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Variation in the *N*-Terminal Sequence of Heavy Chains of Immunoglobulin G from Rabbits of Different Allotype

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The sequences of the *N*-terminal peptides prepared by Pronase digestion of the heavy chain of rabbit immunoglobulin G of allotype Aa1, Aa2 and Aa3 were determined and were shown to be related to the allotype. An *N*-terminal fragment of about 34 residues was also prepared from the allotype heavy chains, by cleavage with cyanogen bromide; the yield varied with the allotype. The sequences of the cyanogen bromide fragments from the Aa1 and Aa3 heavy chains contain allotype-related variations similar to those found in the *N*-terminal Pronase peptides, and these sequences are thought to be representative of the whole heavy-chain populations. There is about 60% homology between the two sequences, and superimposed on the differences between them there are a number of positions within each sequence at which at least two amino acids are present.

Two major sets of allotypic specificities have been recognized in rabbit immunoglobulins; these are controlled by two non-linked loci, *a* and *b*, at each of which there are three alleles, Aa1, Aa2 and Aa3, and Ab4, Ab5 and Ab6 respectively (Dray, Dubiski, Kelus, Lennox & Oudin, 1962). The *a* specificities are carried by the Fd fragment of the heavy chains and the *b* specificities are carried by the light chains (Oudin, 1966). The structure of the Fd fragment is of particular interest because it is expected that amino acid-sequence variation in this part of the molecule is responsible for the formation of the antibody combining site (Haber, 1964; Whitney & Tanford, 1965; Porter & Weir, 1966). In any study of the Fd fragment one may thus expect heterogeneity that is due to both antibody and allotypic specificity and possibly also to other causes such as the subclass variants of the heavy chain that may be expected by analogy with the human heavy chain (see Cohen & Milstein, 1967). Accordingly it becomes necessary to define those areas of sequence variation that are related to each source of heterogeneity to obtain an insight into the position and nature of the combining site.

It has been shown (Wilkinson, Press & Porter, 1966) that the *N*-terminal amino acid of rabbit heavy chain is PCA* and that this is followed by a mixed sequence, and also that an *N*-terminal fragment, of molecular weight about 4000, can be prepared in 30% yield from Fd fragment by cleavage with cyanogen bromide (Press, Givol, Piggot, Porter & Wilkinson, 1966*a*); this fragment has at least one of the *N*-terminal sequences of heavy chain. Prahl & Porter (1968) found an allotype-related sequence variation between Aa1 and Aa3 heavy chains and also showed that the *N*-terminal cyanogen bromide fragments from the three allotypes differ considerably in their yield and amino acid composition. The present paper reports a reinvestigation of the *N*-terminal Pronase peptides from the allotype heavy chains that reverses the previous conclusion that they are not allotype-related (Wilkinson *et al.* 1966), and also the sequences of the *N*-terminal cyanogen bromide fragments from Aa1 and Aa3 heavy chains, these showing a number of differences between the two allotypes and also a considerable heterogeneity within each sequence. A preliminary account of this work was given by Wilkinson (1968).

* Abbreviations: PCA, pyrrolid-2-one-5-carboxylic acid; Aec, *S*-β-aminoethylcysteine; Cmc, *S*-carboxymethylcysteine; Hsr, homoserine; Glx, glutamic acid or glutamine; Asx, aspartic acid or asparagine; IgG, immunoglobulin G.

MATERIALS

Rabbit IgG. Sera, pooled from groups of rabbits homozygous for one of the allotypes at the *a* locus, but not the *b* locus, were kindly provided by Dr A. S. Kelus. IgG was prepared by precipitation with 18% (w/v) Na_2SO_4 followed by reprecipitation of the dissolved precipitate with 14% (w/v) Na_2SO_4 (Kekwick, 1940). The IgG was purified by chromatography on DEAE-Sephadex A-50 in 0·07M-sodium phosphate buffer, pH 6·3. The IgG so prepared was free from β-globulins as judged by electrophoresis on cellulose acetate strips.

Other materials. The enzymes used were as described by Press, Piggot & Porter (1966*b*). Dithiothreitol was purchased from Calbiochem (Los Angeles, Calif., U.S.A.) and ethyleneimine from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Anhydrous hydrazine was prepared from hydrazine hydrate (Kusama, 1957).

METHODS

Heavy and light chains of IgG. IgG at a concentration of 50mg./ml. in 0·5M-tris–HCl buffer, pH 8·2, was reduced with 0·01M-dithiothreitol for 1hr. at room temperature, the reduction mixture was then cooled at 0°, and solid iodoacetamide was added to a final concentration of 0·02M. After 1hr. the reaction mixture was dialysed in the cold for 1hr. against 0·15M-NaCl and then overnight against M-propionic acid. The peptide chains were separated on Sephadex G-75 in M-propionic acid by the method of Fleischman, Pain & Porter (1962).

