The Interchain Disulfide Bonds of a Human Pathological Immunoglobulin*

L. A. Steiner† and R. R. Porter‡

ABSTRACT: The amino acid sequence in the heavy chain of a human pathological immunoglobulin, subclass IgG1, between the approximate residue positions 200–240 from the N-terminal end has been established. By an indirect radioactive-labeling technique and by the direct isolation of a disulfide cross-linked fragment containing sections of both heavy chains and both light chains, it has been demonstrated that the immunoglobulin molecule contains four interchain disulfide bonds, all originating within this section of the heavy chain.

One such bond joins each heavy chain to one light chain and two bonds join the heavy chains to each other.

Evidence has been obtained to show that there is one disulfide bond between the light and heavy chains of IgG[1] of several species (see Cohen and Porter, 1964). The half-cystine residue from the interchain bond in the κ and λ chains of myeloma and normal human IgG has been found to be in C-terminal and penultimate C-terminal positions, respectively (Milstein, 1965). A tetrapeptide containing a half-cystine residue participating in disulfide bonding to the light chain has also been isolated from the heavy chain of a myeloma protein, but its position in the chain was not placed (Pink and Milstein, 1967). We wish to report the isolation of peptides containing both the heavy–light and heavy–heavy interchain disulfide bonds from a human pathological immunoglobulin of subclass IgG1 and the position of these half-cystine residues in the heavy chain.

Materials and Methods

Pathological Daw Immunoglobulin IgG1. This was prepared as described by Press *et al.* (1966b).

Heavy Chains Substituted with [^{14}C]Carboxymethyl Groups on Easily Reduced Half-Cystine Residues. The procedure for obtaining Daw IgG1 heavy chains was the same as that used in the preparation of ^{14}C-labeled rabbit IgG heavy chains (J. J. Cebra, L. A. Steiner, and R. R. Porter, submitted for publication) except that 1 M acetic acid and Sephadex G-100 were used in the separation of the chains by gel filtration. The ratio of heavy to light chains recovered, as determined by absorbance at 280 mμ, was 7:3.

Before digestion with trypsin, the heavy chains were extensively reduced by reaction with 0.05 M dithiothreitol (DTT)[2] (0.1 M sulfhydryl) in 7 M guanidine, 0.2 M Tris-Cl (pH 8.2), for 4 hr at 37°. The reaction was stopped by adding a neutral solution of [^{12}C]-iodoacetic acid in 1.5 M excess relative to the sulfhydryl concentration. The reduced alkylated heavy chains were dialyzed exhaustively against 0.015 M Tris-Cl (pH 8.2) and then against water.

Digestion of Reduced, Alkylated Heavy Chain with Trypsin. The reduced, alkylated heavy chains (600 mg in 30 ml) were digested with 12 mg of trypsin at pH 8.2, 37°, in an autotitrator. After 4 hr, 3 mg of trypsin was added and there was no increase in the rate of uptake of base. After a further 1.5 hr, the solution, which had been clear at the beginning of the digestion, contained a small amount of gelatinous precipitate. The digestion was now stopped by lowering the pH to 3.5 with acetic acid.

Cyanogen Bromide Fragment 2a''. Fragment 2a'' was isolated as a dimer from 10 μmoles of Daw IgG essentially by the procedure described by Piggot and Press (1967), except that the Fab and Fc fragments were not separated or reduced prior to cleavage with cyanogen bromide. The products of cyanogen bromide treatment were subjected to gel filtration on Sephadex G-100 in 6 M urea–0.2 M sodium formate (pH 3.3). The fraction containing fragment 2a'' was first desalted by gel filtration on coarse Sephadex G-25 in 1 M acetic acid and purified further by gel filtration on Sephadex G-50 in 0.05 M NH_3. To achieve final purification, the fragment was reduced in 0.05 M DTT (0.1 M sulfhydryl) (pH 8.2) and alkylated with iodo-

* From the Department of Immunology, St. Mary's Hospital Medical School, London, W.2, England. *Received August 28, 1967.*

† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.

‡ Present address: Department of Biochemistry, University of Oxford, Oxford, England.

[1] The nomenclature used is based on that recommended by the World Health Organization (1964, 1966).

[2] Abbreviations used: DTT, dithiothreitol; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; CM-Cys, *S*-carboxymethylcysteine.

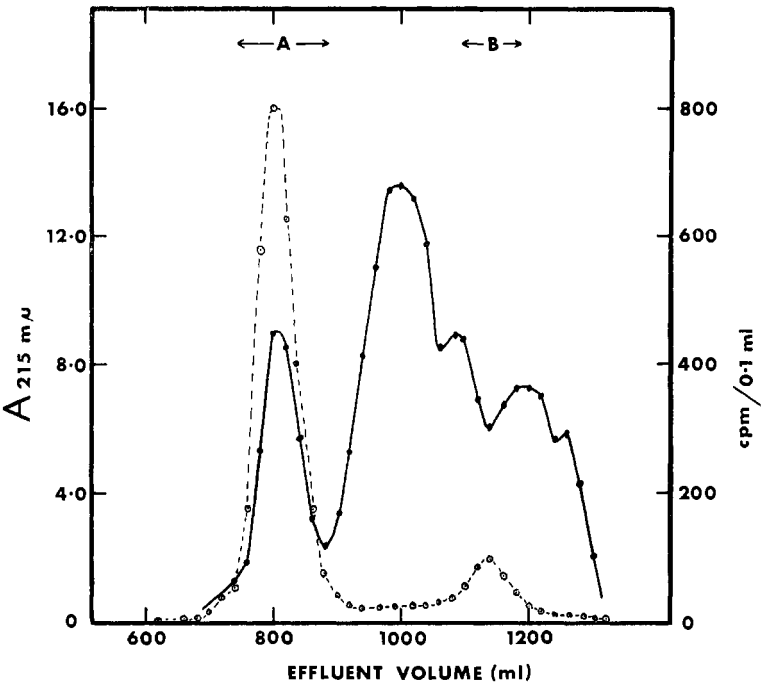


FIGURE 1: Gel filtration of the acid-soluble peptides from a tryptic digest of Daw heavy chains on a column (190 × 3.5 cm) of Sephadex G-50 in 0.05 M NH_4HCO_3. The labile half-cystine residues had been reduced and alkylated with [^{14}C]iodoacetate prior to complete reduction and digestion wtih trypsin. (—●—) Absorbance at 215 mμ; (- -⊙- -) cpm/0.1 ml of effluent.

acetic acid in 1.2 M excess relative to the sulfhydryl concentration and containing 10 μc of [^{14}C]iodoacetate. After successive gel filtration steps on Sephadex G-50, 0.05 M NH_3, and Sephadex G-50, 0.05 M acetic acid, fragment 2a″, reduced and alkylated, was obtained in pure form, as judged by amino acid analysis.

Digestion of Fragment 2a″ with Trypsin. Fragment 2a″, reduced and alkylated (4.3 μmoles), was dissolved in 2.0 ml of 0.1 M NH_4HCO_3 (pH 8.1), and 0.25 mg of trypsin was added in two aliquots. After 6 hr at 37° the digestion was stopped by freezing.

Digestion of Daw IgG with Pepsin. Daw IgG (460 mg in 20 ml) was dialyzed against 0.9% NaCl–0.01 N HCl. The pH was then adjusted to 2.0 and the temperature to 37°, and 19 mg of pepsin was added. The pH was maintained at 2.0 by adding 0.2 N HCl in an autotitrator. After 7 hr, 4 mg of pepsin was added. The digestion was stopped at 8 hr by freezing.

