The *N*-Terminal Sequence of the Heavy Chain of Rabbit Immunoglobulin IgG

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The absence of an *N*-terminal amino acid with a free α -amino group from the heavy chain of rabbit immunoglobulin IgG has been confirmed and no evidence could be found of a blocking formyl, acetyl or propionyl group. The *N*-terminal amino acid appears to be pyrrolid-2-one-5-carboxylic acid (PCA) in all molecules. A mixed amino acid sequence follows in the approximate proportions: PCA-Ser-Val-Glu-Glu-Ser-Gly-Gly-Arg, 50%; PCA-Ser-Leu-Glu, 20%; PCA-Glu(NH₂), 20%. The heavy chains of a purified antibody, namely anti-(human serum albumin), and of immunoglobulin IgG from a rabbit homozygous at the allotypic loci both showed a similar mixed *N*-terminal sequence.

The nomenclature used for immunoglobulin IgG and its fragments is that recommended by the World Health Organisation (1964).

Evidence is accumulating that suggests that the specific affinity of antibody for the antigen is due to the conformation of the antibody-combining site and that this, in turn, depends only on the amino acid sequence of the constituent peptide chains (Haber, 1964; Whitney & Tanford, 1965). The combining site is present in the papain-digest fragment Fab (Porter, 1959) and this is formed from the light chain and the *N*-terminal half of the heavy chain (Porter, 1962; Fleischman, Porter & Press, 1963). The site is not in the light chain, but there is some uncertainty as to whether it is in the heavy chain or is formed jointly by both chains. In the view of Weir & Porter (1966) the combining site is probably contained in the heavy chain, though the light chain plays some part, possibly indirectly.

An attempt is therefore being made to determine the amino acid sequence of the *N*-terminal half of the heavy chain of rabbit IgG. Much difficulty arises because all preparations of IgG from any species are complex and contain mixtures of molecules with different amino acid sequences. All attempts to isolate homogeneous fractions of either 'inert' IgG or specific antibody have been unsuccessful. However, the *C*-terminal end of the heavy chain of rabbit IgG (Givol & Porter, 1965) and of human IgG (Piggot & Press, 1966) appears to have only one amino acid sequence, and work has therefore been commenced to find if the multiplicity of sequences is confined to a relatively small part of the Fd fragment of the heavy chain or whether it extends through most of this fraction. In the present paper we report that the *N*-terminal

residue of the heavy chain of rabbit IgG is PCA* [as in a pathological human IgG (Porter & Press, 1965; Press, Piggot & Porter, 1966)] and that several different sequences follow this residue. IgG from the serum of a rabbit, homozygous with respect to the allotype (A₁A₅), and from a purified antibody, namely anti-(human serum albumin), showed the same mixed sequence.

MATERIALS AND METHODS

Rabbit IgG. This was prepared from serum purchased from a rabbit farm. Fractionation by precipitation with Na₂SO₄ (Kekwick, 1940) was followed by chromatography on DEAE-Sephadex A-50 in 17.5 mm-sodium phosphate buffer, pH 6.3. The unretarded protein was collected, concentrated by pressure dialysis and freeze-dried. Only IgG was present, as judged by electrophoresis on cellulose acetate strips or by immunoelectrophoresis.

Preparation of IgG from the serum from a single rabbit of allotype A₁A₅ was carried out similarly. The serum was given by Dr A. S. Kelus.

Purified anti-(human serum albumin). This was prepared by dissolving the specific precipitate in 0.05 M-NaCl-HCl, pH 2.0. The solution was passed through a Sephadex G-200 column. Three peaks were present: serum albumin, aggregate and dissociated antibody, the last-named running in the position of IgG. This peak was collected, concentrated and freeze-dried; 85% of this fraction could be precipitated by specific antigen.

Heavy and light chains of IgG. These were prepared as described by Fleischman, Pain & Porter (1962). The papain-digest fragment Fab (Porter, 1959) was dissociated to give the *N*-terminal half of the heavy chain, the Fd fragment (Fleischman *et al.* 1963).

