Cyanogen Bromide Cleavage and Partial Sequence of the Heavy Chain of a Pathological Immunoglobulin G

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The heavy chain of a pathological immunoglobulin G (Daw) of type L, subclass γ_{2b} (We) and Gm(a+)(f-), has been cleaved with cyanogen bromide. The five fragments resulting from the cleavage have been isolated and analysed. The papain-digest fragments, Fab and Fc, have also been cleaved with cyanogen bromide and the products analysed and compared with those from the heavy chain. The order of the five fragments in the heavy chain has been established and the location of some of the inter-chain and inter-fragment disulphide bonds has been determined. The sequence of the *N*-terminal fragment consisting of 34 residues is reported.

All the immunoglobulins have a basic four-chain structure (Fig. 1) and the antibody-combining sites are contained in the Fab fragments (see Cohen & Porter, 1964). Although the evidence is not conclusive, it seems likely that the Fd section of the heavy chain plays a major role and the light chain a minor role in the formation of the combining site (Porter & Weir, 1966). The work of Haber (1964) and Whitney & Tanford (1965) suggested that the amino acid sequence alone determined the combining specificity of the antibody and therefore the sequence of the Fd section of the heavy chain is of considerable interest.

The obvious complexity of both the heavy and light chains of immunoglobulin preparations, whether from normal serum or purified antibodies,

makes a direct study of amino acid sequence difficult and hence the sequence of the heavy chain of a pathological human immunoglobulin G (Daw), which is believed to be homogeneous, is being investigated.

The identification of the *N*-terminal tripeptide, pyrrolid-2-one-5-carboxyl-valylthreonine (PCA-Val-Thr)* and the sequence of the *C*-terminal octadecapeptide have been reported (Press, Piggot & Porter, 1966a). The latter peptide was obtained by cleavage of the heavy chain with cyanogen bromide. In the present work, we describe the fractionation of the other products of cyanogen bromide cleavage, their arrangement in the heavy chain and the amino acid sequence of the *N*-terminal fragment consisting of 34 residues. A preliminary account of part of this work has already been given (Press, Givol, Piggot, Porter & Wilkinson, 1966b).

MATERIALS

Pathological IgG (Daw). This was prepared as described by Press *et al.* (1966a).

Enzymes. The enzymes used were described by Press *et al.* (1966a).

Other materials. Anhydrous hydrazine was prepared from hydrazine hydrate by the method of Kusama (1957). Ethyleneimine was purchased from Koch-Light Laboratories Ltd. β -Mercaptoethanol was redistilled, b.p. 153–157°. Phenyl isothiocyanate was redistilled under reduced pressure and stored at -20° . Pyridine was redistilled at 115° and stored at -20° . *n*-Butyl acetate was redistilled at 125° . Iodoacetamide was recrystallized from ethanol.

Fig. 1. Structure of immunoglobulin G. Nomenclature recommended for human immunoglobulins by the World Health Organisation (1964).

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; Glx, glutamic residue, either glutamine or glutamic acid; PCA, pyrrolid-2-one-5-carboxylic acid.

METHODS

Heavy chain. The heavy chain was prepared as described by Fleischman, Pain & Porter (1962) except that degassing and saturation of the solution with nitrogen were omitted.

Papain fragments. The Fab, Fc and Fd fragments of the pathological IgG were prepared as described by Press *et al.* (1966a).

The methods for total reduction of heavy chain and fragments, for starch-gel electrophoresis, for cyanogen bromide cleavage and for high-voltage paper electrophoresis have been described by Press *et al.* (1966a). Amino acid analysis was also as described by Press *et al.* (1966a), except that the analysis of the heavy chain was derived from duplicate 24 hr. acid hydrolysates of the partially reduced and alkylated chains and of performic acid oxidized heavy chains. Hexosamine was estimated by the method of Cessi (Fleischman, Porter & Press, 1963) and also on the 15 cm. column of the amino acid analyser used for the basic amino acids.

Dansyl method. This method, for the determination of the *N*-terminal amino acid of peptides, was used essentially as described by Gray (1967). The peptide (about 5 μ moles) was taken to dryness in a Pyrex tube 80 mm. $\times$ 10 mm. It was dissolved in 10 μ l. of 0.2M-NaHCO₃, freshly made up in NH₃-free water, and 10 μ l. of DNS chloride (2.5 mg./ml.) in acetone was added. The mixture was allowed to react for 1 hr. at 37°, and hydrolysed overnight with 0.05 ml. of constant-boiling HCl at 110°; a 6 hr. hydrolysis was used where proline was suspected. After removal of the acid *in vacuo*, the dansyl derivatives were extracted once with 0.05 ml. of ethyl acetate saturated with water. The extract was evaporated in a stream of nitrogen, dissolved in 10 μ l. of aq. 2N-NH₃ soln. and identified by thin-layer chromatography on silica-gel G. The chromatography systems of Morse & Horecker (1966) were used. These systems did not satisfactorily resolve DNS-Ser from DNS-Thr nor DNS-Val from DNS-Leu in our hands. The system methyl acetate-propan-2-ol-aq. NH₃ soln. sp. gr. 0.88 (9:7:4, by vol.) of Seiler & Weichmann (1964) was also used for the identification of DNS-Pro and of DNS-Arg; DNS-Arg was not extracted by the ethyl acetate. It was found advantageous to make up fresh solvents before each run. Internal standards were always used. Plates were viewed under a u.v. lamp (Camag TL 900) while still damp.