Digestion of Fragment P with Trypsin. Fragment P (4.3 μmoles), obtained by digestion of Daw IgG with pepsin, was dissolved in 4 ml of 0.05 M acetic acid. The pH was adjusted to 7.2, with 2 M NH_3, and the temperature to 37°; 0.54 mg of trypsin was added in three aliquots during a 4-hr period. After 4 hr the pH was lowered to 4.8 by adding 1 M acetic acid and the sample was added to a Sephadex G-25 column in 0.05 M acetic acid.

Digestions with Chymotrypsin. Digestions with chymotrypsin were carried out in 0.1 M NH_4HCO_3 adjusted to pH 8.2–8.4 at 37° for 4–6 hr. The enzyme: substrate ratio was varied from 0.06 to 0.1 mg of enzyme per μmole of peptide.

Digestions with Carboxypeptidase A. Digestions with carboxypeptidase A were done in 0.1 M NH_4HCO_3 (pH 7.9) at room temperature with 1 mg of enzyme/μmole of peptide. For "prolonged" digestion, the time of incubation was 15 hr. "Brief" digestion of peptide T2-C4 was for 1 min. At the end of the period of digestion the pH was lowered to 2.2 and the samples were subjected to amino acid analysis. Sometimes the residual peptide was freed of low molecular weight products by gel filtration and also analyzed.

Reduction and Alkylation of Peptides Containing Cystine. Peptides containing intact disulfide bonds were reduced at 37° for 2 hr in buffer containing 0.05–0.1 M DTT (0.1–0.2 M sulfhydryl), 0.5 M Tris-Cl, and 0.002 M EDTA (pH 8.2). Following reduction, the sulfhydryl groups were blocked by adding either iodoacetic acid or iodoacetamide in 1.2–1.8 M excess with respect to sulfhydryl concentration. Alkylation was for 1 hr at room temperature.

Dansyl-Edman Technique. The method described by Gray (1967) was used, except that the dansyl derivatives were identified by thin layer chromatography, as described by Piggot and Press (1967). When CM-Cys was a possible N-terminal residue, the dansylated sample was evacuated before hydrolysis. In the case of peptides of fewer than 12 residues, identification of the N-terminal residue was usually confirmed by amino acid analysis of the residual peptide.

TABLE I: Amino Acid Compositions of [^{14}C]Tryptic Peptides Derived from Daw Heavy Chain.

	T1	T2	T3
Lys	1.8	2.8	1.0
His	0.85	0.93	
CM-Cys	1.4	2.6	0.80
Asp		1.1	1.0
Thr	1.9	1.9	
Ser	1.2	1.8	0.79
Glu	1.1	1.3	
Pro	7.7	7.9	
Gly	1.9	1.9	
Ala	1.0	1.0	
Val	1.0	1.0	
Leu	2.7	2.6	
Phe	1.8	1.7	
Sp act.[a] (cpm/μmole)	61,000	87,000	32,000

[a] Specific activity is expressed in terms of micromoles of alanine (T1 and T2) or lysine (T3).

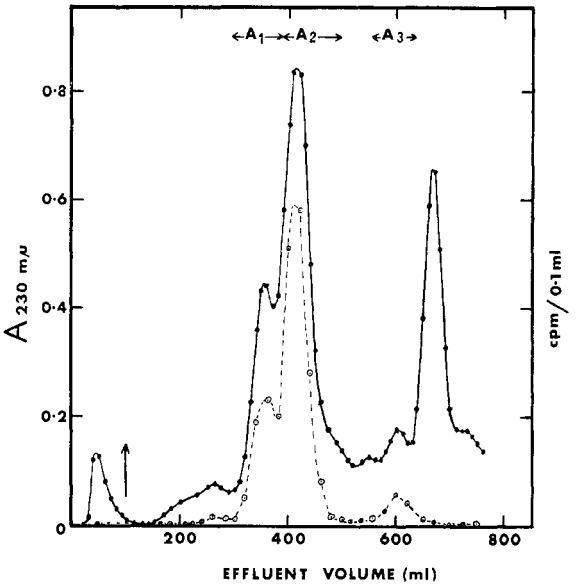


FIGURE 2: Ion-exchange chromatography of fraction A (Figure 1) on a column of DEAE Sephadex A-25 (28 × 1.6 cm). The column was equilibrated with 0.005 M NH_4HCO_3 (pH 8.5) and after 100 ml had been collected (arrow) a linear gradient to 0.3 M NH_4HCO_3 was applied using 500 ml in each chamber. (—●—) Absorbance at 230 mμ; (- -⊙- -) cpm/0.1 ml of effluent.

High-Voltage Paper Electrophoresis. Electrophoresis was carried out as described by Press *et al.* (1966b).

Paper Chromatography. Descending paper chromatography was used with the solvent system described by Baglioni (1961), pyridine–3-methylbutan-1-ol–water (7:7:6).

Amino acid analyses were carried out as described by Press *et al.* (1966b). Values reported are uncorrected for losses occurring during the 20–24-hr hydrolyses and are expressed to two significant figures relative to a suitable residue chosen as 1.0. Fractional residues are generally omitted.

Counting of [^{14}C]Peptides. Aqueous samples were dissolved in hydroxide of Hyamine 10-X (Rohm and Haas) and the solvent described by Kinard (1957), and were counted in a Nuclear-Chicago liquid scintillation counter.

Enzymes and Reagents. The enzymes used were those described by Press *et al.* (1966b), and pepsin which had been purified by chromatography (Ryle and Porter, 1959). DTT (A grade) was obtained from Calbiochem, and dansyl chloride from the British Drug Houses, Ltd. Guanidine hydrochloride was prepared by the method of Anson (1941). Iodoacetic acid and iodoacetamide were recrystallized from petroleum ether and ethanol, respectively. [^{14}C]Iodoacetic acid and [^{14}C]iodoacetamide (each 7.0 mc/mmole) were obtained from the Radiochemical Centre, Amersham.

Results

Isolation and Properties of [^{14}C]Tryptic Peptides from Daw Heavy Chain. Heavy chains, prepared as described, contained 3.9 moles of CM-Cys/mole of heavy chain. The specific activity of the heavy chain pool, expressed in counts per minute per micromole, was 3.6-fold greater than that of the light chain pool.

The heavy chains were extensively reduced, alkylated with [^{12}C]iodoacetic acid, and digested with trypsin. At the end of the digestion, the pH was lowered to 3.5 and a copious precipitate was removed by centrifugation. The supernatant contained 30% of the protein (by absorbance at 280 mμ) and 90% of the radioactivity of the initial heavy chain pool. It was fractionated by gel filtration on a column of Sephadex G-50 (Figure 1); 75% of the radioactivity applied to the column was recovered in the larger peak (A) and 7% in the smaller peak (B).

The material in peak A was fractionated further by chromatography on DEAE Sephadex A-25 (Figure 2); 56% of the radioactivity applied to this column emerged in peak A2, 22% in a partially resolved peak (A1), and 6% in a small peak (A3). The material in A2 showed only a single component on paper electrophoresis at pH 6.5 (mobility, −0.09) and on paper chromatography in pyridine–3-methylbutan-1-ol–water. The amino acid analysis of this material, now designated T2, is shown in Table I. When A1 was subjected to paper electrophoresis at pH 6.5 it appeared as an elongated spot with average mobility −0.04. On paper chromatography in pyridine–3-methylbutan-1-ol–water, A1 separated into two components, the smaller corresponding to T2. Accordingly, A1 was rechromatographed on the same A-25 column with a shallower linear

TABLE II: Procedures for Isolating Chymotryptic Peptides Derived from T1, T2, and 2a''-T1.