Enzymes. Carboxypeptidase A, papain and trypsin were purchased from Worthington Biochemical Corp. (Freehold,

* Abbreviation: PCA, pyrrolid-2-one-5-carboxylic acid.

N.J., U.S.A.) and Pronase from Kaken Chemical Co. Ltd. (Tokyo, Japan). Trypsin was treated with chloromethyl L-(2-phenyl-1-toluene-*p*-sulphonamido)ethyl ketone to decrease chymotryptic activity (Kostka & Carpenter, 1964).

Methods. The following methods were used as described by Press *et al.* (1966): total reduction and oxidation of peptide chains; enzyme digestion; determination of *N*-terminal amino acids by the fluorodinitrobenzene, 'dansyl' and phenyl isothiocyanate methods; electrophoresis on cellulose acetate and on paper. Amino acids were estimated on the automatic analyser modified as described by Benson & Patterson (1965) and with a high-sensitivity unit (Evans Electroselenium Ltd.) incorporated. Satisfactory analysis could be carried out with 0.02 μ mole of amino acid.

Hydrazinolysis was carried out according to the method of Bradbury (1956).

Amide determination was carried out by the procedure of Sanger, Thompson & Kitai (1955), except that after hydrolysis the content of ammonia was estimated on the 5 cm. column of the automatic analyser. Blank runs were carried out in parallel to correct for any ammonia contamination of the solutions used.

Estimation of acetyl groups was by the micro-method of Ludowieg & Dorfman (1960).

RESULTS

With the fluorodinitrobenzene method, only trace amounts of amino acids with a free α -amino group could be detected in the heavy chain of rabbit IgG (Fleischman *et al.* 1962). The presence of an *N*-terminal half-cystine residue was excluded by reaction with fluorodinitrobenzene after oxidation (Bettleheim, 1955), and further examination with the phenyl isothiocyanate method also confirmed the absence of any significant content of free α -amino groups.

Other proteins in which an *N*-terminal amino acid could not be detected have been shown to have an acetylated α -amino group. Winstead & Wold (1964) have listed ten such proteins. An attempt was therefore made to estimate the acetyl content of the Fd fragment of rabbit IgG heavy chain. As only one acetyl group/mol. was expected, it was essential to have a low blank value, and as both the heavy chain and Fd fragment are usually prepared by dissociation in acetic acid or propionic acid the papain-digest Fab fragment was used. It was isolated by fractionation on CM-cellulose columns, as described by Porter (1959), but a phosphate buffer was used instead of acetate. Fab-fragment II was used rather than Fab-fragment I, as the carbohydrate content is lower in the former and this would be expected to contain acetyl residues due to the presence of *N*-acetylated glucosamine. The results obtained with the method of Ludowieg & Dorfman (1960) are given in Table 1. Ovalbumin gave the expected four acetyl groups/mol., but Fab-fragment II gave only 0.3 mol./mol. in excess of the glucosamine content. This was confirmed both

Table 1. *Content of acetyl groups and hexosamine residues in rabbit IgG fragment-Fab II and ovalbumin*

	Ovalbumin (mol./mol. of mol.wt. 45000)	Rabbit IgG fragment-Fab II (mol./mol. of mol.wt. 42000)
Hexosamine residues	2.8	0.2
Acetyl groups	4.2	0.5

with the *N*-terminal peptides, isolated as described below, and with the whole molecule. It was therefore concluded that the unreactive α -amino groups could not be acetylated and, as the method used would also detect propionyl groups, these too must be absent. The presence of formyl groups was not excluded as the colour yield with the ferric perchlorate-perchloric acid reagent is substantially less than that of either acetyl or propionyl residues. However, *N*-formyl groups are hydrolysed by methanolic hydrochloric acid at room temperature (Waley, 1953) and exposure of the *N*-terminal peptides to these conditions did not expose a free α -amino group. It was therefore unlikely that the α -amino group of the heavy chain was acylated.