Dansyl-Edman technique. In general the dansyl technique was used in conjunction with the stepwise Edman degradation. The method of Gray (1967) was used. Peptide was dissolved in 0.2 ml. of aq. 50% (v/v) pyridine, 0.2 ml. of a 5% (v/v) solution of phenyl isothiocyanate in pyridine was added, and the mixture incubated for 60 min. at 45°. Reagents were removed *in vacuo* at 60°. No extraction was performed before cyclization with trifluoroacetic acid for 45 min. at 45°; trifluoroacetic acid was removed *in vacuo*. The sample was then suspended in 0.15 ml. of water and extracted three times with 1.5 ml. of redistilled *n*-butyl acetate. The aqueous residue was dried *in vacuo*, redissolved in aq. 50% pyridine and a suitable portion removed for identification of the *N*-terminal amino acid by the dansyl method. The remainder of the residual peptide was then subjected to a further step of the Edman degradation.

Alkylation with ethyleneimine (Raftery & Cole, 1963, 1966). Cyanogen bromide-treated heavy chain at 40 mg./ml. was reduced with 0.5M- β -mercaptoethanol in 6M-guanidine-0.5M-tris-HCl buffer, pH 8.2, for 5 hr. at 37°. The solution

was then cooled in ice-water and the ethyleneimine was added to give a twofold molar excess over mercaptoethanol (1 ml. of ethyleneimine is 19.2 m-moles). The pH was kept at 8.2 by periodic additions of 6N-HCl. After 1 hr. the reaction mixture was applied to a column of Sephadex G-50 in 0.05N-NH₃ to separate the desired fragment 2b. The guanidine in the applied sample did not affect the separation.

Removal of urea. Urea was removed from protein solutions by chromatography on a column of coarse Sephadex G-25 in 1.0N-acetic acid. Batches of 80 ml. of protein solution could conveniently be chromatographed on a column 60 cm. $\times$ 3.5 cm.

RESULTS

Fractionation of cyanogen bromide-cleavage products of the heavy chain. The heavy chain of the pathological IgG (Daw) contains four methionine residues (Table 1) and thus should be split by cyanogen bromide into five fragments. The isolation of the *C*-terminal cyanogen bromide peptide on a column of Sephadex G-100 in 1.0N-acetic acid has already been described (Press *et al.* 1966a). This system did not, however, satisfactorily resolve the other fragments, although one other fragment, D, was isolated in low yield (10% of theoretical). Chromatography of cyanogen bromide-treated heavy chain on a column of Sephadex G-100 in 6M-urea-0.2M-formate, pH 3.3, separated five components (Fig. 2a). Components 1, 2 and 3 moved as single bands on electrophoresis in a urea-formate starch gel (Fig. 2b). Component 4 corresponded to D of the previous system, but now was isolated in higher yield (65%). Component 5 was the *C*-terminal peptide. Neither component 4 nor 5 was detected on electrophoresis in starch gel, probably because of their small size. Components 2 and 3 were incompletely resolved on the G-100 Sephadex column, but could be purified by chromatography on CM-cellulose in 6M-urea by gradient elution from 0.05M-acetate, pH 5.15, to 0.2M-phosphate, pH 7. Component 2 was split into two fragments, 2a and 2b, by complete reduction in 6M-guanidine and alkylation followed by gel filtration on a column of Sephadex G-50 in 0.05N-ammonia. Incubation of component 2 in 6M-guanidine for 5 hr. at 37°, without reduction and alkylation, did not release the two fragments 2a and 2b. It is assumed therefore that they are joined by a stable disulphide bond in the partially reduced heavy chain. The amino acid analyses of all the fragments are shown in Table 1. Component 1 alone contained some unchanged methionine and on total reduction fragment 2b could be separated from it. Furthermore, the molecular weight of the reduced component 1, after removal of 2b (fragment 1 in Table 1), based on a homoserine content of 1, was 25 000, which is in agreement with its behaviour on gel filtration (Fig. 2a). Since, however, it represents only about 12% of the whole digest, it most

Table 1. *Amino acid analyses of Daw heavy chain and of the products of cyanogen bromide cleavage*

Analysis of fragment 5 is based on 1 glycine residue/mol. Analyses of the other fragments are based on 1 homoserine residue/mol.