Peptide	Sephadex G-25 Gel Filtration Solvent (M)	Elution Vol.[a]	Paper Electrophoresis[c] Mobility[b]
T2-C1	NH_3 (0.05)	1.0	
T2-C2	NH_3 (0.05)	1.5	+0.15
T1-C2	NH_3 (0.05)	1.5	+0.15
2a''-T1-C2	NH_3 (0.05)	1.5	+0.15
T2-C3	NH_3 (0.05)	1.6	+0.7
T1-C3	NH_3 (0.05)	1.6	+0.7
2a''-T1-C3	NH_3 (0.05)	1.6	+0.7
T2-C4	NH_3 (0.05)	1.2	
T1-C1	NH_3 (0.05)	1.0	
	Acetic acid (0.05)	1.4	
T1-C12	NH_3 (0.05)	1.0	
	Acetic acid (0.05)	1.3	
2a''-T1-C1	NH_3 (0.05)	1.0	

[a] The elution volume is expressed as the ratio of the column volume at which the sample is eluted to the void volume of the column. [b] The mobility of each peptide is expressed in terms of reference amino acid markers. The position of valine was taken as 0, lysine as +1, and aspartic acid as −1. The mobility of a cationic peptide is expressed as a positive fraction, the ratio of the distance moved by that peptide relative to the distance moved by lysine. Similarly, the mobility of an anionic peptide is expressed as a negative fraction relative to the mobility of aspartic acid. [c] At pH 3.5.

gradient (0.005 M NH_4HCO_3–0.2 M NH_4HCO_3, pH 8.5). The major peak eluted contained 62% of the radioactivity applied to the column and behaved as a single component on paper electrophoresis at pH 6.5 (mobility, −0.02) and on paper chromatography in the same buffer system as before. The analysis of the material in this peak (T1) is also shown in Table I. A3 contained less than 5% of the radioactivity of the heavy chain pool and was not studied further.

The material in peak B (Figure 1) was fractionated by gel filtration on Sephadex G-25 and paper electrophoresis. It contained a single radioactive component, T3 (mobility at pH 6.5, −0.46), that yielded the amino acid analysis shown in Table I.

The sum of the amino acid compositions of T1 and T3 was equal to that of T2 (Table I) and it seemed likely that T1 and T3 were tryptic digest products of peptide T2. Although amino acid analyses for CM-Cys were not considered reliable because of partial destruction of this residue during acid hydrolysis, the compositions in Table I suggested that peptides T1, T2, and T3 contained, respectively, 2, 3, and 1 mole of CM-Cys per mole of peptide. Table I also shows the specific activity of these peptides expressed as counts per minute per micromole of alanine (T1 and T2) or lysine (T3). The ratio of the specific activities of T1, T2, and T3 was approximately 2:3:1. This suggests that the three residues of CM-Cys were each labeled to an equivalent extent in the preparation of the heavy chains.

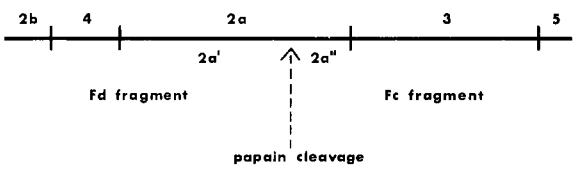


FIGURE 3: Alignment of the fragments derived from cyanogen bromide cleavage of Daw heavy chain. This is taken from Piggot and Press (1967).

Partial Sequence of T1, T2, and T3

The sequence of T3, determined by the dansyl–Edman technique, was Ser-CM-Cys-Asp-Lys. The N-terminal sequence of T2 was found to be Ser-CM-Cys-Asp-Lys-Thr- and as threonine was the N-terminal residue of T1 this confirmed that T1 and T3 were derived by tryptic digestion of T2 and these peptides can now be aligned as shown in Figure 6.

Chymotryptic Peptides Derived from T1 and T2. Upon digestion with chymotrypsin, three peptides were obtained from T2 in high yield. These together accounted for the composition of the whole of T2. The method of isolation of these peptides is shown in Table II; 90% of the radioactivity was found in T2-C1 and none was found in T2-C2 or T2-C3. The order of the three peptides was apparent from their amino acid compositions (Table III). T2-C1 contained N-terminal serine, the only aspartic acid residue, and also lysine and from the N-terminal sequence above must come from the N-terminal end of T2. T2-C3 contained both the other lysine residues and hence must be C terminal, leaving T2-C2 between T2-C1 and T2-C3. The complete sequences of T2-C3 and T2-C2 were determined

TABLE III: Amino Acid Compositions of Chymotryptic Peptides Derived from T2 and T1.

	T2-C1	T2-C2	T2-C3	T2-C4	T1-C1	T1-C12
Lys	0.73		2.0	1.0		
His	0.70			0.88	1.3	1.1
CM-Cys	2.5			0.75	1.7	0.80
Asp	0.88			1.0		
Thr	1.7			0.94	1.8	1.7
Ser	0.83	0.96		0.93		1.0
Glu	1.2				1.0	1.4
Pro	4.1	1.1	2.9		4.6	4.9
Gly		2.0				1.8
Ala	1.0				1.0	1.0
Val		1.1				0.91
Leu	1.9		1.0		2.1	1.9
Phe		1.0	1.0			0.86

TABLE IV: Amino Acid Compositions of Reduced, Alkylated Fragment 2a'' and Its Tryptic and Chymotryptic Peptides.

	2a''	2a''-T1	2a''-T2	2a''-T1-C1	2a''-T1-C2	2a''-T1-C3
Lys	2.1	1.6				2.1
CM-Cys	1.9	1.6		1.6		
Asp	1.0		0.98			
Thr	1.8	0.81	0.93	0.77		
Ser	0.93	0.96			0.87	
Homoserine	1.0		1.0			
Glu	0.96	0.96		0.94		
Pro	7.1	7.0		3.9	1.1	3.4
Gly	2.1	2.2			2.0	
Ala	1.0	1.0		1.0		
Val	1.0	0.93			0.99	
Leu	4.0	2.8	0.98	2.0		1.0
Phe	2.0	1.7			1.0	0.97

by the dansyl–Edman method. Prolonged digestion of T2-C1 with carboxypeptidase A released 2 moles of leucine/mole of peptide; the residual peptide was purified by gel filtration and had the same amino acid composition as T2-C1, except for the absence of leucine. These results established the sequence of the 15 C-terminal residues of T2, as shown in Figure 6.

A further peptide (T2-C4) was isolated in low yield; it contained 10% of the radioactivity of T2. Its composition (Table III) corresponded with that of the N-terminal five residues of T2 plus histidine. Dansylation of this peptide indicated that serine was N terminal and digestion with carboxypeptidase A confirmed that histidine was the C-terminal residue with threonine in the penultimate position. (Prolonged treatment with carboxypeptidase released histidine and threonine, each in 95% yield. Brief treatment with the enzyme released histidine only, in 45% yield.) Hence, the N-terminal sequence of T2 was extended to six residues, leaving the sequence of the nine subsequent residues in T2 to be determined.

The chymotryptic peptides of T1 were isolated as shown in Table II. Peptides T1-C2 and T1-C3 were identical with T2-C2 and T2-C3, the equivalent peptides from T2; the composition of T1-C1 (Table III) was the same as that of T2-C1 except for the absence of the N-terminal tetrapeptide. An additional peptide (T1-C12), with composition equal to that of T1-C1 plus T1-C2, was evidently the result of partial chymotryptic cleavage of T1. The isolation of this peptide confirmed the alignment shown in Figure 6.

