The *N*- and *C*-Terminal Amino Acid Sequences of the Heavy Chain from a Pathological Human Immunoglobulin IgG

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The heavy chain of a pathological human immunoglobulin IgG and also the Fd fragment have been isolated. No free α-amino group was present on either and the *N*-terminal sequence of both has been identified as pyrrolid-2-one-5-carbonyl-valylthreonine. Splitting at the four methionine residues of the heavy chain with cyanogen bromide gave five fractions. The fraction from the *C*-terminal end of the chain was isolated in high yield and the amino acid sequence was:

His-Glu-Ala-Leu-His-Asp(NH_2)-His-Tyr-Thr-Glu(NH_2)-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly

These results give strong support to the view that the heavy chain of immunoglobulin is a single peptide chain.

The gross structure of the immunoglobulin is summarized in Fig. 1, showing the nomenclature recommended by the World Health Organisation (1964).

Knowledge of the structure of the immunoglobulins has increased sufficiently rapidly to offer the hope that detailed amino acid sequences of the peptide chains will soon become available. The principal difficulty lies in the known complexity of all preparations of immunoglobulin from normal animals, and hence work has commenced on myeloma or myeloma-like immunoglobulins, which are much more homogeneous and may well have a single unique sequence. Success has been achieved rapidly with Bence-Jones proteins from the urine of myeloma patients. These proteins are readily available and were shown by Edelman & Gally (1962) to be probably identical with the light chains of the myeloma globulin of the same patient. Thus Hilschmann & Craig (1965) and Titani, Whitley, Avogardo & Putnam (1965) have worked out much of the sequence of several Bence-Jones proteins, and Milstein (1965) has placed some of the disulphide bonds as well as the *C*-terminal sequence of the two types of chain.

We have been able to obtain serum from a patient with idiopathic hyper-γ-globulinaemia. This serum contains an IgG fraction that is present in large amounts and that is apparently homogeneous by such criteria as have been used. The heavy chain has no free terminal amino group and hence serves as a model for the heavy chain of normal rabbit IgG, which also has no free α-amino group. The nature of the terminal acid and the *C*- and *N*-terminal amino acid sequences have been determined. A preliminary account of the *N*-terminal sequence has been given by Porter & Press (1965).

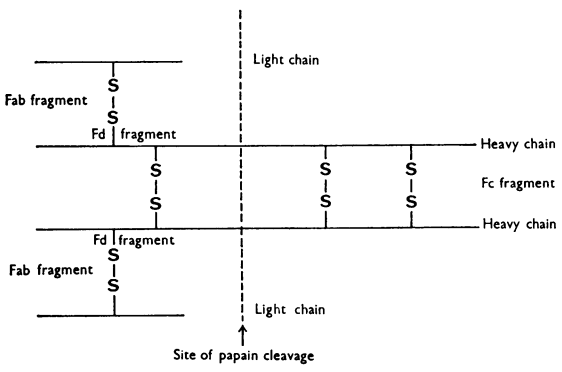

Fig. 1. Structure of immunoglobulin G.

MATERIALS

Pathological IgG. This was present at 7–8g./100ml. in the idiopathic hyper-γ-globulinaemic serum, Daw 28 (see Cohen, 1963). The serum was dialysed against 100 vol. of 17·5mM-sodium phosphate, pH6·2, at 2° and the precipitate collected. This was washed three times with 3·5mM-sodium phosphate buffer, pH6·2, at 2° and resuspended in 0·15M-NaCl at room temperature. Electrophoresis at pH8·9 on cellulose acetate strips showed traces of α- and β-globulin to be still present, but these could be removed by one precipitation with 12% (w/v) Na_2SO_4. Electrophoresis showed only one sharp band and it was identified

by reaction with specific sera to be a type L IgG protein. The yield was 6–7 g./100 ml.

Enzymes. The enzymes used were as follows. Papain, carboxypeptidase A, trypsin and chymotrypsin were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and Pronase P from Kaken Chemical Co. Ltd., Tokyo, Japan. Carboxypeptidase A was treated with di-isopropyl phosphorofluoridate and dissolved as described by Hirs, Moore & Stein (1960). Chymotryptic contamination of trypsin was reduced by treatment with chloromethyl L-(2-phenyl-1-toluene-*p*-sulphonamido)ethyl ketone (Kostka & Carpenter, 1964). Leucine aminopeptidase was prepared from hog kidneys and had specific activity $C_1=40$ (Hill, Spackman, Brown & Smith, 1958). It was stored frozen and was dialysed against 0·01 M-tris–HCl buffer, pH 7·5, containing $MnCl_2$ (2 mM); 0·1 μmole of peptide was digested with 30 μg. of enzyme in 0·1 M-NH_4HCO_3–2·5 mM-$MnCl_2$.

Antiserum. Antiserum specific for Fd fragment was prepared by adsorbing rabbit anti-(human Fab fragment) with human immunoglobulin light chains.

Other materials. Coumassie dye for staining cellulose acetate strips was a gift from Imperial Chemical Industries Ltd., High Holborn, London, W.C.1. Anhydrous hydrazine was prepared from hydrazine hydrate (Kusama, 1957). Silica gel G (E. Merck A.-G., Darmstadt, Germany) was used for thin-layer chromatography.

METHODS

Peptide chains. The peptide chains of Daw 28 IgG were prepared as described by Fleischman, Pain & Porter (1962). The yield (from the extinction at 280 mμ) was 70–72% of heavy chain and 28–30% of light chain.