Amino acid content (moles/mole)								Residues recovered 2a+2b+ 3+4+5
Heavy chain	Fragments							
	1	2a	2b	3	4	5		
Lys	30	15	12	0	13	3	1	29
His	9	3.7	3	0	3	0	3	9
Arg	11.5	4.1	2	2	5	2	0	11
Asp	35	19	14	0	17	7	1	39
Thr	36	20	17	7	11	3	1	39
Ser	48	26	24	4	15	5	3	51
Glu	36	18	10	4	18	6	2	40
Pro	34	18	17	2	12	3	1	35
Gly	27	16	15	3	7	3	1	29
Ala	19	12	9	1	5	5	1	21
Val	39	24	19	2	19	4	0	44
Met	3.9	0.1	0	0	0	0	0	0
Ile	8	4.4	2.6	0	3.5	2	0	8.1
Leu	36	17	14	5	11	7	3	40
Tyr	16	9.6	8	0	7	2	1	18
Phe	12	6.3	6	2	4.5	0	0	12.5
Cys	8.5	0	0	0	0	0	0	0
Cys·CH ₂ ·CO ₂ H	4.5	5.4	6	1	3	1	0	11
Homoserine	0	1.0	1	1	1	1	0	4
Trp	9	—	2	0	3	3	0	8
Total residues	422.4	219.6	181.6	34	158	57	18	448.6
Yield (% of theoretical)		20	65	70	80	65	65

Fig. 2. (a) Fractionation of the products of cyanogen bromide cleavage of a pathological human heavy chain by gel filtration on a column (135 cm. $\times$ 3.3 cm.) of Sephadex G-100 in 6M-urea-0.2M-sodium formate, pH 3.3. (b) Starch-gel electrophoresis in 8M-urea-sodium formate buffer, pH 3.3, of the whole cyanogen bromide digest, H, and of the fractions separated by gel filtration, 1, 2 and 3.

probably results from incomplete reaction of cyanogen bromide with one of the methionine residues. The sum of the analyses of fragments 2a, 2b, 3, 4 and 5 agrees well with the analysis of the whole heavy chain (Table 1), and fragment 1 is probably a combination of fragments 2a and 4. Hexosamine was present in fragment 3 but could not be detected in any of the other fragments.

Alignment in the whole heavy chain of the five fragments derived from cyanogen bromide cleavage. Fragment 2b was the *N*-terminal fragment of the heavy chain, as its Pronase digest contained the peptide PCA-Val-Thr, previously shown to be the *N*-terminal sequence of the heavy chain (Porter & Press, 1965). Fragment 5 was the *C*-terminal fragment and fragment 3 was identified by starch-gel electrophoresis as a product of the cyanogen bromide cleavage of the Fc fragment. A cyanogen bromide digest of the Fc fragment was totally reduced and fractionated on a column of Sephadex G-50; in addition to the *C*-terminal fragment 5, fragment 3 and a small peptide, which is presumably the *C*-terminal half of one of the other fragments of the heavy chain, were isolated.

Fab fragment was cleaved by cyanogen bromide,

totally reduced with mercaptoethanol in 6M-guanidine hydrochloride, alkylated with iodoacetamide and the products were fractionated on a column of Sephadex G-100 with 6M-urea-formate buffer. Three components were isolated, the first was identified as whole light chain; the light chain of Daw IgG contains no methionine residue and is therefore not cleaved by cyanogen bromide. The second component was eluted immediately after the light chain and the third component was eluted in the same position as fragment 4. On rechromatography on Sephadex G-50 in 0.05N-ammonia solution, the third component was resolved into two components which were identical in amino acid composition with fragments 2b and 4. Since therefore the whole of fragment 4 must be within the Fd fragment, the ordering of the fragments must be as shown in Fig. 3. The second component eluted

from the Sephadex G-100 column immediately after the light chain is therefore presumably the part of fragment 2a which is within the Fd fragment and will be referred to as 2a'. The small peptide isolated from the Fc fragment is then the C-terminal portion of fragment 2a and will be referred to as 2a''.

Papain is known to cleave the heavy chains of native immunoglobulins at about the midpoint, with the release of a few small peptides. Evidence in favour of the origin of fragments 2a' and 2a'' was obtained by comparison of the amino acid analyses of these fragments with that of 2a (Table 2). Fragment 2a'' contains homoserine and its analysis is based on 1 homoserine residue/mol. The analysis of 2a', which contains no homoserine since it was cleaved at its C-terminus by papain, is based on the assumption that, for each amino acid, the number of residues in 2a $\geq$ the residues in (2a' + 2a''); on this basis 2a' cannot have more than 132 residues. A comparison of the compositions of Fd and Fc fragments with the cyanogen bromide-cleavage products is shown in Table 3.

Inter-fragment disulphide bonds. It has been shown that fragment 2b is linked to fragment 2a by a disulphide bond. This must be a relatively stable intra-chain bond since, when heavy chain which has been prepared by reduction of the IgG with 0.2M-mercaptoethanol is cleaved with cyanogen bromide, no trace of free fragment 2b can be

Fig. 3. Alignment in the heavy chain of the fragments derived from cyanogen bromide cleavage.