The Relation of T2 to the Fragments of Daw Heavy Chain Obtained by Cleavage at the Methionine Residues with Cyanogen Bromide

Five fragments have been obtained from the heavy chain by cyanogen bromide cleavage at the four methionine residues and they have been aligned as shown in

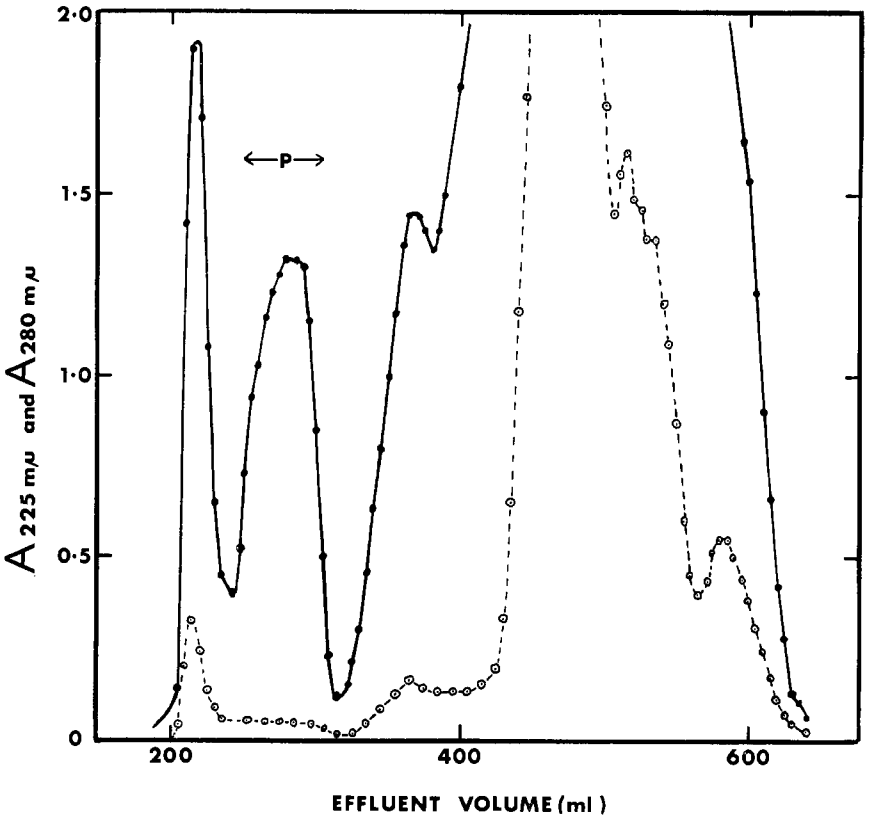


FIGURE 4: Gel filtration of the products of peptic digestion of Daw IgG on a column of Sephadex G-50 (114 × 2.6 cm) in 0.05 M acetic acid. (—●—) Absorbance at 225 mμ; (- -⊙- -) absorbance at 280 mμ.

TABLE V: Edman Degradation of 2a''-T1-C1.

		Edman Step[a]							
	2a''-T1-C1	1	2	3	4	5	6	7	8
CM-Cys	1.6	1.7	*1.0*	0.93	0.78	*0.18*	0.20		
Thr	0.77	*0.17*	0.14						
Glu	0.94	1.1	1.1	1.1	1.1	1.1	1.2	1.0	1.1
Pro	3.9	3.9	3.8	*2.6*	2.5	2.5	*1.6*	1.6	*0.68*
Ala	1.0	1.0	1.0	1.0	1.0	1.0	1.0	*0.32*	0.36
Leu	2.0	2.0	2.1	2.0	1.9	2.0	2.1	2.0	2.0
Sp act. (cpm × 10^{-3}/μmole)[b]	113	117	82	73	62	28	28	28	40
Dansyl	Thr	CM-Cys	Pro	Pro	CM-Cys	Pro	Ala	Pro	Glu

[a] The residue in italics is the one presumed to be lost after each Edman step. After the fourth step there was no significant decrease in any residue and the italics are omitted. The residue in the fourth position, proline, was therefore determined by the dansyl method alone. The failure to demonstrate a decrease in this residue is probably due to the technical difficulty in accurate determination of proline by amino acid analysis. [b] Specific activity is expressed in terms of micromoles of alanine (steps 0–6) or leucine (steps 7–8).

Figure 3 (Press *et al.*, 1966a; Piggot and Press, 1967). When the whole IgG molecule is digested with papain to produce two fragments Fab and one fragment Fc, hydrolysis of the heavy chain occurs within fragment 2a. Thus after cyanogen bromide treatment, fragment 2a' can be isolated from Fab and fragment 2a'' from Fc. Fragment 2a'' as isolated from Fc was a dimer and hence it was probable that it contained disulfide bonds

connecting the heavy chains (Piggot and Press, 1967) and was perhaps related to T2. Fragment 2a'' was isolated as described above and its analysis (Table IV) suggested that T2 was indeed derived from the same section of the chain. After hydrolysis with trypsin two fractions were obtained from fragment 2a'' by gel filtration on Sephadex G-25. The composition of the smaller fraction (2a''-T2) is shown in Table IV and it was evidently a single peptide; its sequence, determined by the dansyl–Edman method, was Asp-Thr-Leu-homoserine. The presence of homoserine established that this tetrapeptide was derived from the C-terminal section of 2a''. The larger peptide (2a''-T1) was separated from a small amount of undigested material on a Sephadex G-50 column and its composition (Table IV) corresponded to T2 less the N-terminal hexapeptide sequence. Its N-terminal residue was threonine as in whole 2a''. The sequence of the N-terminal 14 residues of 2a''-T1 was determined directly by the dansyl–Edman technique and gave a satisfactory overlap with the C-terminal sequence of T2 given above, thus completing the sequence of T2. Whether the aspartic and glutamic residues in T2 occur as the free acid or as the amide was determined in studies described below (see section on pepsin digestion of Daw IgG).

Chymotryptic Peptides of 2a''-T1. Confirmation of the sequence of T2 was obtained by the isolation of several peptides from a chymotryptic digest of 2a''-T1. Peptide 2a''-T1-C1 was eluted early from a Sephadex G-25 column in 0.05 M NH_3 and contained all the radioactivity. Two other peptides (2a''-T1-C2 and 2a''-T1-C3) were isolated, as shown in Table II, and had the amino acid compositions shown in Table IV. They were identical with C2 and C3 obtained from both T1 and T2. The sequence of 2a''-T1-C1 was determined by the dansyl–Edman technique and by difference analysis (Table V) and confirmed the sequence of this section that had been obtained previously.

The results place the position of T2 in relation to the cyanogen bromide fragment 2a'', as shown in Figure 6. It is clear that T2 overlaps the position(s) of the hydrolysis by papain that occurs in the preparation of the Fab and Fc fragments. If few or no free amino acid residues or peptides are released by the papain hydrolysis, the C-terminal portion of 2a' should correspond with the N-terminal section of T2. Indeed, a tryptic hydrolysate of 2a' contained the tetrapeptide (Ser,CM-Cys,Asp,Lys) (N. Hogg, unpublished data) in agreement with the N-terminal sequence of T2. Thus, at most two residues (Thr,His) are split out during brief papain hydrolysis of Daw IgG.