Fab fragment and Fc fragment. These were prepared by incubating the IgG (50 mg./ml.) in sodium phosphate buffer, pH 7·0, containing cysteine (10 mM) and EDTA (2 mM), with 2% by weight of papain at 37° for 8 min. The digest was cooled to 0° and iodoacetamide added to give a concentration of 50 mM. Chromatography on Sephadex G-100 in 0·15 M-NaCl–25 mM-potassium phosphate buffer, pH 7, gave one major peak and separated about 10% of undigested IgG which was present. The main peak was concentrated and the Fab and Fc fragments were separated by zone electrophoresis in a column of cellulose powder (vol. 450 ml.) in a borate–phosphate buffer, pH 8·9, at 6 v/cm. for 48 hr. (Porath, 1956). Fab and Fc fragments were recovered in yields of 60–65% and 30–35% respectively.

Fd fragment. This was prepared as described for rabbit Fd fragment (Fleischman, Porter & Press, 1963), except that the solvent for chromatography was 0·2 N-acetic acid–15 mM-NaCl. The first peak eluted (40% of the total) was Fd fragment, as was shown by reaction with specific anti-(Fd fragment) serum. No light chains could be detected on starch-gel electrophoresis of this peak (Fig. 2) or by reaction with anti-(light chain) serum, and only traces of *N*-terminal amino acid were present.

Total reduction of peptide chains. Freeze-dried preparations were dissolved in 6 M-guanidine in 0·5 M-tris–HCl buffer, pH 8·2, and reduced with 0·5 M-β-mercaptoethanol for 5 hr. at 37°, followed by alkylation with a 1·2-fold molar excess of iodoacetamide at 0° for 1 hr. and dialysis against water for 40 hr.

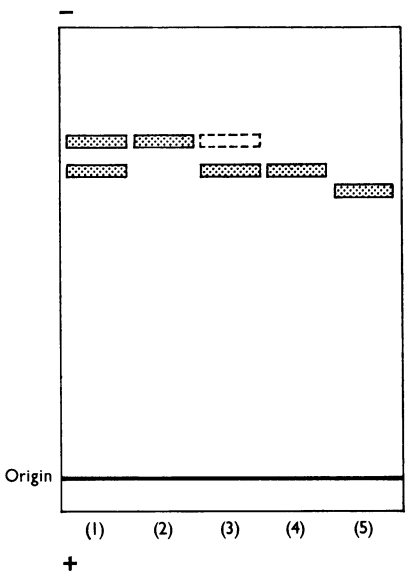

Fig. 2. Electrophoresis in 8 M-urea–formate/starch gel, pH 3·5, of (1) reduced Fab fragment, (2) and (3) first and second fractions obtained by gel filtration of reduced Fab fragment on Sephadex G-100 with 0·2 N-acetic acid–15 mM-NaCl, (4) light chain and (5) Fab fragment, non-reduced.

Oxidation of peptide chains (*Hirs*, 1956). A 30 mg. sample of freeze-dried protein was dissolved in 1·4 ml. of 99% (v/v) formic acid, 0·3 ml. of methanol was added and the mixture was immediately cooled to −10°. Then 2 ml. of performic acid at 0° was added and the mixture allowed to react for 2½ hr. at −10°. The oxidized solution was then diluted with 10 vol. of ice-cold water, dialysed at 2° against water for 2 days and freeze-dried.

Electrophoresis on cellulose acetate strips. The cellulose acetate strips (16 cm. × 2·5 cm.) were soaked in tris–EDTA–boric acid buffer, pH 8·9 (Aronsson & Grönwall, 1958), for several hours and 2 μl. of protein solution was applied as a narrow band. A voltage of 11 v/cm. was applied for 3 hr. The strips were not dried but immersed in 10% (w/v) trichloroacetic acid for 1 min., and stained with 0·5% coumassie dye for 10 min. and washed with water for 1 hr. (Fazekas de St Groth, Webster & Datyner, 1963).

Starch-gel electrophoresis. This was carried out in vertical trays with 8 M-urea in 50 mM-formate buffer, pH 3·5, as described by Edelman & Poulik (1961), at 5 v/cm. for 18 hr.; the protein concentration used was 10 mg./ml. The gels were sliced and stained with Amido Black (Smithies, 1955).

N-Terminal amino acid of peptide chains. The 1-fluoro-2,4-dinitrobenzene method of Sanger, as described by Porter (1957), was used. Bis-DNP-histidine was extracted from the other water-soluble DNP derivatives and amino acids with ethyl acetate. The remaining aqueous fraction was examined for the presence of DNP-arginine, as suggested by Biserte, Holleman, Holleman-Dehove & Sautière (1959), by separating the DNP-amino acids on a talc column and treating with fluorodinitrobenzene again to convert the

ε-DNP-lysine into bis-DNP-lysine. The phenylisothiocyanate method of Edman (1950) was also used. Reaction with phenylisothiocyanate was carried out by the method of Eriksson & Sjöquist (1960); cyclization (for 1½–2hr.) and extraction of the PTH* derivative was carried out as described by Margoliash (1962). The yield of PTH derivative and its identification were as described by Crumpton & Wilkinson (1965); in addition, the reaction mixture was examined for the presence of the PTH derivatives of histidine and arginine, as described by Fraenkel-Conrat, Harris & Levy (1955).

Amino acid analyses. These were performed with an automatic amino acid analyser (Spackman, Stein & Moore, 1958) with 55cm. and 8cm. columns for the separation of neutral and acidic amino acids and basic amino acids respectively. Corrections were made for destruction, during acid hydrolysis, of serine, threonine, tyrosine and histidine. For analysis of peptide chains after treatment with CNBr, the homoserine lactone was converted into homoserine by treatment of the hydrolysates with pyridine–acetate buffer, pH6·5, as described by Ambler (1965), immediately before applying to the 150cm. column, on which homoserine is eluted between serine and glutamic acid. The colour yield was 87% of the 'average standard HW constant'.

Analyses of small peptides were calculated on the assumption that the average of the amounts of the different amino acids present was integral; amino acids present in trace amounts were ignored.