Isolation of blocked N-terminal peptides

As no blocking group could be detected an attempt was made to isolate peptides without a free α -amino group. Fully reduced heavy chain was digested with Pronase at pH 8.2 at an enzyme/protein ratio of 1:25 (w/w) at 37° in the cell of a pH-stat for 3 hr., when digestion was complete as judged by the rate of alkali uptake. In a typical experiment 300 mg. of reduced heavy chain was used and it was estimated that about 20% of all peptide bonds were broken. The digest was run through a column (20 cm. $\times$ 1 cm.) of Dowex 50 (X2; H⁺ form) that had been well washed with water. Almost all the peptides were adsorbed and the small fraction coming through was concentrated and fractionated on a Sephadex G-25 column (145 cm. $\times$ 1.4 cm.) in aq. 0.02N-ammonia. The elution diagram (Fig. 1) shows four peaks. The first (*A*) was identified by analysis as the glycopeptide described by Press (1965) and was not examined further. Peak *D* contained no amino acids after hydrolysis and is probably a contaminant from the Dowex 50 column, and this was discarded. Peaks *B* and *C* were further fractionated by electrophoresis on paper at pH 3.5. Three fractions were identified that gave no colour with ninhydrin but stained with the starch-iodide reagent and hence were probably peptides lacking a free α -amino group. In several experiments trace amounts of a component with the same mobility as PCA were also

Fig. 1. Separation of *N*-terminal peptides on a Sephadex G-25 column (145 cm. $\times$ 1.4 cm.) with aq. 0.02N-NH₃ and subsequent electrophoresis on paper at pH 3.5 at 4000 v for 45 min. The mobilities are measured relative to PCA.

Table 2. Yield of amino acids in the combined peaks B and C from fractionation on Sephadex G-25 of a Pronase digest of rabbit IgG heavy chain

Expt. no.	Yield of amino acid (mol./mol. of heavy chain)				
	1	2	3	Mean	4*
Ser	0.56	0.76	0.63	0.65	0.84
Glu	1.40	1.76	1.31	1.49	1.66
Val	0.28	0.40	0.30	0.33	0.34
Leu	0.13	0.09	0.09	0.10	0.15

* In this experiment the Pronase digest was treated with fluorodinitrobenzene in the titrator cell before fractionation.

detected. Peak B contained fractions 2 and 3, and peak C fractions 1 and 2, together with traces of the PCA-like component when this was present. Analysis of the peptides after elution from paper showed that fraction 1 contained equal amounts of serine and glutamic acid, fraction 2 contained only glutamic acid, and fraction 3 contained glutamic acid, serine, valine and leucine in the approximate

molar proportions 2:1:0.8:0.2. As fraction 3 appeared to be a mixture, it was fractionated further by chromatography on paper with pyridine-3-methylbutan-1-ol-water as solvent (Baglioni, 1961), when it resolved into two components with the compositions: fraction 3a, (Glu₂Ser,Val); fraction 3b, (Glu₂Ser,Leu). The recovery of peptides on elution from paper is uncertain and hence the yields of these peptides were calculated from analysis of the combined B and C peaks from the Sephadex column. These are shown in Table 2.

The presence of glutamic acid in all the peptides suggested that the absence of an *N*-terminal amino acid was due to a terminal PCA residue, which might be an artifact formed in the acidic condition on the Dowex 50 column. Hence a further Pronase digestion of reduced heavy chain was carried out and was followed by the immediate addition of fluorodinitrobenzene to the titrator cell to block all free amino groups (Press *et al.* 1966). It had been established previously that under these conditions glutamine did not give any significant amount of PCA (Press *et al.* 1966). The reaction mixture was acidified to pH 2 by the addition of 99% (v/v) formic acid and most of the DNP-peptides were extracted with ethyl acetate and ether. The aqueous phase was passed through a Dowex 50 (X2) resin column and a Sephadex G-25 column, as before, and peaks B and C were again collected. Traces of DNP-peptide were, however, still present and were not satisfactorily separated by electrophoresis on paper at pH 3.5, and so they were removed by passage through a small column (8.5 cm. $\times$ 0.5 cm.) of talc (Sanger, 1949) in *N*-acetic acid. The DNP-peptides, but not other peptides, were retarded. Electrophoresis now gave the same three fractions as before, and on elution and analysis they were found to have the same composition and were present in approximately the same yield. The yield of amino acids from peaks B and C is given in Table 2. Hence it was concluded that these peptides were derived from the *N*-terminal sequence and were not artifacts of the method of isolation.