Pepsin Digestion of Daw IgG

The above results establish a sequence of 34 residues, approximately, 210–240 from the N-terminal end of the heavy chain (Piggot and Press, 1967). The three CM-Cys residues found within this sequence were radioactively labeled after reduction under conditions believed to break mainly interchain bonds and alkylation with [^{14}C]iodoacetate. It seemed probable that one of these CM-Cys residues participates in disulfide bonding with the light chain and the remaining two in bonding to the other heavy chain. However, under any conditions of reduction there is a risk of disulfide interchange, and hence to establish this point definitely it was necessary to reisolate peptides from this section of the molecule without reduction and with the disulfide bonds still present. As the three radioactive CM-Cys residues were present in a sequence of only ten residues an attempt was made to isolate a peptide containing the equivalent section from both heavy and both light chains still held together by disulfide bonds. Sephadex column fractionations were carried out in dilute acetic acid since it has been shown that disulfide interchange is less likely to occur in acid (Ryle and Sanger, 1955).

Daw immunoglobulin was digested with $^1/_{20}$ its weight of pepsin at pH 2.0 for 8 hr. A small amount of precipitate (less than 7% of the total absorbance units) formed during the digestion and was removed. The digest was then fractionated on a column of Sephadex G-50 in 0.05 M acetic acid (Figure 4). The material in the second peak (P), obtained at elution volume 1.3, was thought perhaps to contain the hypothetical tetrameric disulfide cross-linked peptide. Indeed, despite several obvious differences, the amino acid composition

TABLE VI: Isolation Procedures and Amino Acid Compositions of Fraction P and Related Peptides Obtained by Reduction and Alkylation.

	P	P1	P2	P3
Sephadex G-50[d] Elution volume[a]		1.5	2.0	2.0
Paper electrophoresis[b] Mobility[a]		−0.10	−0.70	−0.20
Lys	4.4	5.0		1.1
His	1.0	1.0		
CM-Cys		2.2	0.44	0.48
Asp	2.1	2.1		
Thr	2.6	1.9	0.95	1.9
Ser	1.5	1.0	1.0	1.1
Glu	2.1	2.0	1.0	1.1
Pro	4.7	5.4	0.95	1.1
Ala	1.0	1.0		1.0
Cys	2.7			
Val	2.1	1.8		1.0
Leu	0.97	1.0		
Sp act. (cpm/μmole)[c]		316,000	98,000	101,000

[a] Defined in Table II. [b] At pH 6.5. [c] Specific activity is expressed in terms of micromoles of alanine (P1 and P3) or glutamic acid (P2). [d] 0.05 M NH_3.

TABLE VII: Isolation Procedures and Amino Acid Compositions of Tryptic Peptides Derived from Fraction P.

	P-T2	P-T3a	P-T3b	P-T4	P-T5a	P-T5b	P-T5c	P-T5d
Sephadex G-50[a]								
Elution volume[b]	1.2	1.4	1.4	1.5	1.6–1.7	1.6–1.7	1.6–1.7	1.6–1.7
Paper electrophoresis								
pH		3.5	3.5	6.5	3.5	3.5	3.5, 6.5	3.5, 6.5
Mobility[a]		+0.25	+0.50	−0.25	+0.50	+0.55	+0.85, +0.45	+0.85, +0.50
Lys		1.1	2.0	1.1	1.0	1.0	2.4	2.1
His	1.2							
Asp		0.98	1.2	1.0	0.79			1.0
Thr	2.0	1.6	1.7	0.94				
Ser		1.9	1.8	1.7				
Glu	1.1	0.92	0.90	0.96		0.92	0.97	
Pro	4.3	1.2	0.94	1.1		1.0	0.98	
Ala	1.0	1.0	1.0					
Cys	1.7	1.5	1.1	1.3				
Val		0.84	0.93		0.83	0.92	1.0	0.90
Leu	1.0							

[a] 0.05 M acetic acid. [b] Defined in Table II.

of pool P (Table VI) did resemble that of peptide T2-C1 (Table III). The material in P was then reduced and alkylated. Of several resulting peptides the three principal ones were isolated by methods outlined in Table VI; their amino acid compositions and specific activities are also shown in Table VI. Peptides P2 and P3 were identical in composition with the C-terminal five and nine amino acid residues of the Daw light chain (λ type) (Milstein, 1965). Peptide P1 resembled T2-C1 except that it contained one less residue of leucine and nine additional residues: four residues of lysine, two of valine, and one each of proline, glutamic acid, and aspartic acid. It seemed likely that P1 was composed of T2-C1 (except for a C-terminal leucine) plus an N-terminal section consisting of the nine additional residues and this was substantiated when the N-terminal sequence of P1 was shown to be Lys-Val-Asp-Lys-Lys-Val-Glu-Pro-Lys-Ser-.

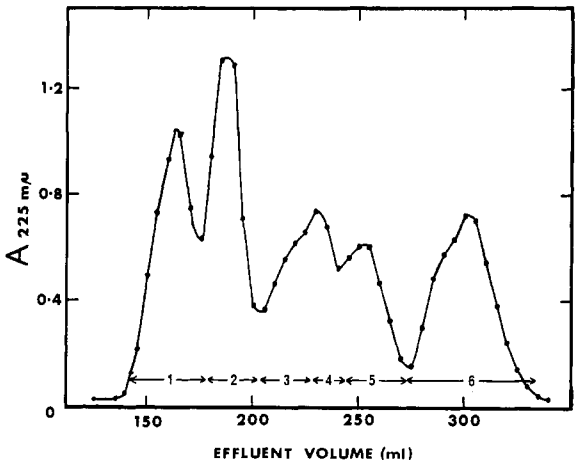


FIGURE 5: Gel filtration of the peptides obtained by tryptic digestion of fraction P (Figure 4) on a column (138 × 2.1 cm) of Sephadex G-25 in 0.05 M acetic acid.

From the nature of the peptides released by reduction and alkylation (P1, P2, and P3) it could be inferred that fraction P was composed of peptides derived from both the light and heavy chains and that these peptides were held together by disulfide bonds. However, fraction P was not homogeneous: not only was its elution pattern asymmetric (Figure 4), but after reduction it yielded small amounts of a number of peptides that were apparently related to but distinct from P1, P2, and P3.

Tryptic Peptides from Fragment P. Rather than attempt to resolve such a large and probably complex group of peptides as those in fraction P, we elected instead to split P into several components by digestion with trypsin, and then to purify each of these smaller components. Accordingly, fraction P was digested with trypsin and the digest fractionated on a column of Sephadex G-25 in 0.05 M acetic acid. The resulting elution pattern is shown in Figure 5.

Fraction 1 consisted largely of partially digested material and was not characterized further. Fraction 2, after rerunning on the same Sephadex column, contained a single peptide, P-T2. Fraction 3 was rerun on the same Sephadex column; it contained two peptides (P-T3a and P-T3b) that were separated by paper electrophoresis and differed only in their lysine content. Fraction 4 consisted mainly of a single peptide, P-T4, that was separated from small amounts of contaminating peptides by electrophoresis. Fraction 5 contained four peptides (P-T5a to P-T5d) that were resolved by paper electrophoresis. Fraction 6 did not contain significant amounts of ninhydrin-positive ma-

TABLE VIII: Isolation Procedures and Amino Acid Compositions of Peptides Obtained by Reduction and Alkylation of Peptides P-T2, PT3b, and P-T4.[a]

	P-T2-r	P-T3b-r1	P-T3b-r2	P-T4-r1	P-T4-r2
Sephadex column					
Type	G-25	G-15	G-15	G-25	G-25
Solvent (M)	Acetic acid (0.05)	Acetic acid (0.05)	Acetic acid (0.05)	NH_3 (0.02)	NH_3 (0.02)
Elution volume[b]	1.4	1.0	1.2	1.1	1.2
Paper electrophoresis[c]					
Mobility[b]	−0.05	−0.20	−0.45	−0.40	0
Lys		1.0	1.0		1.0
His	1.0				
CM-Cys	1.6	0.68	0.76	0.66	0.65
Asp			1.0		1.0
Thr	1.8	1.8		1.0	
Ser		1.1	0.80	1.0	0.83
Glu	1.0	1.0		0.98	
Pro	3.8	0.95		1.1	
Ala	1.0	1.0			
Val		0.92			
Leu	1.0				

[a] Following reduction, peptides P-T2-r, P-T4-r1, and P-T4-r2 were alkylated with iodoacetamide; peptides P-T3b-r1 and P-T3b-r2 with iodoacetic acid. [b] Defined in Table II. [c] At pH 6.5.

terial. The methods of isolation and the compositions of these peptides are summarized in Table VII.