C-*Terminal amino acid of peptide chains.* (1) With carboxypeptidase A. About 0·5μmole of peptide chain was incubated with 1mg. of carboxypeptidase A at 37° for various times in 20mM-tris–HCl buffer, pH8·1. The reaction was stopped by the addition of acetic acid and freeze-drying. The freeze-dried material was resuspended in citrate buffer, pH2·2 (Moore & Stein, 1954), and analysed on the amino acid analyser. Control solutions containing only substrate or enzyme were also incubated and analysed.

(2) By hydrazinolysis (Niu & Fraenkel-Conrat, 1955). About 0·5μmole of peptide chain was dried *in vacuo* over P_2O_5, 0·5ml. of anhydrous hydrazine was added and the mixture was heated at 100° for 9hr. Excess of hydrazine was removed by evaporation over H_2SO_4. The dry residue was suspended in 1ml. of water, shaken for 2hr. with 0·3ml. of benzaldehyde to remove hydrazides and centrifuged to separate the aqueous layer. The benzaldehyde was washed with a further 1ml. of water, and the combined aqueous solution was evaporated to dryness and resuspended in citrate buffer, pH2·2 (Moore & Stein, 1954), for amino acid analysis. Corrections for destruction of amino acids during hydrazinolysis were calculated from the recovery of a mixture of amino acids after 9hr. at 100° in the presence of hydrazine and extraction with benzaldehyde.

Cyanogen bromide cleavage. A 400mg. sample of heavy chain was dissolved in 10ml. of 70% (v/v) formic acid, 1g. of CNBr was added and the mixture was incubated for 24hr. at 25°. The reaction mixture was diluted with 9 vol. of water and freeze-dried to remove the CNBr, which was collected in an acetone–solid CO_2 cold trap and destroyed with NaOH.

High-voltage paper electrophoresis. The apparatus described by Katz, Dreyer & Anfinsen (1959) was used. The solvents and method have been described by Crumpton & Wilkinson (1965); peptides were stained with ninhydrin and with the sodium hypochlorite/starch–iodide method of Pan & Dutcher (1956). The Pauly stain for histidine and the α-nitroso-β-naphthol stain for tyrosine were also used (Smith, 1960). When high-voltage electrophoresis was used for preparative separations of peptides, the mixture was applied to Whatman 3MM paper in a band 3–6cm. wide and, after electrophoresis and drying at room temperature, a strip was cut off the side and stained to locate the peptides, which were then eluted from the remainder of the paper with N-acetic acid for neutral and basic peptides and with 0·1N-NH_3 solution for acidic peptides.

N-*Terminal amino acid sequence of peptides.* The Edman method was used, as described above, except that extraction with ethyl acetate was omitted, as some peptides were soluble in this solvent. A sample of the aqueous layer containing about 0·05μmole of peptide was hydrolysed for amino acid analysis of the residual peptide and the remainder was subjected to a further Edman degradation. In addition to the solvents used by Crumpton & Wilkinson (1965) for chromatographic identification of the PTH derivatives, we have used chloroform–formic acid (20:1, v/v) for histidine, chloroform for leucine and proline, and chloroform–methanol–formic acid (70:30:7, by vol.) for histidine (Brenner, Niederwieser & Pataki, 1961). Although identification of the PTH derivative of serine is unsatisfactory, it gave a characteristic pattern of spots with the solvent system chloroform–methanol (9:1, v/v) and chloroform–formic acid (20:1, v/v). It was, however, always confirmed by the 'dansyl' method or subtractive Edman techniques.

'*Dansyl*' *method for the determination of* N-*terminal amino acid of peptides.* A 0·005μmole sample of peptide was allowed to react with 1-dimethylaminonaphthalene-5-sulphonyl chloride for 3hr. at room temperature, dried *in vacuo* and hydrolysed, as described by Gray & Hartley (1963*a*,*b*). After removal of the acid *in vacuo*, the 1-dimethylaminonaphthalene-5-sulphonyl derivative was extracted into acetone and identified by thin-layer chromatography on silica gel G (method developed by J. M. Wilkinson in this Laboratory). The chromatograms were first developed with chloroform–acetic acid (7:3, v/v) and viewed under a u.v. lamp (Camag TL.900) while still damp. The fluorescent spots were compared with standard 1-dimethylaminonaphthalene-5-sulphonyl derivatives of the amino acids. The thin-layer plate was then dried and developed with ethanol–aq. NH_3 (sp.gr. 0·88) (9:1, v/v) and again viewed under the u.v. lamp while damp. The 1-dimethylaminonaphthalene-5-sulphonyl derivatives used as standards were prepared by J. M. Wilkinson, by the method described by Boulton & Bush (1964), and excess of reagent was removed by fractionation on a column of Dowex 50 (X8; H^+ form).

N-*Acetyl derivatives.* The *N*-acetyl derivatives of glutamic acid, glutamine and glutamylalanine were prepared by acetylation with acetic anhydride in alkali, as described by Greenstein & Winitz (1961).

Preparation of PCA-valine. tert.-Butyl ester of PCA-valine. PCA (1·72g.) and valine *tert.*-butyl ester (1·83g.) were dissolved in 25ml. of warm dimethylformamide and the solution was cooled to 2° before the addition of dicyclohexylcarbodi-imide (2·5g). The reaction mixture was stirred at 2° overnight and was then allowed to warm to room temperature. Acetic acid (0·2ml.) was added to decompose

* Abbreviations: PTH, phenylthiohydantoin; PCA, pyrrolid-2-one-5-carboxylic acid.

the excess of dicyclohexylcarbodi-imide, and the insoluble dicyclohexylurea was filtered off (2·36g.), m.p. 226°; concentration of the filtrate at 30–40° gave an oil, which was dissolved in ethyl acetate, washed with $NaHCO_3$ and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the resultant oil was taken up in diethyl ether. Some insoluble material was removed at this stage. The ether was evaporated and the oil that remained was crystallized from diethyl ether–light petroleum (1:1, v/v) to give the *tert.*-butyl ester of PCA-valine (546mg.), m.p. 105°. A sample recrystallized for analysis from diethyl ether–light petroleum had m.p. 107° (Found: C, 59.1; H, 8·3; N, 9·7. Calc. for $C_{14}H_{24}N_2O_4$: C, 59·2; H, 8·4; N, 9·8%).

