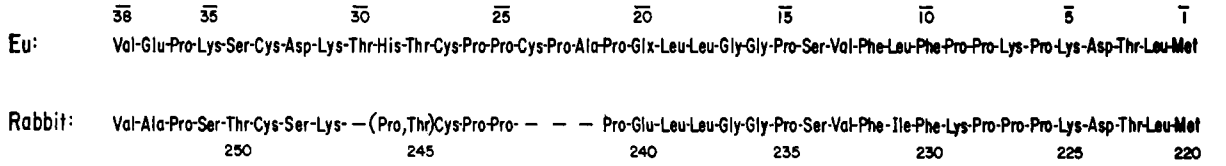

FIGURE 10: Comparison of the sequence of Eu heavy chain the region of the interchain disulfide bonds to the corresponding sequence of rabbit γG-immunoglobulin (Hill *et al.*, 1966b; Cebra, 1967; Smyth and Utsumi, 1967). The sequence of the rabbit protein has been interrupted at positions marked by the dashes, and the three resulting stretches have been aligned to maximize the homology between the two proteins. The sequence of the rabbit protein is numbered according to Hill *et al.* (1966b).

tryptic digests. The small amount of radioactivity incorporated was diffusely distributed and no single peptide fraction contained significant amounts of radioactivity. The disulfide bond between the light chain and the heavy chain (Figure 9) was found in disulfide-linked peptide, L–H-1. Reduction and alkylation of L–H-1 yielded two peptides, Ser-CMCys-Asp-Lys and Gly-Glu-CMCys. The peptide Ser-CMCys-Asp-Lys was also isolated from the heavy-chain fragments Fd(t) and H_4, and Gly-Glu-CMCys was the carboxyl-terminal tryptic peptide of the light chain (Cunningham *et al.*, 1968); this confirms the location of the light-chain–heavy-chain disulfide bond.

Both of the heavy-chain–heavy-chain disulfide bonds were located in tryptic peptide H–H-1, which contained two half-cystine residues per minimal molecular weight. The molecular weight of H–H-1 was twice the minimal molecular weight, and its molecular weight decreased by a factor of two when the peptide was reduced (Table IV). These data indicate that H–H-1 is a symmetrical dimer linked by two disulfide bonds. It is unlikely that the decrease in molecular weight resulted from the cleavage of two intrachain bonds. Considerable strain is required in order to form an intrachain disulfide bond between two cysteines separated by two proline residues (Figure 8) as indicated by the construction of a space-filling model of the sequence Thr-Cys-Pro-Pro-Cys-Pro-Ala. In Figure 9 we have shown the heavy chains linked by disulfide bonds in a parallel fashion, *i.e.*, Cys $\overline{24}$ to Cys $\overline{24}$ and Cys $\overline{27}$ to Cys $\overline{27}$. At present there is no evidence in favor of this arrangement as opposed to the antiparallel arrangement.

Our studies on the amino acid sequence of the region containing the interchain disulfide bonds indicate that one of the points of proteolysis by trypsin to yield Fab(t) and Fc(t) is at lysine $\overline{31}$. This cleavage point lies between Cys $\overline{27}$, which participates in the heavy-chain–heavy-chain disulfide bond, and Cys $\overline{33}$, which participates in the light-chain–heavy-chain disulfide bond (Figure 8). The bond between leucine at position $\overline{11}$ and phenylalanine at position $\overline{12}$ was not cleaved by tryptic hydrolysis of the whole molecule, but was cleaved during tryptic hydrolysis of fraction I.

A comparison of the sequence of Eu in the region of the interchain disulfide bonds with comparable regions of another human γG-immunoglobulin and with rabbit immunoglobulin supports the current view that the constant portion of the heavy chain is homologous among mammalian species (Hill *et al.*, 1966a; Press *et al.*, 1966a,b; Cebra, 1967; Waxdal *et al.*, 1968a,b). Steiner and Porter (1967) have recently presented evidence for the existence of two heavy-chain–heavy-chain disulfide bonds in a human γG-immunoglobulin (Daw) of a different antigenic type, and have reported the sequence of 43 residues of the heavy chain in the region of the interchain bonds. Eu and Daw are identical from residues $\overline{1}$ to $\overline{38}$, indicating that this sequence is part of the constant portion of the heavy chain and that the constant portion extends into the COOH-terminal portion of the Fd region.

A comparison of the amino acid sequence of a region of heavy chains from pooled rabbit γG-immunoglobulin (Cebra, 1967; Hill *et al.*, 1966b; Smyth and Utsumi, 1967) with the corresponding sequence from Eu (Figure 10) shows that there is a high degree of homology in this region of the two proteins. The sequence in Eu from $\overline{28}$ to $\overline{38}$ is similar to the sequence in rabbit heavy chains. The half-cystine residue at position $\overline{33}$ in Eu heavy chain is the point at which the light chain is attached by a disulfide bond. By analogy, the corresponding residue in the rabbit heavy chain should be linked to the light chain. The sequence from $\overline{1}$ to $\overline{21}$ is identical except for the replacement of leucine at $\overline{11}$ in Eu by isoleucine in rabbit heavy chains and a difference in the order Pro-

Pro-Lys ($\bar{9}$–$\bar{7}$) in Eu as opposed to Lys-Pro-Pro (229–227) in rabbit heavy chain. As suggested by Smyth and Utsumi (1967), the half-cystine residue 244 (Figure 10) in rabbit immunoglobulin probably participates in the heavy-chain–heavy-chain disulfide bond. In contrast, the sequence in Eu from $\overline{22}$ to $\overline{27}$, which contains the two heavy-chain–heavy-chain disulfide bonds, is three residues longer than the analogous sequence in rabbit. A possible explanation for this difference in the sequence between the rabbit and the human proteins is that during evolution a misalignment occurred in a precursor gene followed by unequal crossing-over. The crossing-over could have occurred in two ways: (a) an insertion to change the sequence from Cys-Pro-Pro- to Cys-Pro-Pro-Cys-Pro-Pro- followed later by a single-step mutation of the final proline to alanine, or (b) a deletion to change the sequence from Cys-Pro-Pro-Cys-Pro (Ala or Pro) to Cys-Pro-Pro. The determination of a homologous sequence of a γ chain from an amphibian species (Marchalonis and Edelman, 1966), might suggest whether the precursor heavy chain contained one or two half-cystine residues at this point.

Acknowledgments

We wish to thank Mr. Paul Gottlieb for the determination of the sequence of some of the peptides. Mrs. Elaine Shown and Miss Catherine Volin have provided invaluable technical assistance. We also thank Mrs. Helvi Hjelt for assistance with the amino acid analyses.

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