Amino acid sequence

Fraction 1. The composition was (Glu,Ser) and hydrazinolysis gave serine in 70% yield. Amide determination gave only 0.2 mol. of ammonia/mol. of peptide and hence the sequence was:

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

Fraction 2. This fraction was present in both peaks B and C and contained only glutamic acid after hydrolysis. Amide determination gave 0.46 mol. of ammonia/mol. of glutamic acid. This,

together with its mobility at pH 3.5 and pH 6.5 and its relative size as judged by the order of elution from the Sephadex G-25 column, suggests that its sequence is:

Fraction 3a. The composition was (Glu₂,Ser,Val). Carboxypeptidase A failed to release any amino acid, the peptide being recovered unchanged. Hydrazinolysis gave only glutamic acid, but in poor yield (10%). After incubation with half its weight of Pronase for 5 hr. at 37° the hydrolysate was fractionated on a Dowex 50 column, the peptides with a blocked *N*-terminal group being unadsorbed and others eluted with aq. *N*-ammonia. (A control experiment with twice the weight of Pronase showed that the peptides had not arisen from autolysis.) Electrophoresis at pH 3.5 of the unadsorbed fraction gave only one peptide, in the position of fraction 1, which was found by analysis to be (Glu,Ser). It again gave only serine on hydrazinolysis and hence its sequence was [Glu-Ser].

Electrophoresis at pH 6.5 of the second fraction, eluted by aq. *N*-ammonia, gave a single ninhydrin-positive spot and its composition on elution was (Glu,Val). Edman degradation gave *N*-terminal valine with only glutamic acid in the aqueous phase. Hence the sequence was Val-Glu, and this was confirmed by amide analysis and the mobility at pH 6.5. The sequence of fraction 3a is therefore:

Fraction 3b. Fractions 3a and 3b are indistinguishable except by chromatography on paper in pyridine-3-methylbutan-1-ol-water. It seemed likely therefore that the leucine was replacing the valine and this was confirmed when a Pronase digest gave:

by using the methods described above. The sequence of fraction 3b is therefore:

***N*-Terminal-blocking group.** The complexity of the *N*-terminal peptide has made a study of the blocked α -amino group difficult, but, after the demonstration that PCA was *N*-terminal in the heavy chain of a pathological human IgG (Porter & Press, 1965; Press *et al.* 1966), alkali-lability (Dekker, Stone & Fruton, 1949) was used to decide if PCA was also *N*-terminal in the heavy chain of rabbit IgG.

It was clear that the terminal dipeptide was either [Glu-Ser] or [Glu-Glu(NH₂)], as the tetrapeptide gave rise to [Glu-Ser] on further digestion with Pronase. Hence about 0.25 μ mole of these peptides was dissolved in 0.2 ml. of *N*-sodium hydroxide, kept at room temperature for 24 hr. and

Table 3. *Alkali-lability of the N-terminal blocking agent*

Hydrolysis was measured after 24 hr. at room temperature in *N*-NaOH.

	Hydrolysis of <i>N</i> -terminal bond (%)	Hydrolysis of peptide bond (%)
<i>N</i> -Acetyl-Glu	5	—
PCA-Val	44	11
[Glu-Ser]	34	90
[Glu-Glu(NH ₂)]	50	< 10

then run through a column of Dowex 50 (H⁺ form) and well washed with water. Two fractions were collected: (a) material that has no free amino group and is not adsorbed; (b) material with a free amino group that was adsorbed and subsequently eluted with aq. *N*-ammonia. Controls of *N*-acetylglutamic acid and PCA-Val were treated similarly. The percentage hydrolysis of the blocking group is expressed as the percentage of the glutamic acid in fraction (b) for *N*-acetylglutamic acid, PCA-Val and [Glu-Ser]. The percentage hydrolysis of the peptide bond in the latter two compounds is derived from the relative content in fractions (a) and (b) of peptide and free amino acids.