PCA-Valine. *tert.*-Butyl ester of PCA-valine (200mg.) was incubated with 1ml. of trifluoroacetic acid for 1½hr. at room temperature and the reagent removed by evaporation at room temperature over NaOH overnight. The product dissolved in ethyl acetate, from which it crystallized on cooling (126mg.), m.p. 126°, R_F 0·65 in butan-1-ol–acetic acid–water (12:3:5, by vol.). A sample recrystallized for analysis from ethyl acetate had m.p. 174° (Found C, 52·8; H, 6·8; N, 12·1. Calc. for $C_{10}H_{16}N_2O_4$: C, 52·7; H, 7·0; N, 12·3%).

RESULTS

Analyses of the heavy and light chains of Daw 28 IgG are given in Table 1. *N*-Terminal amino acid analysis by either the fluorodinitrobenzene or the phenylisothiocyanate method revealed only trace amounts (0·05 μmole each of glycine and aspartic acid) of terminal acids in the isolated heavy chain. Hence an attempt was made to isolate the terminal peptide, with a view to characterizing the supposed blocking agent.

Table 1. *Amino acid analysis of the peptide chains of Daw 28 IgG*

	Amino acid content (moles/mole)	
	Heavy chain	Light chain
Assumed mol. wt. ...	50000	20000
Lys	29	10
His	8·9	2·1
Arg	11	3·6
Asp	34	12
Thr	35	18
Ser	47	26
Glu	36	16
Pro	34	16
Gly	26	17
Ala	20	14
Val	41	14
Met	3·6	0
Ile	8·1	5·1
Leu	36	13
Tyr	17	8·0
Phe	13	3·5
CyS	3·7	3·3
$CyS{\cdot}CH_2{\cdot}CO_2H$	4·4	0·8
Trp	7·5	3·4
Total residues	415	186

N-Terminal peptide. Heavy chain was totally reduced in 6M-guanidine and the thiol groups were alkylated with iodoacetamide. The guanidine was dialysed away and 100mg. of the insoluble protein in about 8ml. of water was adjusted to pH 8·1 at 37° in the titration cell of a pH-stat. Then 2mg. of Pronase was added and the pH maintained automatically with 0·5N-sodium hydroxide. Digestion had ceased after 3hr., when about 25% of the total peptide bonds were estimated to have been broken. The solution, now clear, was run down a column (0·9cm. × 15cm.) of Dowex 50 (X2) in water. The resin was in the H^+ form and had been washed until neutral. About 0·5% (by ninhydrin analysis after alkaline hydrolysis; Hirs, Moore & Stein, 1956) of the peptide material added was not retained on the column. This was concentrated and, when run on a Sephadex G-25 column (2cm. × 150cm.) in N-acetic acid, it was eluted as a single peak. High-voltage paper electrophoresis at pH 6·5 resolved the peptide material into an acidic spot and a neutral spot, both of which were stained by the hypochlorite/starch–iodide technique but failed to react with ninhydrin reagent. The two spots were eluted with 0·1N-ammonia solution, hydrolysed with constant-boiling hydrochloric acid and analysed on the amino acid analyser. The neutral spot contained only trace amounts of amino acids, less than 2% of the total, but the acidic spot contained equimolar amounts of glutamic acid, valine and threonine and proved to be a tripeptide, the yield of which was 0·75mole/mole of heavy chain. Since this peptide was not adsorbed on the Dowex 50 column and did not stain with ninhydrin, it presumably lacked a free *N*-terminal amino acid and was therefore probably the *N*-terminal peptide of the heavy chain.

The peptide was digested with half its weight of carboxypeptidase A at pH 8 overnight at 37°, and a sample was acidified and analysed on the amino acid analyser. The only amino acid present in the digest was threonine, in about 85% yield. The remainder of the digest was put on a column of Dowex 50 (X2; H^+ form) and eluted with water. Acid hydrolysis and analysis of a small sample of the eluate gave equimolar amounts of glutamic acid and valine and no threonine. The dipeptide was then dried over phosphorus pentoxide and treated with dry hydrazine for 7½hr. at 100°, the hydrazine was removed *in vacuo* over concentrated sulphuric acid and the residue was suspended in water and put on a column of Amberlite IRC-50 (H^+ form) to remove the hydrazides (Lay &

Polglase, 1957). Ninhydrin-positive material was eluted from the column with water and analysis showed that valine had been released in 60% yield. The sequence is therefore deduced to be:

[Glu-Val-Thr

where [Glu indicates that the *N*-terminal glutamyl residue is blocked.