Table 2. Amino acid analyses of the cyanogen bromide-cleavage products 2a, 2a' and 2a''

Analyses of 2a and 2a'' are based on 1 homoserine residue/mol. Analysis of 2a' is based on the assumption that for each residue the number of residues in 2a $\geq$ the number of residues in (2a' + 2a'').

	Amino acid content		
	Fragment 2a (moles/mole)	Fragment 2a'	Fragment 2a'' (moles/mole)
Lys	12	8	2
His	3	2.4	0
Arg	2	0.8	0
Asp	14	10	1.5
Thr	17	14	2
Ser	24	21	1.7
Glu	10	6	1.3
Pro	17	9	8
Gly	15	13	3
Ala	9	8	1.2
Val	19	14	1.6
Met	0	0	0
Ile	2.6	2	0
Leu	14	9	3
Tyr	8	6	0
Phe	6	3.7	1.7
Cys	0	0	0
Cys·CH ₂ ·CO ₂ H	6	3.4	1
Homoserine	1	0	1
Trp	2	2	0
Total residues	181.6	132.3	29.0

Table 3. *Comparison of the amino acid analyses of fragments Fd and Fc and the cyanogen bromide-cleavage products derived from these fragments*

Analysis of Fd fragment is based on 20 valine residues/mol. Analysis of Fc fragment is based on 21 valine residues/mol. Analyses of the other fragments are as in Tables 1 and 2.

	Amino acid content			
	Fd fragment (moles/mole)	Fragments 2b+4+2a'	Fc fragment (moles/mole)	Fragments 2a''+3+5
Lys	12	11	16	16
His	2	2.4	5.4	6.0
Arg	4.3	4.8	4.9	5.0
Asp	16	16.7	19	19.5
Thr	23	24	14	14
Ser	30	30	19	19.7
Glu	15	15.6	21	21.3
Pro	15	14	20	21
Gly	18	19	10	11
Ala	13	14	6.7	7.2
Val	20	20	21	20.6
Met	1.5	0	1.9	0
Ile	3.8	4	3.5	3.5
Leu	19	21	17	17
Tyr	7.7	8	8.0	8.0
Phe	5.5	5.7	6.0	6.2
Cys	—	0	—	—
Cys·CH ₂ ·CO ₂ H	1.4	5.4	0	4.0
Homoserine	0	2.0	0	2.0
Trp	5.0	5.0	3.0	3.0

detected at the appropriate elution volume on gel filtration of the products on a Sephadex G-100 column in 6M-urea-formate buffer, pH 3.3. Fragment 2b contains 1 *S*-carboxymethylcysteine residue/mol. (Table 1) and it is presumably this residue which joins it to fragment 2a.

Fragment 4, on the other hand, is obtained in a yield of up to 65% without reduction of all the disulphides in the heavy chain. It contains only one *S*-carboxymethylcysteine residue which might be derived from a cysteine residue having a free thiol group in the whole molecule. Alternatively, it could have resulted from the splitting of an inter- or intra-chain disulphide bond, during the gentle reduction necessary to separate the chains. However, when a cyanogen bromide digest of Fab fragment is chromatographed on Sephadex G-100 in urea-formate, without first reducing the disulphide bonds, 85% of the cyanogen bromide-cleavage products are eluted as a single peak in the breakthrough volume, with no trace of any free fragment 4. (Components of molecular weight > 30000 are eluted in the breakthrough volume in 6M-urea-formate on Sephadex G-100.) Therefore, fragment 4 must be linked through a disulphide bond to one of the other components of the Fab fragment. If it had been joined through its cysteine residue to the light chain, as in Fig. 4(a), then two

Fig. 4. (a) and (b) Alternative arrangements of disulphide bonds in the Fab fragment. Vertical bars indicate positions of cyanogen bromide cleavage and the numbers refer to the cyanogen bromide-cleavage fragments.

components would have been readily resolved on the G-100 column, namely a component (2a'+2b), linked through a disulphide bond, of molecular weight approx. 18000 and a larger component

(light chain + fragment 4) of molecular weight approx. 26000. But as the cyanogen bromide-cleavage products were eluted at the breakthrough volume, fragment 4 must be linked to fragment 2a' by a disulphide bond and the light chain also joined to fragment 2a' as shown in Fig. 4b. This was confirmed by subjecting the fraction eluted from the G-100 column to total reduction and alkylation and rechromatography, when light chain, fragments 2a', 4 and 2b were resolved and identified as described in the previous section.

The small fragment 2a'', derived from the cyanogen bromide cleavage of the Fc fragment after total reduction and alkylation, contained one *S*-carboxymethylcysteine residue, which must presumably be involved in an inter- or intra-chain bond in the unreduced Fc fragment. When cyanogen bromide-cleavage products of the Fc fragment were chromatographed without prior reduction, the three components, 2a'', 3 and 5, were resolved, but the fragment 2a'' was recovered as a dimer, (2a'')₂, indicating that the *S*-carboxymethylcysteine residue in 2a'' is part of an inter-heavy-chain disulphide bond in the Fc fragment. On the other hand, fragment 3 was recovered as the monomer, and as fragment 5 does not contain any cysteine residue, the only disulphide bond between the heavy chains of the Fc fragment is near the *N*-terminal end, in fragment 2a''.