With the peptide [Glu-Glu(NH₂)] the position is more difficult, but, as hydrolysis of the peptide bond was found to be less than 10%, this was ignored and it was assumed that all the glutamic acid remained as dipeptide in both fractions, and hence the percentage hydrolysis of the blocking group is derived from the ratio of the amounts of glutamic acid in fractions (b) and (a). This gives a maximal value as any hydrolysis of the peptide bond will increase this ratio.

Table 3 shows that the lability of the blocked glutamic acid in both peptides is close to that of PCA-Val and much higher than that of *N*-acetylglutamic acid. It is therefore likely that in the rabbit IgG, as in the pathological human IgG, the absence of a free α -amino group is due to an *N*-terminal PCA residue.

Isolation of an *N*-terminal tryptic peptide. In an attempt to extend the *N*-terminal sequence in the rabbit IgG heavy chain the products of a tryptic digest of oxidized Fd fragment were examined for the presence of peptides that would not react with ninhydrin. A 60 mg. portion of oxidized Fd fragment was digested with 1.2 mg. of trypsin at 40° at pH 8.2 in a pH-stat. Another 1.2 mg. of trypsin was added after 2 hr. Digestion appeared to be complete after 4 hr., when about 13 peptide bonds were broken, as judged by alkali uptake and in agreement with the known content of arginine and lysine. The solution was fractionated on a Sephadex G-50 column in

Table 4. *Electrophoresis of peptides adsorbed on a column of Dowex 50 (H⁺ form) from a Pronase digest of tryptic N-terminal peptide*

The positive mobilities are expressed relative to lysine and the negative mobilities relative to aspartic acid. N-Terminal groups were estimated by the 'dansyl' technique (Gray & Hartley, 1963).

Peptide	Mobility	Composition	N-Terminal group
P2c	-0.68	(Glu,Ser)	Glu
P2d	-0.61	(Glu,Val)	Val
P2e	-0.35	(Glu ₂ ,Ser,Gly ₂ ,Arg)	Glu
P2g	+0.51	(Ser,Gly ₂ ,Arg)	Ser
P2h	+0.69	(Gly ₂ ,Arg)	Gly

N-acetic acid. Four fractions were collected and were subjected to paper electrophoresis. The third fraction gave a component, negative to ninhydrin but positive with the starch-iodide reagent, that moved slightly slower than the neutral amino acids at pH 3.5. It was eluted and on analysis had the composition (Ser 1.8, Glu 3.0, Gly 2.2, Val 1.1, Arg 1.0) and was present in about 0.25 mol. of peptide/mol. of Fd fragment. On digestion with Pronase and fractionation on a column of Dowex 50 (X2; H⁺ form), the water eluate was found to contain two ninhydrin-negative peptides by electrophoresis at pH 3.5, having the same mobility as PCA-Ser and PCA-Ser-Val-Glu, suggesting that the tryptic peptide was an extension of this tetrapeptide isolated from a Pronase digest of the whole heavy chain. The identity of the di- and tetra-PCA-peptides was confirmed as described above. The ninhydrin-positive peptides eluted from the Dowex 50 column with aq. ammonia were also fractionated by electrophoresis at pH 6.5, and Table 4 summarizes the results. If the assumption is made that arginine is the C-terminal amino acid, these peptides and the blocked N-terminal peptide could only be derived from the sequence:

PCA-Ser-Val-Glu-Glu-Ser-Gly-Gly-Arg

The mobilities of peptides P2c, P2d and P2e show that the glutamic residues are not present as amides.