As glutamic acid was *N*-terminal, it was possible that it was present as PCA and hence might have been formed from an internal peptide released by digestion. The rapid conversion in acid conditions of free glutamine, or of glutamine when *N*-terminal in a peptide, into PCA was observed by Sanger & Thompson (1953) in their investigation of the amino acid sequence of insulin, and by most subsequent workers in this field. At neutrality, however, this reaction is slow and incubation of glutamine in the titration cell under the same conditions as used for digestion (at pH 8·1 and 37° for 3hr. and a Pronase concentration of 2mg./ml.) showed that less than 3% was converted into PCA. The digest of the reduced heavy chain (100mg.) was therefore repeated, but, when the alkali uptake stopped after 3hr., 0·2ml. of fluorodinitrobenzene was added. There was a rapid reaction and further fluorodinitrobenzene was added over 3hr., when the rate of uptake of alkali became constant owing to hydrolysis of the reagent. Under these conditions all free amino groups were blocked, including any *N*-terminal glutamine that might have been present. The yellow solution was extracted with ether to remove excess of reagent, and after acidification with hydrochloric acid it was extracted several times with ethyl acetate, taking out most of the DNP-amino acids and DNP-peptides. The aqueous residue was poured through a column of Dowex 50 as before. The peptide material was collected in the first 50ml. of eluate and much of the yellow material that was eluted later was discarded. The peptide eluate was neutralized with aq. ammonia and concentrated. Chloride was removed on a column of Dowex 1 (X8; OH⁻form) and peptides were eluted with formic acid. Electrophoresis at pH 6·5 on paper removed traces of DNP derivatives still present and showed a main component with the same mobility as the tripeptide eluted previously. Analysis showed glutamic acid, valine and threonine to be present again in equal amounts and the overall recovery of this peptide was 0·6mole/mole of heavy chain. In view of the additional steps required in purification this is considered to be comparable with the 75% recovery when reaction with fluorodinitrobenzene was omitted. It is therefore concluded that the [Glu-Val-Thr sequence was present in the protein before digestion and hence must represent the *N*-terminal peptide of the heavy chain.

Proof that the terminal glutamic acid was present as PCA was obtained as follows. Electrophoresis on paper at pH 6·5 of the dipeptide [Glu-Val, together with synthetic PCA-valine, *N*-acetylglutamic acid, *N*-acetylglutamine and *N*-acetylglutamylalanine, showed that by mobility (Table 2) the dipeptide might be either PCA-valine or an acylated glutaminylvaline. Amide ammonia was therefore estimated as described by Sanger, Thompson & Kitai (1955). *N*-Acetylglutamine gave 0·9mole of ammonia/mole, whereas the tripeptide under the same conditions gave only 0·25mole/mole. This was considered to have arisen from contamination and hence it was concluded that the peptide must have an *N*-terminal PCA. Further evidence in support of this was given by the alkali-lability of blocked glutamic acid. Dekker, Stone & Fruton (1949) found that the PCA ring is opened by treatment with N-sodium hydroxide for 24hr. at 25°, whereas *N*-acetylglutamic acid is not hydrolysed under these conditions (Blombäck & Doolittle, 1963). We have compared the alkali-lability of synthetic PCA-valine, the terminal dipeptide and *N*-acetylglutamic acid, by heating at 100° in N-sodium hydroxide for 15min. The extent of hydrolysis of *N*-acetylglutamic acid, PCA-valine and dipeptide was 14, 65 and 61% respectively. Chromatographic comparison of the dipeptide with synthetic standards (Table 3) also

Table 2. *Electrophoretic mobilities of amino acid and peptide derivatives relative to PCA at* pH 6·5

	Relative mobility	Net negative charge
PCA	1·0	1
PCA-Val	0·7	1
[Glu-Val	0·7	—
[Glu-Val-Thr	0·6	—
N-Acetyl-Glu(NH_2)	0·8	1
N-Acetyl-Glu	1·3	2
N-Acetyl-Glu-Ala	1·1	2

Table 3. *R_F values of peptides on thin-layer chromatography*

Solvent 1, butan-2-ol–aq. 3% NH_3 (35:11, v/v); solvent 2, butan-1-ol–acetic acid–water (12:3:5, by vol.).

	R_F	
	Solvent 1	Solvent 2
PCA	0·35	0·50
PCA-Val	0·40	0·65
[Glu-Val	0·40	0·65
N-Acetyl-Glu(NH_2)	0·23	0·45
N-Acetyl-Glu-Ala	0·10	0·62

agreed with the conclusion that the terminal acid was PCA. Hence it is concluded that the *N*-terminal sequence of the heavy chain is:

PCA-Val-Thr

This terminal peptide was also isolated from the Fd fragment (see Fig. 1) by similar procedures, confirming that the Fd fragment is formed from the *N*-terminal end of the heavy chain, as suggested by Fleischman *et al.* (1963).

C-*Terminal peptide.* Digestion of the heavy chain with carboxypeptidase A before or after oxidation with performic acid released only traces of amino acids. However, hydrazinolysis gave yields, after correction for losses, of 0·9 mole of glycine and 0·1 mole of serine/mole of heavy chain. The Fc fragment (see Fig. 1) also gave 1·1 moles of glycine/mole. Hence the *C*-terminal amino acid of the heavy chain is glycine, with perhaps proline in a penultimate position in view of the resistance to digestion by carboxypeptidase. Recovery of the small amount of serine may be due to degradation of serine hydrazides, which has been reported by Bradbury (1958) to occur, especially with proteins having a high concentration of serine. However, some of the serine may have arisen from contamination with light chain, as it was found that this chain had *C*-terminal serine, in agreement with the results of Milstein (1965) with myeloma type L light chains.

The heavy chain contains 4 methionine residues/mole (Table 1) and hence cyanogen bromide cleavage was investigated as a technique for obtaining large fragments. A variety of conditions have been suggested by previous workers to achieve complete reaction without non-specific acid hydrolysis. Gross & Witkop (1961, 1962) used 0·1 N-hydrochloric acid at 20° for 24 hr., Bargetzi, Thompson, Sampath Kumar, Walsh & Neurath (1964) used 70% (v/v) trifluoroacetic acid, and Steers, Craven, Anfinsen & Bethune (1965) used 70% (v/v) formic acid. These conditions were used with the heavy chain, and controls were included in which the cyanogen bromide was omitted. Behaviour on electrophoresis in urea–formate gels was taken as a test of non-specific hydrolysis, and analysis for methionine and homoserine showed whether the reaction was complete. The 70% formic acid caused little or no non-specific hydrolysis but almost complete reaction of the methionine residues (Table 4). There appeared to be substantial non-specific cleavage with 70% trifluoroacetic acid as solvent. Hence 70% formic acid was used, and after freeze-drying the reaction mixture was dissolved in N-acetic acid and fractionated on a column of Sephadex G-100 in the cold room. Five fractions were collected (Fig. 3) and freeze-dried. On electrophoresis in starch gel fractions A and B contained the same two components, whereas fraction C contained a third component that could be purified by re-running. Fractions D and E could not be detected, presumably as their molecular weights were too low. Thus there were five components present, as would be expected from the presence of four methionine residues in the chain. Fraction E could be freed from fraction D by running on a column of Sephadex G-50 in 0·1 M-ammonium hydrogen carbonate, pH 8·2. The u.v. spectrum resembled that of tyrosine, and on amino acid analysis (Table 5) this fraction, peptide E, was found to contain molar ratios of amino acids but no homoserine, suggesting that this was the