It is, of course, not possible to draw any conclusion from these results about the presence or absence of a second disulphide bond between the heavy chains in the Fd portion or in that part of the heavy chain which is digested by papain. It should be emphasized also that this arrangement of disulphide bonds relates to the Fab and Fc fragments as isolated. As they are obtained by digestion with a sulphhydryl enzyme (papain), a simultaneous catalysis of disulphide interchange cannot be excluded, though the cleanness of the products obtained suggests that this has not occurred.

Amino acid sequence of fragment 2b

Fragment 2b could conveniently be prepared by total reduction in 6*M*-guanidine and alkylation with iodoacetamide of the whole cyanogen bromide digest of the heavy chain; the guanidine solution was applied directly to a column of Sephadex G-50 in 0.05*N*-ammonia, which resolved fragments 4, 5 and 2b from the other fragments. Fragment 2b had no absorption at 280*mμ* and therefore contained neither tyrosine nor tryptophan. It was detected by its absorption at 215*mμ*. The amino acid sequence of the fragment was largely worked out by studying the chymotryptic peptides derived from it. A less detailed study of the tryptic peptides derived from 2b and from 2b which had been alkyl-

ated with ethyleneimine (and not iodoacetamide) established the necessary overlaps to complete the sequence.

Chymotrypsin. Fragment 2b at 10mg./ml. in 0.2*M*-ammonium hydrogen carbonate, pH 8.4, was digested with 1% by wt. of α -chymotrypsin for 3 hr. at 37°. The digest was freeze-dried, redissolved in 0.02*N*-ammonia and fractionated on a column of Sephadex G-25 in 0.02*N*-ammonia (Fig. 5). The digest was resolved into four peaks; peak 1 did not contain peptide material. Peaks C2, C3 and C4 were each resolved into several peptides by high-voltage paper electrophoresis at pH 6.5 or 3.5. The analyses of the five chymotryptic peptides which together account for all of fragment 2b are shown in Table 4. In addition, two peptides, C2 β 2 and C4 α , were isolated which were apparently more extensive digestion products of the larger peptide C2 α (Table 5).

Peptide C3 δ . This stained with hypochlorite-starch-iodide stain but not with ninhydrin. On digestion with Pronase, a peptide was released having the same mobility at pH 6.5 and staining characteristics as PCA-Val-Thr. Thus C3 δ was taken to be the *N*-terminal peptide of fragment 2b and has the composition:

Peptide C2 γ . This peptide is the *C*-terminal peptide of fragment 2b since it contains a homoserine residue. It is an acidic peptide at pH 6.5

Fig. 5. Fractionation of the chymotryptic digest of fragment 2b on a column (148 cm. $\times$ 1.8 cm.) of Sephadex G-25 in 0.02*N*-ammonia.

Table 4. *Amino acid composition of significant chymotryptic peptides derived from fragment 2b*

	Amino acid content (moles/mole)					Residues recovered C2 α +C2 γ + C3 γ +C3 δ +C4 β	Fragment 2b (moles/mole)
	C2 α	C2 γ	C3 γ	C3 δ	C4 β		
Arg	2.0	—	—	—	—	2.0	1.8
Thr	2.0	1.0	3.0	1.0	(0.3)	7.3	7.1
Ser	1.0	2.0	—	—	0.9	3.9	4.4
Glu	2.0	0.8	—	1.0	—	3.8	4.1
Pro	2.1	—	—	—	—	2.1	2.2
Gly	1.0	1.1	—	—	1.0	3.1	3.3
Ala	0.8	—	—	—	—	0.8	1.2
Val	1.0	—	—	1.0	—	2.0	2.2
Leu	2.1	0.9	1.0	1.0	—	5.0	5.2
Phe	—	—	1.0	—	1.0	2.0	2.0
Homoserine	—	1.1	—	—	—	1.1	1.1
Cys·CH ₂ ·CO ₂ H	—	—	0.5	—	—	0.5	0.8
Yield (% of theoretical)	26	33	20	17	25		
pH of electrophoretic purification	6.5	6.5	3.5	3.5	3.5		
Mobility of peptide*	basic 0.25	acidic 0.2	neutral —	acidic 0.4	neutral —		

* Mobility of basic peptide relative to lysine as 1.0; mobility of acidic peptides relative to aspartic acid as 1.0.