Since the digestion with trypsin was carried out at neutral pH the possibility that disulfide interchange may have occurred during the course of the digestion was considered (Ryle and Sanger, 1955). Accordingly, the tryptic digestion was repeated under the same conditions except that the reaction mixture was made 0.0025 M in iodoacetamide. After gel filtration on Sephadex G-25 an elution pattern similar to that in Figure 4 was obtained. Moreover, the yield of peptide P-T2 was similar to that found previously.

Peptide P-T2 was identical in composition with T1-C1 except that it contained one less residue of leucine and two residues of half-cystine replaced the two residues of CM-Cys. Upon incubation of the peptide with carboxypeptidase A, 0.9 mole of leucine was released/mole of alanine. After reduction and alkylation with iodoacetamide, the electrophoretic mobility of this peptide (Table VIII, P-T2-r) indicated that the glutamic residue was in the free acid (not amide) form. Other properties of this peptide will be considered below.

The amino acid compositions of P-T4, P-T3a, and P-T3b suggested that these peptides were dimers composed of a common section from the heavy chain, the tetrapeptide Ser-Cys-Asp-Lys, linked to a section of the light chain consisting, respectively, of the C-terminal five, eight, or nine residues of the λ chain. This hypothesis was supported by the results of reduction and alkylation of these peptides as shown in Table VIII.

When peptide P-T4 was reduced and blocked with iodoacetamide, peptides P-T4-r1 and P-T4-r2 were obtained. The composition of the former was the same as that of the five C-terminal residues of the λ chain (Milstein, 1965), and the composition and sequence of the latter the same as that of the four N-terminal residues of T2 (Figure 6). The electrophoretic mobility of P-T4-r2 indicated that aspartic acid, not asparagine, was present. Similarly, the glutamic residue in P-T4-r1 was present in the acid form. Peptide P-T3b was reduced and blocked with iodoacetic acid, producing peptides P-T3b-r1 and P-T3b-r2. The former had the same composition as the C-terminal nine residues of the λ chain (Milstein, 1965); the latter was identical with P-T4-r2. The identification of P-T4-r1 and P-T3b-r1 with the C-terminal five and nine residues of the λ chain was confirmed by the determination of the sequence of these peptides. Thus, P-T4-r1 had the sequence Pro-Thr-Glu-CM-Cys-Ser and P-T3b-r1 the partial sequence Lys-Thr-Val-Ala-Pro-Thr-Glu-(CM-Cys,Ser). The isolation of a peptide identical with P-T4 had previously been reported by Pink and Milstein (1967).

Peptides P-T5a, P-T5b, P-T5c, and P-T5d were evidently derived from a section of the heavy chain corresponding to the N-terminal portion of P1. The sequences of these peptides were P-T5a, Val-Asp-Lys; P-T5b, Val-Glu-Pro-Lys; P-T5c, Lys-Val-Glu-Pro-Lys; and P-T5d, Lys-Val-Asp-Lys. Therefore these peptides are derived from the N-terminal sequence of P1 (Figure 6). On paper electrophoresis at pH 6.5, both P-T5a and P-T5b were neutral components, indicating that the aspartic and glutamic residues in these peptides are in the free acid form.

Since peptides P1 and T2 overlap, the sequence of the 43 residues in this portion of the heavy chain was now complete (Figure 6).

Properties of the Peptide P-T2. This peptide contained two residues of half-cystine per residue of alanine. It seemed probable that each of these residues participates in a disulfide bond with a half-cystine residue in the other heavy chain, *i.e.*, that P-T2 is a dimer cross-linked by two cystine residues. Evidence in support of this hypothesis was the following. (1) Peptide P-T2-r was obtained from P-T2 by reduction and alkylation with iodoacetamide. An estimate of the size of peptides P-T2 and P-T2-r was obtained from their elution volumes on a column of Sephadex G-25 that had been calibrated with several other peptides of known size (Figure 7). The deduced molecular weights of P-T2 and P-T2-r were 2300 and 1350, respectively. From its amino acid composition (Table VIII) peptide P-T2-r has a minimum molecular weight of 1360. The calculated molecular weight of the dimer P-T2 is 2480. Therefore the change in molecular weight after reduction of peptide P-T2, as measured by the change in elution volume, is compatible with the transition of dimer to monomer. (2) P-T2 contained no free SH groups since alkylation with [^{14}C]iodoacetamide in the absence of reducing agents did not result in significant radioactive labeling of this peptide. (3) Neither of the half-cystine residues is joined to other amino acid residues or to other peptides. This is evident from the amino acid analysis of P-T2 (Table VII). (The presence of 2 moles of half-cystine/mole of alanine was confirmed by amino acid analysis of P-T2 after performic acid oxidation (Moore, 1963); 1.7 moles of cysteic acid was found/mole of alanine.) Therefore there are two residues of cystine spanning the heavy chains of Daw IgG and the arrangement of the interchain disulfide bonds is probably that shown in Figure 8.

Discussion

The results establish the amino acid sequence in the heavy chain of this pathological IgG between the approximate positions 200 and 240 from the N-terminal end. This numbering is based on the known sequence from 1 to 84 and the number of residues calculated to be in fraction 2a′ from its amino acid analysis (Piggot and Press, 1967; Press, 1967). The three residues of half-cystine in this section have been shown to be involved in interchain bonds both indirectly by their ease of reduction with subsequent separation of the peptide chains and directly by isolation of the tetrameric peptide that contains sections of both light chains and both heavy chains. The yield of this tetramer as judged by recovery of the single histidine residue in this section was greater than 90%. The tetramer was fragmented by digestion with trypsin into peptides