Table 4. *Partial analysis of the heavy chain after reaction with cyanogen bromide in* 70% *formic acid for* 24 *hr. at* 25°

CNBr used (mg./100 mg. of heavy chain)	Amino acid content (moles/mole of heavy chain)			
	Methionine	Homoserine	Proline	Isoleucine
0	3·6	0·0	34	8·2
270	0·13	3·9	34	8·2
330	0·05	4·1	34	7·9
250	0·26	3·7	34	8·1

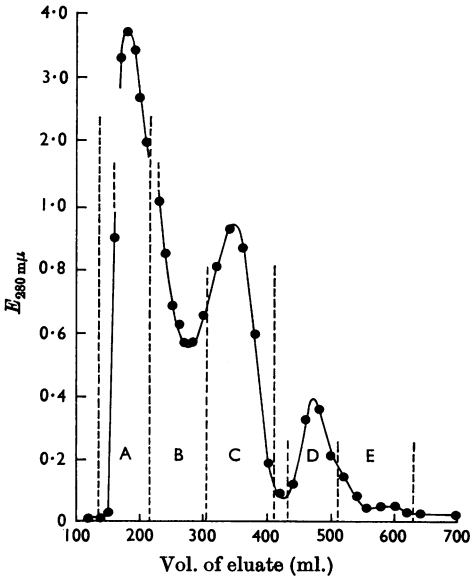

Fig. 3. Fractionation of CNBr-treated heavy chain on a column (122 cm. × 2·5 cm.) of Sephadex G-100 in N-acetic acid. The eluate was divided into five fractions, A, B, C, D and E, as marked.

Table 5. *Amino acid composition of peptide E and tryptic peptides derived from it*

	Amino acid content			
	Peptide E		Peptide ET1	Peptide ET2
	(μmole)	(moles/mole)	(moles/mole)	(moles/mole)
Lys	0·16	1·0	—	1·0
His	0·47	3·0	—	3·0
Asp	0·18	1·1	—	1·1
Thr	0·16	1·0	—	1·0
Ser	0·45	2·9	3·1	—
Glu	0·30	2·0	—	1·9
Pro	0·15	1·0	0·8	—
Gly	0·17	1·1	1·0	—
Ala	0·17	1·1	—	1·0
Leu	0·45	2·9	2·0	1·0
Tyr	0·13	0·8	—	0·9
Homoserine	0·0	0·0	—	—
Total residues		18	7	11

C-terminal peptide. In agreement with this, hydrazinolysis showed glycine to be *C*-terminal, as in the whole heavy chain. Electrophoresis on paper at pH 3·5 showed only a single basic component, which stained positive for histidine but negative for tryptophan. If there is one glycine residue present, then the peptide contains 18 amino acid residues. The yield was 3·3 mg. of peptide/100 mg. of heavy chain, which is 83% of the theoretical value. These weights were uncorrected for moisture and ash.

Amino acid sequence of peptide E. The *N*-terminal sequence was determined by three successive steps with the phenylisothiocyanate reaction. Subtractive analysis (Table 6) shows a successive loss of histidine, glutamic acid and alanine. There is also loss of lysine and, in the third step, of serine, but non-specific loss of these amino acids has been reported by Margoliash (1962) and Smyth, Stein & Moore (1962). Subsequent findings that all three serine residues are in the *C*-terminal end of this peptide confirmed that the loss of serine here was non-specific and therefore the *N*-terminal sequence is:

His-Glu-Ala-

Identification of the PTH residues after the three steps confirmed this sequence.

Peptide E was hydrolysed with trypsin for 3½ hr. at pH 8·0 and 37°, and fractionated on a column of Sephadex G-25 in 1% formic acid. The two peptides were located in the eluate by their absorption at 280 and 235 mμ (Fig. 4), and electrophoresis on paper showed each to be a single component.

Table 6. *Amino acid analyses of peptides after successive stages of the Edman degradation of peptide E*

	Amino acid content (moles/mole)			
Stage ...	0	1	2	3
Lys	1·0	0·8	0·7	0·7
His	3·0	2·3	2.2	2·0
Asp	1·1	1·2	1·2	1·3
Thr	1·0	1·0	1·0	1·0
Ser	2·9	3·0	2·9	2·3
Glu	2·0	2·0	1·5	1·5
Pro	1·0	0·9	0·9	0·8
Gly	1·1	1·2	1·4	1·4
Ala	1·1	1·0	1·2	0·6
Leu	2·9	3·0	3·0	2·7
Tyr	0·8	0·6	0·8	0·8

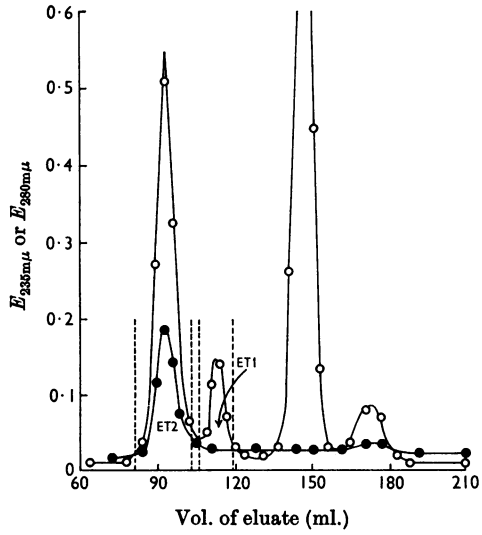

Fig. 4. Separation of the tryptic peptides of peptide E on a column (144 cm. × 1·2 cm.) of Sephadex G-25 in 1% formic acid. ○, $E_{235\,m\mu}$; ●, $E_{280\,m\mu}$. Fractions ET1 and ET2 were combined as shown.