Table 5. *Amino acid composition of chymotryptic peptides derived from peptide C2 α*

	Amino acid content (moles/mole)				
	C2 α	C2 β 2	C4 α (C2 α C3)	C2 α C1	C2 α C2
Arg	2.0	1.0	0.7	0.9	1.0
Thr	2.0	—	2.2	1.1	1.3
Ser	1.0	1.0	—	—	(0.35)
Glu	2.0	0.9	1.1	—	0.9
Pro	2.1	1.0	1.0	1.0	1.0
Gly	1.0	1.1	—	—	(0.25)
Ala	0.8	1.0	—	—	—
Val	1.0	—	1.0	0.9	1.1
Leu	2.1	1.0	1.0	—	(0.4)
Yield (% of theoretical)	—	4	6	—	—
pH of electrophoretic purification	6.5	6.5 ; 3.5	3.5	6.5	6.5
Mobility of peptide*	basic 0.25	neutral ; basic — 0.5	basic 0.6	basic 0.6	basic 0.5

* Mobility of basic peptides relative to lysine as 1.0.

(Table 4) and therefore the glutamic residue must be present as glutamic acid. Successive steps of the dansyl-Edman technique and analysis of the residual peptides after steps 1 and 3 of the Edman degradation established the sequence:

Ser-Leu-Ser-Gly-Glu-Thr-Homoserine

Peptide C2 α . This was the largest chymotryptic

peptide isolated. It contained both the prolines, both the arginines and the only alanine of fragment 2b. Peptides C2 β 2 and C4 α (Table 5) must therefore have resulted from enzymic splitting of C2 α .

Hydrazinolysis showed that leucine is the C-terminal residue of C2 α (and of C4 α). The analysis of the residual peptide after one step of the Edman degradation established that arginine was the N-

terminal residue and successive steps of the dansyl-Edman technique established the *N*-terminal sequence of peptide C2 α as:

The presence of glycine and alanine in this sequence proves that the *N*-terminal seven residues of C2 α correspond to C2 β 2 and therefore residue 3 must be serine and residue 7 must be leucine (Table 5). Residue 8 is valine, since the second leucine residue of C2 α has been shown to be *C*-terminal. Peptide C2 β 2 was neutral at pH 6.5 and thus the glutamic residue in C2 β 2 cannot be amidated. Peptide C4 α was basic at pH 6.5 and therefore the glutamic residue in C4 α must be amidated. Thus far we have for C4 α , the *C*-terminal half of C2 α , the sequence:

Overnight chymotryptic digestion of peptide C2 α yielded, in addition to peptides neutral at pH 6.5, three basic peptides, C2 α C1, C2 α C2 and C2 α C3 (Table 5). C2 α C3 was identical with C4 α . Treatment with DNS chloride showed that valine was *N*-terminal for all three peptides. They thus represented different portions of the same region of peptide C2 α :

The sequence of peptide C4 α was completed by successive steps of the dansyl-Edman technique and thus the sequence of C2 α established as:

Peptide C3 γ . This was the only chymotryptic peptide containing *S*-carboxymethylcysteine. Thus it was distinct from the other peptides so far described. Hydrazinolysis showed that phenylalanine was *C*-terminal and the first three residues were identified by the dansyl-Edman method, giving the sequence:

S-Carboxymethylcysteine was tentatively identified as the fourth residue, but its recovery as α -DNS-*S*-carboxymethylcysteine was very low. No residue was observed after four steps of the Edman degradation. As iodoacetamide had been used for alkylation, the cysteine residue in this peptide was present as *S*-carboxamidomethylcysteine. Possibly this residue, when *N*-terminal, ringed on itself in an analogous fashion to glutamine

and prevented further steps of the Edman degradation.

Peptide C4 β . Hydrazinolysis showed this to have phenylalanine as *C*-terminal residue. It contained glycine and not *S*-carboxymethylcysteine, and thus could not be part of, or overlap with, peptide C3 γ . The dansyl-Edman technique established the sequence:

The five chymotryptic peptides, C3 δ , C2 α , C3 γ , C4 β and C2 γ , account for all the residues of fragment 2b. To order the chymotryptic peptides and complete the sequence, the tryptic peptides of the fragment were also studied.

Trypsin. Fragment 2b which had been alkylated with iodoacetamide was digested with 2% of its weight of chloromethyl L-(2-phenyl-1-toluene-*p*-sulphonamido)ethyl ketone treated-trypsin in 0.2M-NH₄HCO₃ for 3.5 hr. at 37°. The digest became very viscous, but was diluted with water, centrifuged and the supernatant fractionated on a column of Sephadex G-25 in 0.1M-NH₄HCO₃, pH 8.3. Only one peptide, T1, was recovered in good yield and this was further purified by paper chromatography with the solvent system pyridine-3-methylbutan-1-ol-water (7:7:6, by vol.).

Peptide T1. This had the composition shown in Table 6, was ninhydrin-negative, hypochlorite-starch-iodide stain positive and was thought to be the *N*-terminal pentapeptide. It was digested with pronase for 1.5 hr. at pH 8.2, 37° and the digest fractionated by paper electrophoresis at pH 6.5. The products were: free arginine (56% recovery), free leucine (58%), free threonine (20%), a peptide (Glu 0.9; Thr 1.1; Val 1.0; 58%) with the same mobility and staining characteristics as authentic PCA-Val-Thr, and a trace of material having the same mobility and staining characteristics as synthetic PCA-Val. Thus the peptide has the sequence:

and represents the *N*-terminal sequence of fragment 2b and of the heavy chain. T1 overlaps the chymotryptic peptides C3 δ and C2 α , thus establishing the order of these two peptides (Fig. 6).