Pronase digest of the heavy chain of rabbit anti-(human serum albumin) IgG and IgG allotype A₁A₅. To find out whether any of the variation of sequence was related to either the allotypic or antibody specificity, the N-terminal peptides were isolated from a Pronase digest of heavy chain from antibody IgG and allotype A₁A₅, as described above, and the analyses of the combined fractions B and C from the Sephadex column are given in Table 5. Qualitatively and quantitatively the N-terminal peptides were similar to those isolated from the inert pooled rabbit IgG heavy chain and hence there appears to

Table 5. *Yield of amino acids in the combined peaks B and C from fractionation on Sephadex G-25 of a Pronase digest of heavy chain from rabbit IgG allotype A₁ and of specific rabbit anti-(human serum albumin) IgG*

	Inert IgG allotype A ₁	Pooled anti-(human serum albumin)	Inert pooled IgG (mean)
Ser	0.63	0.69	0.65
Glu	1.33	1.96	1.49
Val	0.38	0.29	0.33
Leu	0.08	0.17	0.10

be no significant correlation of N-terminal sequence with either property.

DISCUSSION

The evidence for an N-terminal PCA residue in the heavy chain of a pathological human IgG has been given previously (Porter & Press, 1965; Press *et al.* 1966) and, although the position in the rabbit IgG is more complex because of the mixed sequence, the following evidence supports the view that PCA is the terminal residue in this case also: (1) the absence of acetyl, propionyl or formyl groups; (2) the alkali-lability of the blocking agent; (3) the mobility of the N-terminal peptides at pH 3.5 and pH 6.5; (4) the evidence that PCA was not an artifact of isolation procedures.

The yields (mol./mol. of heavy chain) of the peptides contained in the terminal peptide were deduced to be: PCA-Ser-Val-Glu, 0.33; PCA-Ser-Leu-Glu, 0.10; PCA-Ser, 0.22; PCA-Glu(NH₂), 0.21; total, 0.86. As PCA-Ser has been shown to arise from the Pronase digest of the tetrapeptides, this dipeptide may be attributed to these sequences to give the mixed N-terminal sequence in the approximate proportions: PCA-Ser-Val-Glu, 50%, PCA-Ser-Leu-Glu, 20%, PCA-Glu(NH₂), 20%. The fluorodinitrobenzene method always shows the presence of traces of aspartic acid, glutamic acid, serine, threonine, alanine and leucine, with a free α -amino group, none being present in more than 0.05 mol./mol. of heavy chain. These may account for the remaining 10% of terminal peptides, but they are more likely to arise from contamination of the heavy chain with the light chain or slight non-specific hydrolysis during the reaction with the fluorodinitrobenzene or traces of adsorbed peptides.

The recovery of the terminal tryptic nonapeptide was only about 0.25 mol./mol. of heavy chain, whereas the tetrapeptide PCA-Ser-Val-Glu, which arises from the same sequence, is believed to be present in about 50% of the molecules. This might mean that there are further variations from the fifth to the ninth amino acid residue, but as isolation of the tryptic peptide required more extensive

fractionation, including elution from paper, it seems more likely that losses occurred and that, in fact, about half the molecules have the same *N*-terminal sequence for at least the first nine amino acid residues. In the other 50% of the molecules, it is probable that the first arginine or lysine residue occurs later, as no other peptide with a blocked *N*-terminal amino acid was found among the smaller peptides from tryptic hydrolysis.

Though no firm conclusions can be drawn until much longer sequences are established, the evidence available suggests that there is a variability of sequence at the *N*-terminal end of the heavy chain that is unrelated to either the allotypic specificity or the antibody specificity. The amino acid sequence in that part of the Fd fragment that is controlled by the *a* locus should be a single sequence in IgG from a rabbit homozygous at that locus. The relation between antibody specificity and sequence may be more difficult to determine as the isolation of an antibody preparation with a single specificity has not yet been convincingly demonstrated and, even if this is achieved, the assumed relationship of sequence to specificity may not be simple.

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