Analysis (Table 5) confirmed the purity of these products.

Peptide ET1. This peptide contains glycine and no lysine and was therefore the *C*-terminal heptapeptide. The sequence of the three *N*-terminal residues was established by a sequential Edman degradation. The PTH derivative of serine was tentatively identified after the first step and leucine after the second step. Treatment of the residual

peptide by the 'dansyl' method and hydrolysis proved the third residue to be serine. Thus the partial sequence was:

Ser-Leu-Ser-(Ser,Pro,Leu)-Gly

Peptide ET1 was digested by chymotrypsin for 16hr. at pH 8·2 at 37°. Three peptides were separated by paper electrophoresis at pH 3·5 and analysed: peptide ET1Ca, residual undigested peptide ET1; peptide ET1Cb, (Ser, 2·2; Pro, 0·7; Gly, 0·9; Leu, 1·2); peptide ET1Cc, (Ser, 1·1; Leu, 0·9).

Peptide ET1Cb. This must be the *C*-terminal pentapeptide since it contains glycine. By using the 'dansyl' method it was established that serine was *N*-terminal. After one Edman degradation step the residual peptide was analysed and had the composition (Ser, 1·2; Pro, 0·7; Gly, 1·0; Leu, 1·1), confirming that serine was the *N*-terminal residue of peptide ET1Cb. Treatment of this tetrapeptide by the 'dansyl' method established leucine as the next residue and this was supported by the identification of the PTH derivative of leucine after a further Edman degradation. Hence the partial sequence is:

Ser-Leu-(Ser,Pro)-Gly

Peptide ET1Cc. By using the 'dansyl' method it was established that serine was *N*-terminal and after Edman degradation only free leucine was recovered. The sequence is therefore:

Ser-Leu

Thus the partial sequence of peptide ET1 is:

Ser-Leu-Ser-Leu-(Ser,Pro)-Gly

Leucine aminopeptidase digestion of peptide ET1 (50 μg. to 0·4 μmole of peptide) for 16hr. at pH 8·2 at 37° yielded the free amino acids serine and leucine, and a peptide ET1L was recovered by paper electrophoresis of the digest at pH 3·5.

Peptide ET1L. This had the composition (Ser, 1·0; Pro, 1·0; Gly, 1·0). It is therefore the *C*-terminal tripeptide. Serine was shown to be the *N*-terminal residue of peptide ET1L by using the 'dansyl' method. After one Edman degradation step the residual peptide had the composition (Ser, 0·1; Pro, 0·9; Gly, 1·1), and after a further Edman degradation the PTH of proline was identified and free glycine recovered, establishing the sequence:

Ser-Pro-Gly

The sequence of peptide ET1 is therefore:

Ser-Leu-Ser-Leu-Ser-Pro-Gly

Table 7. *Amino acid analysis of chymotryptic peptides of peptide ET2*

	Amino acid content (moles/mole)			
	Peptide ET2	Peptide ET2C1	Peptide ET2C2	Peptide ET2C3
Lys	1·0	—	1·0	—
His	3·0	2·2	0·2	1·5
Asp	1·1	1·2	—	0·5
Thr	1·0	—	0·9	—
Glu	1·9	—	1·0	1·0
Ala	1·0	—	0·1	1·0
Leu	1·0	—	0·1	1·0
Tyr	0·9	0·7	—	0·4

Peptide ET2. This peptide is the *N*-terminal undecapeptide of peptide E (Table 5) and, as the three *N*-terminal residues have been identified (see above) and also it is the product of tryptic digestion, the partial sequence can be written as:

His-Glu-Ala-(His_2,Asp,Thr,Glu,Leu,Tyr)-Lys

Peptide ET2 was digested with α-chymotrypsin at pH 8·3 for 3½hr. at 37°. The digest was fractionated by paper electrophoresis at pH 3·5 and three basic peptides were recovered (see Table 7). Peptide ET2C2 must be the *C*-terminal peptide of peptide ET2 as it contains lysine. Peptide ET2C1 contains neither glutamic acid nor alanine, which were shown to be in the *N*-terminal sequence, and therefore it is the middle peptide of peptide ET2. Peptide ET2C3 is apparently a mixture of two peptides, the *N*-terminal (see above) His-Glu-Ala-Leu and a tripeptide, in about half the molar yield (His,Asp,Tyr), presumably derived from peptide ET2C1. The three chymotryptic peptides can therefore be arranged:

His-Glu-Ala-Leu-(His_2,Asp,Tyr)(Thr,Glu)-Lys

ET2C3 ET2C1 ET2C2

Peptide ET2C1. This was subjected to three Edman degradations; the PTH derivative after the first step was identified as that of histidine, after the second step as that of asparagine and after the third step as that of histidine. Analysis of the residual peptides after each of the three steps (Table 8) established the sequence:

His-Asp(NH_2)-His-Tyr

Peptide ET2C2. This was also degraded by the Edman technique and the PTH derivatives identified after each step were those of threonine and glutamine respectively. Analysis of the residual

Table 8. *Amino acid analyses of residual peptides after successive Edman degradations of peptide ET2C1*

	Amino acid content (moles/mole)			
Stage ...	0	1	2	3
His	2·2	1·1	1·1	0·0
Asp	1·2	1·1	0·2	0·0
Tyr	0·7	0·9	0·9	1·0

peptide after the first Edman degradation step confirmed that threonine was *N*-terminal in this peptide. As it is known that lysine is *C*-terminal, the sequence is:

Thr-Glu(NH_2)-Lys

Peptide ET2C3. This peptide contained the *N*-terminal tetrapeptide of peptide E and this was confirmed by identification of the PTH of histidine after the first step of the Edman degradation and PTH-glutamic acid after the second step. After the third step PTH-alanine was identified and free leucine recovered.