Fragment 2b which had been alkylated with ethyleneimine was digested with 5% of its weight of chloromethyl L-(2-phenyl-1-toluene-*p*-sulphonamido)ethyl ketone-treated trypsin in 0.1M-NH₄HCO₃, pH 8.3, for 16 hr. at 37°. Trypsin cleaved the peptide bond at the aminoethylcysteine residue, as well as at arginine residue 5, and the digestion products were fractionated by paper electrophoresis at pH 6.5. The analyses of the important peptides are shown in Table 6. T1 is the same peptide as that obtained from tryptic digestion of

Table 6. *Amino acid composition of tryptic peptides derived from fragment 2b*

	Amino acid content (moles/mole)			Residues recovered T1+T2+T3	Amino acid content (moles/mole)	
	T1	T2	T3		Fragment 2b*	Fragment 2b†
Arg	1.0	1.0	—	2.0	2.2	1.8
Asp	—	—	(0.4)	(0.4)	(0.8)	(0.4)
Thr	1.0	4.0	1.9	6.9	6.0	7.1
Ser	0.2	1.1	2.9	4.2	4.1	4.4
Glu	1.0	1.9	1.2	4.1	4.0	4.1
Pro	—	1.8	—	1.8	2.2	2.2
Gly	—	1.2	2.1	3.3	3.2	3.3
Ala	—	1.0	—	1.0	1.3	1.2
Val	1.0	1.0	—	2.0	2.7	2.2
Leu	1.0	2.8	1.2	5.0	4.5	5.2
Phe	—	—	1.7	1.7	2.0	2.0
Homoserine	—	—	0.6	0.6	1.0	1.1
Aminoethylcysteine	—	0.6	—	0.6	1.2	—
Cys·CH ₂ ·CO ₂ H	—	—	—	—	—	0.8
Yield (% of theoretical)	50	20	20			

* Fragment 2b alkylated with ethyleneimine.

† Fragment 2b alkylated with iodoacetamide.

Fig. 6. Amino acid sequence of fragment 2b. The tryptic peptides T2 and T3 were obtained from a preparation of fragment 2b in which the cysteine residue was present as aminoethylcysteine.

iodoacetamide-blocked fragment 2b. T1, T2 and T3 account for all the residues of fragment 2b.

Peptide T3. This contains homoserine and is therefore the *C*-terminal peptide and must consist of the chymotryptic peptide C2γ and five additional residues. As T3 has two glycine, three serine and two phenylalanine residues, it must contain the whole of C4β, which therefore must be adjacent to C2γ (see Fig. 6), and also part of C3γ. This establishes the order of the peptides in fragment 2b (Fig. 6). The sequence of C3γ was completed and the order of the peptides confirmed by subjecting T3 to five steps of the dansyl-Edman technique, which gave Thr-Phe-Ser-Gly-Phe-.

Peptide T2. This contains arginine and aminoethylcysteine (presumably aminoethylcysteine is the *C*-terminal residue) and the composition of the peptide is compatible with the arrangement shown in Fig. 6. Trypsin did not cleave the peptide bond

at the arginine residue, presumably because of the adjacent proline residue.

The possibility was considered that C2α and C3γ might overlap, since the dipeptide sequence Thr-Leu occurs at the *C*-terminus of the former and is *N*-terminal in the latter. However, in view of the amino acid composition of fragment 2b and of peptide T2, this seems very unlikely.

DISCUSSION

Satisfactory cleavage of the heavy chain by cyanogen bromide into five fragments has been achieved. The sum of the residues of the five fragments (Table 1) is 448, giving a molecular weight, including the carbohydrate, of about 51000. This is 26 residues in excess of the whole heavy chain, but as this excess is spread over most of the residues, the discrepancy implies either that

the heavy chain analysis is 8% low, or that the analyses of the fragments are a little high. These latter analyses are based on 1 homoserine residue/mole and therefore, particularly with the two large fragments 2a and 3, low recovery of homoserine would give falsely high molecular weights for these fragments. The number of cysteine residues in the heavy chain is assumed to be 13, since 4.5 residues were found as *S*-carboxymethylcysteine and 8.5 as cysteic acid in the partially reduced heavy chain (Table 1). The sum of the *S*-carboxymethylcysteine residues for the five cyanogen bromide fragments is, however, only 11. This discrepancy is no doubt due to poor recovery of this residue on acid hydrolysis. The small differences in the analysis of the heavy chain given in Table 1 compared with that previously reported (Press *et al.* 1966a) are due to modification of the technique as stated in the Methods section.