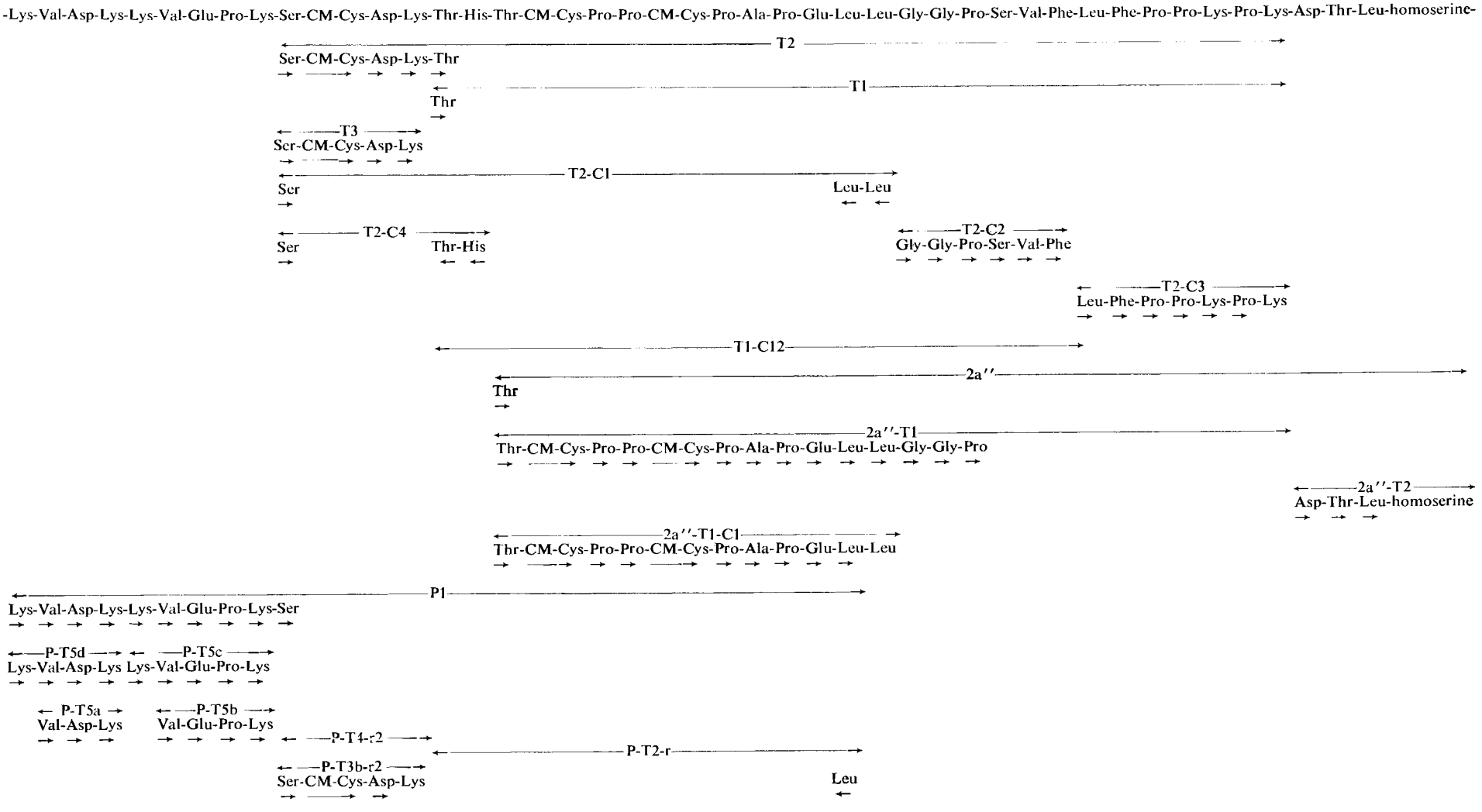


FIGURE 6: Summary of sequence of peptides derived from Daw heavy chain. (→) N-terminal residue released by Edman degradation and identified by dansyl method and/or amino acid analysis of residual peptide; (←) C-terminal residue identified by digestion with carboxypeptidase A.

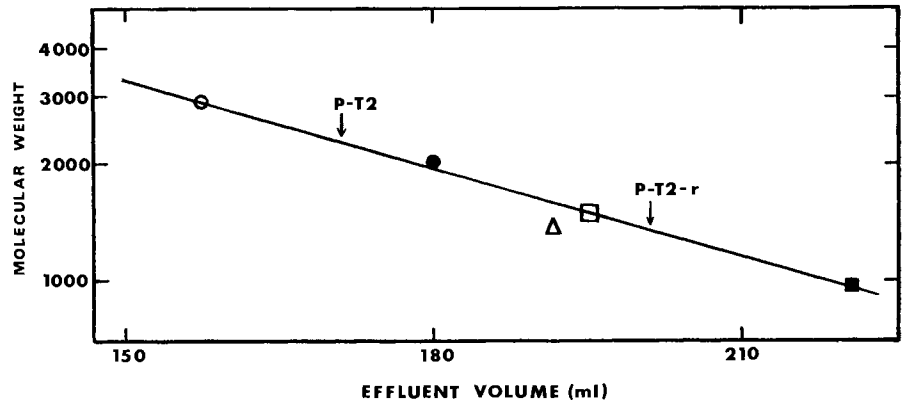


FIGURE 7: Determination of molecular weights of peptides P-T2 and P-T2-r by gel filtration on a column (138 × 2.1 cm) of Sephadex G-25, 0.05 M acetic acid, based on the method of Andrews (1964). Peptides P1 (○), T1-C12 (●), P-T3b (△), T1-C1 (□), and P-T4 (■) were used to calibrate the column and their log (molecular weight) is plotted against their elution volume. An approximately linear relation is obtained. The elution positions of P-T2 and P-T2-r are indicated.

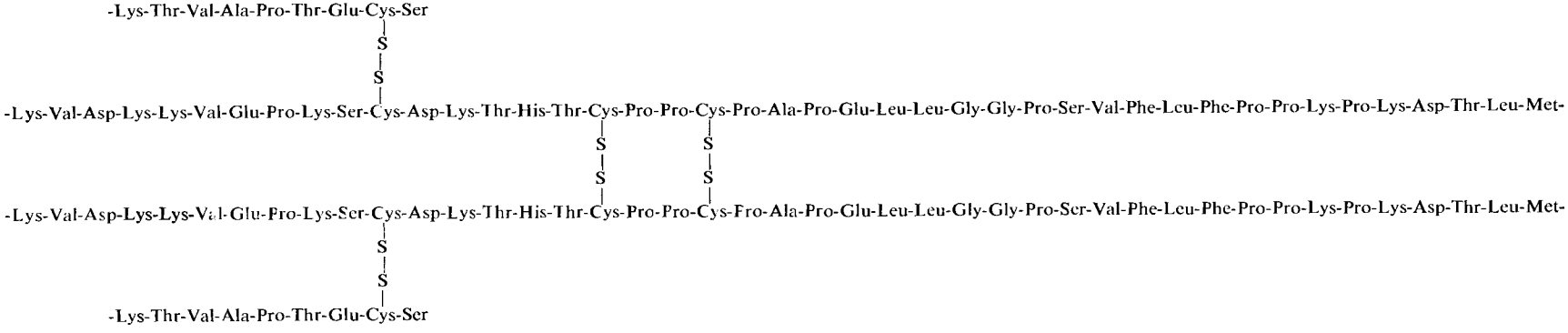


FIGURE 8: Summary of sequence of the section of Daw IgG that contains the interchain disulfide bonds.

Daw IgG, heavy chain	-Lys-Val-Asp-Lys-*Lys*-Val-*Glu*-Pro-Lys-*Ser*-Cys-**Asp**-Lys-*Thr*-His-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-Glu-Leu-
Rabbit IgG, heavy chain	-Thr-Lys-Val-Asp-Lys-*Thr*-Val-*Ala*-Pro-**Ser**-*Thr*-Cys-**Ser**-Lys-*Pro*- Thr-Cys-Pro-Pro- Pro-Glu-Leu-
Daw IgG, heavy chain	Leu-Gly-Gly-Pro-Ser-Val-Phe-*Leu*-Phe-Pro-Pro-Lys-Pro-Lys-Asp-Thr-Leu-Met-
Rabbit IgG, heavy chain	Leu-Gly-Gly-Pro-Ser-Val-Phe- *Ile*-Phe(Pro,Pro,Lys)Pro-Lys-Asp-Thr-Leu-Met-

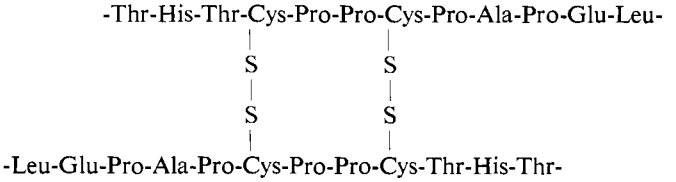
FIGURE 9: Comparison of the amino acid sequences of Daw and rabbit IgG heavy chains in the region approximately 200–240 residues from the N-terminal end of these chains. The data for rabbit IgG heavy chain are abstracted from J. J. Cebra, L. A. Steiner, and R. R. Porter, submitted for publication. Italics: Residues that correspond to codons differing in a single nucleotide base. Boldface: Residues that are not identical and do not correspond to "single base changes."

containing the single disulfide bond between the heavy and light chains, and into a symmetrical dimer containing two disulfide bonds joining the heavy chains to each other. The isolation of peptides containing intact disulfide bonds was carried out in acid or in the presence of a sulfhydryl-blocking agent at neutral pH, conditions that minimize the risk of disulfide interchange (Ryle and Sanger, 1955).