Amide content of ET2. The amide content was tentatively deduced by identification of PTH derivatives isolated during sequential analysis. Confirmation was obtained by digestion of each of the chymotryptic peptides with leucine aminopeptidase at pH 8·2 overnight, followed by analysis of the digests on the 55 cm. column of the analyser. Asparagine was obtained in a yield of 0·6 mole/mole of peptide ET2C1. Peptide ET2C2 contains threonine, which is incompletely resolved from glutamine on the 55 cm. column. However, the yield of threonine plus glutamine was 1·4 moles/mole and only 0·1 mole of glutamic acid/mole was found. Therefore we assume that this glutamic acid residue is amidated. Leucine aminopeptidase digestion of peptide ET2C3 yielded 0·9 mole of glutamic acid/mole. A small amount of amide was also found, owing to contamination of this *N*-terminal tetrapeptide with the tripeptide His,Asp(NH_2),Tyr, as mentioned above. The full sequence of peptide E is therefore:

His-Glu-Ala-Leu-His-Asp(NH_2)-His-Tyr-Thr-Glu(NH_2)-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly

DISCUSSION

Satisfactory isolation of Fd fragment from normal human IgG has not been reported, as conditions for dissociation do not cause dimerization (as for rabbit Fd fragment) and separation of Fd fragment and light chains is very difficult. Success with this pathological IgG has depended on finding that dissociation did occur in weak acid (0·2 N-acetic acid–15 mM-sodium chloride), presumably because the light chains were type L, which are more readily dissociated (Cohen & Gordon, 1965), and because in this weaker acid Fd fragment did behave as a dimer. It is unlikely that this method can be applied generally and, for chemical studies, cyanogen bromide cleavage of the heavy chain is probably preferable, though in this method all biological activity so far investigated is lost.

The isolation of the same *N*-terminal peptide from the heavy chain and the Fd fragment is in agreement with the accepted structure of immunoglobulin G shown in Fig. 1 and, in turn, supports the conclusion that PCA-Val-Thr is indeed the *N*-terminal sequence of the heavy chain. Likewise, the finding that the heavy chain and Fc fragment have the same *C*-terminal amino acid also supports this structure.

The finding that PCA is the sole *N*-terminal acid of the heavy chains from pathological human IgG, and further evidence (J. M. Wilkinson, unpublished work) that it is also the *N*-terminal acid of the heavy chain of normal rabbit IgG, raises the question as to its origin. PCA was first reported to be *N*-terminal in a tripeptide isolated from *Pelvetia fastigiata* (Dekker *et al.* 1949). More recently, it has been found to occur in human and bovine fibrinopeptides B (Blombäck, Blombäck & Edman, 1963; Blombäck & Doolittle, 1963) and in two naturally occurring polypeptides, gastrin (Gregory, Hardy, Jones, Kenner & Sheppard, 1964) and eledoisin (Anastasi & Erspamer, 1963). It has also been reported to be present in a small fraction of commercially isolated ribonuclease (Eaker, King & Craig, 1965), but here the evidence suggests that it is an artifact of the method of isolation of the protein. The isolation of both the rabbit and the human IgG was, however, carried out at neutral pH and low temperatures, conditions that *in vitro* cause very little conversion of glutamine into PCA. Separation of the peptide chains is carried out in acid, but no *N*-terminal glutamine is present in either whole Daw IgG or normal rabbit IgG. It seems unlikely that ring closure has occurred during isolation of the protein and probable that PCA is present in the IgG circulating in the blood. If PCA formation occurs *in vivo*, but after synthesis of the proteins, it would be expected to be an enzymically catalysed reaction, and, indeed, such a specific enzyme (glutamine cyclotransferase) has been isolated by Messer & Ottesen (1964) from crude papain, but there appears to be no evidence at present that such an enzyme occurs in animal tissues. The possibility that PCA accounts for the missing *N*-terminal amino acids in the immuno-

globulins of normal human and other animal sera remains to be investigated.

Biological evidence has been put forward by several authors (see Cohen & Porter, 1964; Stemke & Fischer, 1965) that would be most easily explained if the heavy chain of the immunoglobulin was, in fact, two peptide chains. Chemical evidence was lacking, except insofar as the molecular weight of the heavy chain did not fall after oxidation or total reduction (Fleischman *et al.* 1963), and hence if two separate chains were present they were not held together by disulphide bonds. The present work has now identified the *N*- and *C*-terminal amino acid sequences and only one of each is present/mole of heavy chain, and this makes it much less likely that there are two separate chains present. It is still possible that two peptide chains are synthesized separately and joined after synthesis by an unknown bond which also blocks both the *N*- and *C*-terminal acids. If, in fact, it was a linkage through an α-peptide bond, only synthesis studies *in vitro* could distinguish such a phenomenon.

Another point of interest is that the 19 residues at the *C*-terminal end of this pathological human heavy chain show only two replacements when compared with the same section of the *C*-terminal end of normal rabbit heavy chain (Givol & Porter, 1965). This suggests that Fc fragment from immunoglobulin in mammalian species may have a unique sequence showing relatively little variability from one to the other, the great variability being confined to the Fd fragment part of the chain. Such an arrangement of a variable half and a stable half is already suggested for the light chains from the preliminary results of the amino acid sequence of several Bence-Jones proteins. A similar arrangement in the heavy chains would be of the greatest significance, but its interpretation in terms of mechanisms of antibody synthesis and specific combining power is far from clear.

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