The yields of each of the fragments are satisfactory, especially when allowance is made for the fact that in 20% of the molecules the methionine at residue 91 is not cleaved, as evidenced by the recovery of fragment 1 in 20% yield. The reason for the failure to cleave the heavy chain at this residue is not understood. Fragment 1 has been obtained with as little as 0.06 mole of methionine/mole of homoserine, which suggested that this particular methionine residue had been alkylated in some molecules during preparation of the heavy chains and therefore could not react with cyanogen bromide. However, preparation of heavy chain by reduction with 0.005 M-dithiothreitol and alkylation with 0.01 M-iodoacetamide (instead of the 0.25 M-iodoacetamide usually employed after reduction with mercaptoethanol) did not result in any difference in the yield of fragment 1. Also, the main product of acid hydrolysis of alkylated methionine, *S*-carboxymethyl-homocysteine (Gundlach, Moore & Stein, 1959), could not be detected on amino acid analysis of fragment 1. If this methionine residue had been oxidized to the sulfoxide in some molecules, it would also not react with cyanogen bromide, but on acid hydrolysis it would have reverted to methionine (Ray & Koshland, 1960). If this methionine had just not reacted with cyanogen bromide in some molecules, again fragment 1 would have contained 1 methionine residue/mol. It seems most likely therefore that reaction with cyanogen bromide did take place, but that the peptide bond was not cleaved in some 20% of the molecules. However, the possibility of some limited heterogeneity in the pathological heavy chain involving replacement of methionine at this position cannot be excluded.

The presence of carbohydrate moiety on fragment 3 is in agreement with the known association of carbohydrate with the Fc fragment of human IgG

(Franklin, 1960). However, the fact that none could be detected in fragment 2a (<0.1 mole of hexosamine/mole) was of interest, in view of the fact that papain digestion of normal human IgG releases a small glycopeptide representing about 25% of the total carbohydrate (Dische & Franklin, 1964). Possibly in any homogeneous IgG the carbohydrate is confined to only one part of the heavy chain. In contrast, the cyanogen bromide-cleavage product of normal rabbit heavy chain, C1 (Givol & Porter, 1965), which is similar but slightly larger than the Fd fragment, did contain 1 hexosamine residue/mol., as would be expected, since some 25% of the carbohydrate of normal rabbit IgG is released as a glycopeptide on digestion with papain (Fleischman *et al.* 1963).

The *N*-terminal half of rabbit heavy chains has been presumed to be heterogeneous from studies of tryptic peptide 'maps' (Small, Reisfeld & Dray, 1966) and similar conclusions have been drawn from studies of human heavy chains (Frangione & Franklin, 1965). However, normal rabbit heavy chain has a common sequence in 70% of the molecules for the first nine residues, with only one substitution in position 3 (Wilkinson, Press & Porter, 1966; Press *et al.* 1966b). Also, D. Givol (personal communication) isolated a chymotryptic peptide of 17 residues from the *N*-terminal end of rabbit heavy chain, and when this sequence is compared with the pathological human heavy chain, there is found to be a degree of homology between the two:

Daw heavy chain:

PCA-Val-Thr-Leu-Arg-Glu-Ser-Gly-Pro-Ala-
Leu-Val-Arg-Pro-Thr-Gln-Thr-Leu

Rabbit heavy chain:

PCA - Ser-^{Val}_{Leu}-Glu-Glu-Ser-Gly-Gly-Arg-
Leu-Val-Thr-Pro-Thr-Pro-Gly-Leu

Also, in 30% of the rabbit heavy chain molecules there is a methionine at residue 35 and the *N*-terminal peptides produced by cleavage of rabbit and Daw heavy chains with cyanogen bromide have a similar composition (Press *et al.* 1966b).

Daw light chain, which is a λ -chain, does not have a free α -amino group, and a ninhydrin-negative peptide has been obtained from a Pronase digest having the composition (Glu,Ser) and the same mobility at pH 6.5 as PCA-Ser. Another ninhydrin-negative peptide of composition (Glu,-Ser,Val) was also recovered in low yield. If it is concluded that the *N*-terminal sequence of Daw light chain is PCA-Ser-Val (this sequence was found for human λ -chains by Hood, Gray & Dreyer, 1966), then it resembles the rabbit heavy chain and differs from Daw heavy chain.

Similarities in sequence at the C-terminal end of the rabbit and human heavy chains have been noted previously (Press *et al.* 1966a). A similarity in composition at the N-terminal end of Fc fragment of Daw and rabbit heavy chains is apparent from the composition of fragment 2a" (Table 2). This fragment contains 8 proline residues in a peptide of 29 residues, and Hill, Delaney, Lebovitz & Fellows (1966) found 7 proline residues in this part of rabbit heavy chain, which no doubt imposes a somewhat extended structure in this region of the molecule.

Perhaps the most important point to be established in this work on the sequence of the heavy chain of pathological immunoglobulins is the presence or otherwise of a variable and stable part of the sequence, such as has been found in the light chains. Present evidence suggests that this does occur in the heavy chain, but there is no information, as yet, as to the point where the switch occurs.

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