In the indirect experiment the labile disulfide bonds were reduced and blocked with [^{14}C]iodoacetate; about 80% of the radioactivity was equally distributed among the three residues of CM-Cys in the section of the heavy chain shown in Figure 6, as might be expected if these residues were produced by quantitative cleavage of interchain disulfide bonds. The other radioactive peptides representing the remaining 20% of the radioactivity probably arose from the partial reduction of the intrachain disulfide bond between the half-cystine at position 35 (Press, 1967) and its partner in 2a′, as this bond has been shown to be labile under mildly reducing conditions (Piggot and Press, 1967). It is concluded therefore that the four interchain disulfide bonds shown in Figure 8 are the only interchain bonds in this protein.

This result agrees with the previous finding that fragment 2a″, isolated as a dimer from fragment Fc after cleavage with cyanogen bromide, contains at least one disulfide bond between the heavy chains (Piggot and Press, 1967). The agreement between these data indicates that hydrolysis with papain carried out in the presence of cysteine probably does not here result in significant disulfide interchange.

One uncertainty remains in that the bonds between the heavy chains might be reversed, as shown below,



a possibility suggested by Utsumi and Karush (1965). To distinguish between this form and that shown in Figure 8 it would be necessary to hydrolyze between the two cystine residues and to isolate and characterize the cystine peptides produced.

As has been noted previously for the N-terminal section (Press and Piggot, 1967), there is an obvious homology between the sequence of the Daw heavy chain and that of rabbit IgG. This is illustrated in Figure 9. In the alignment shown here, four residues in the Daw chain (His and Cys-Pro-Ala) are positioned as insertions with respect to the rabbit chain. Of the 43 residues in this section of the Daw heavy chain, 29 residues are identical in the two species, and 7 residues are different. Of these 7, 5 are probably the result of single nucleotide changes according to the genetic code (Brimacombe *et al.*, 1965). The sequence of three residues in the rabbit chain (Pro,Pro,Lys) is uncertain at present.

The homology in position of two of the three interchain half-cystine residues in the Daw heavy chain with two half-cystine residues in the rabbit chain suggests that the interchain disulfide bonds in rabbit IgG may occur in the corresponding positions. If this inference is correct, most rabbit IgG heavy chains would be joined by a single disulfide bond, as has been suggested by Palmer and Nisonoff (1964). The residue participating in the solitary bond between the heavy chains would be the second half-cystine in the rabbit heavy chain sequence shown in Figure 9 and would correspond in position to the second half-cystine in the Daw sequence. An alternative alignment can be made, however, by assuming different deletions, but in either case isolation of a similar tetrameric peptide from the peptic digest of unreduced whole rabbit IgG should be possible and preliminary experiments suggest that this is indeed the case.

Cebra (1967) has drawn attention to the homologies in sequence between this section of rabbit Fd and the C-terminal end of human κ and λ chains. The homology exists therefore between the sequence of the Fd section of Daw heavy chains and the λ chain associated with it. As might be expected, the similarities are less striking than those between the equivalent sections of the heavy chains from the two proteins.

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Added in Proof

Similar conclusions on the sequence around two interchain disulfide bonds between the heavy chains of another human pathological protein of subclass IgG1 have been drawn by B. Frangione and C. Milstein (personal communication).

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A New Assay for Adenosine 3′,5′-Cyclic Monophosphate in Tissue*

John R. Turtle† and David M. Kipnis‡

ABSTRACT: A new, highly sensitive assay for adenosine 3′,5′-cyclic monophosphate (3′,5′-cyclic AMP) has been developed permitting measurement of the nucleotide in as little as 1 mg of tissue. 3′,5′-Cyclic AMP in tissue extracts is first separated from other nucleotides by thin layer chromatography. The isolated 3′,5′-cyclic AMP is then converted to 5′-AMP with 3′,5′-cyclic nucleotide phosphodiesterase. In the presence of γ-labeled [^{32}P]adenosine triphosphate and myokinase, the AMP is converted to [^{32}P]adenosine diphosphate which is then separated by thin layer chromatography. Using this method, the 3′,5′-cyclic AMP concentration has been measured in rat liver ($4.9 \pm 1.3 \times 10^{-9}$ mole/g), skeletal muscle ($1.05 \pm 0.2 \times 10^{-9}$ mole/g), and adipose tissue ($5.05 \pm 0.6 \times 10^{-10}$ mole/g). In isolated rat fat cells the 3′,5′-cyclic AMP concentration increased from 2.3 ± 0.3 to $9.5 \pm 0.5 \times 10^{-11}$ mole/10^6 cells in the presence of epinephrine.

Adenosine 3′,5′-cyclic monophosphate[1] is involved in many biological processes from activation of phosphorylase (Murad *et al.*, 1962), UDPG:glycogen α-4-glucosyltransferase (Sutherland and Rall, 1960), and tryptophan pyrrolase (Rosell-Perez and Larner, 1964) to stimulation of insulin secretion (Turtle *et al.*, 1967), lipolysis (Chytil and Skrivanova, 1963; Rizack, 1964), permeability of the toad bladder to water (Orloff and Handler, 1962), uterine protein synthesis (Creange and Roberts, 1965; Szego, 1965), and steroid hydroxylation (Butcher *et al.*, 1965). Two techniques have been described for measuring 3′,5′-cyclic AMP in tissues. One involves activation of the phosphorylase system by the cyclic nucleotide, as described by Posner *et al.* (1964), Øye *et al.* (1964), and Butcher *et al.* (1965), and the other is based on the enzymatic conversion of the cyclic nucleotide to 5′-AMP and subsequent measurement of the latter nucleotide by an enzymatic cycling procedure (Breckenridge, 1964). In both procedures, the assay of 3′,5′-cyclic AMP in tissue extracts has been limited by poor separation of the cyclic nucleotide from other nucleotides and limited sensitivity secondary to either interference from contaminant nucleotides in the enzymes used or nonspecific stimulation of glycogenolysis (Krebs *et al.*, 1959).

Because of these limitations, a minimum of 60–100

* From the Metabolism Division, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri. *Received July 28, 1967.* Supported in part by U. S. Public Health Service Grant AM-01921.

† Fellow in Metabolism, supported by the Royal Australian College of Physicians. Present address: Department of Medicine, University of Sydney, N.S.W., Australia.

‡ Author to whom reprint requests should be sent.

[1] Abbreviations used: 3′,5′-cyclic AMP, adenosine 3′,5′-cyclic monophosphate; UDPG, uridine diphosphate glucose; ADP and ATP, adenosine di- and triphosphates; TCA, trichloroacetic acid.