Cyanogen bromide cleavage of heavy chain. This was carried out in aq. 70% (v/v) formic acid for 24hr. at room temperature (Steers, Craven, Anfinsen & Bethune, 1965), with a twofold excess, by weight, of CNBr over heavy chain. Excess of CNBr was removed either by dilution of the reaction mixture tenfold and freeze-drying, or by chromatography on a column of Sephadex G-25 (coarse grade) in 0·3M-acetic acid, the separation being followed with a Uvicord I (LKB-Produkter AB, Stockholm, Sweden). After elution of the protein peak the remainder of the eluate was immediately neutralized by allowing it to flow into 10M-NaOH. The fraction C-1 was isolated by chromatography on Sephadex G-100 in M-acetic acid as described by Givol & Porter (1965).

Total reduction of the C-1 *fragment.* The C-1 fragment was reduced in 6M-guanidine–0·4M-tris–HCl buffer, pH 8·2, at 37° for 3hr. with 0·1M-dithiothreitol. The reduction mixture was cooled to 0° and alkylated for 1hr. either with 0·22M-iodoacetic acid dissolved in sufficient 2M-tris to give pH 8, or with 0·4M-ethyleneimine; in the latter case the pH was maintained at 8 by addition of 6M-HCl. The reaction mixture was then applied directly to the appropriate Sephadex column to separate the fragments (see the Results section).

Analytical methods. High-voltage paper electrophoresis was carried out as described by Crumpton & Wilkinson (1965). Peptides containing no free α-amino group were detected by the hypochlorite–starch–iodide method of Pan & Dutcher (1956) and those containing tyrosine by the 1-nitroso-2-naphthol stain (Smith, 1960).

Amino acid analysis was carried out as described by Wilkinson *et al.* (1966). The dried hydrolysates of peptides containing homoserine were dissolved in 1ml. of pyridine–acetate buffer, pH 6·5, and heated at 100° for 1hr. before being run on the analyser (Ambler, 1965). Also, 10μl. of 0·1M-phenol or 0·1M-thioglycollic acid/ml. of 6M-HCl was added before the hydrolysis of peptides containing tyrosine or alkylated cysteine residues respectively, to minimize destruction of these amino acids during hydrolysis (Sanger & Thompson, 1963).

The 'dansyl'–Edman procedure was used as described by Gray (1967), and the 'dansyl'-amino acids were identified by chromatography on thin layers of silica gel G (E. Merck A.-G., Darmstadt, Germany). In general, plates were developed in the solvent A of Morse & Horecker (1966) followed, after drying, by a second development in ethanol–aq. ammonia (sp.gr. 0·88) (9:1, v/v). Hydrazinolysis was carried out by the method of Bradbury (1956), the free *C*-terminal amino acids being identified with the amino acid analyser.

RESULTS

Blocked N*-terminal peptides from heavy chains of different allotype*

The blocked *N*-terminal peptides from Aa1, Aa2 and Aa3 heavy chains were isolated by the same technique as used by Wilkinson *et al.* (1966). Fully reduced heavy chain, alkylated with iodoacetamide, was digested for 3hr. with Pronase at pH 8·2 at 37° in a pH-stat. The digest was immediately applied to a column (20cm. × 1cm.) of Dowex 50 (X2; H^+ form) and eluted with water. The material that was unretarded by the Dowex 50 was rechromatographed on a column of Sephadex G-25 (140cm. × 1·4cm.) in 0·02M-ammonia, and peptides were located by their E_{215}, the elution patterns in all cases differing only slightly from that reported by Wilkinson *et al.* (1966). The eluate was divided into four fractions, *A*, *B*, *C* and *D*. As with the pooled heavy chain, fraction *A* contained glycopeptide and fractions *B* and *C* contained the *N*-terminal peptides. Fraction *D* did not contain either peptide or amino acid. Samples of fractions *B* and *C* were analysed for their constituent amino acids and these are shown, for each allotype, in Table 1.

Electrophoresis of fraction *B* at pH 3·5 gave two bands, *B*1 and *B*2; re-electrophoresis of band *B*2 at pH 6·5 gave two further bands, *B*2a and *B*2b, these three bands being present in amounts that varied from allotype to allotype. Electrophoresis of fraction *C* at either pH gave only one band, *C*1, and also a small amount of free PCA. Analysis of fraction *C*1 and its mobility showed it to be PCA-

Table 1. *Amounts of amino acids present in fractions B and C from the fractionation on Sephadex G-25 of Pronase digests of heavy chain from rabbits of different allotype*

	Yield of amino acid (moles/mole of heavy chain)		
Allotype	Aa1	Aa2	Aa3
Fraction *B*			
Ser	0·41	0·15	0·25
Glu	0·96	0·61	1·27
Val	0·33	0·09	—
Leu	0·09	0·10	0·24
Fraction *C*			
Ser	0·25	0·37	0·25
Glu	0·49	0·51	0·56

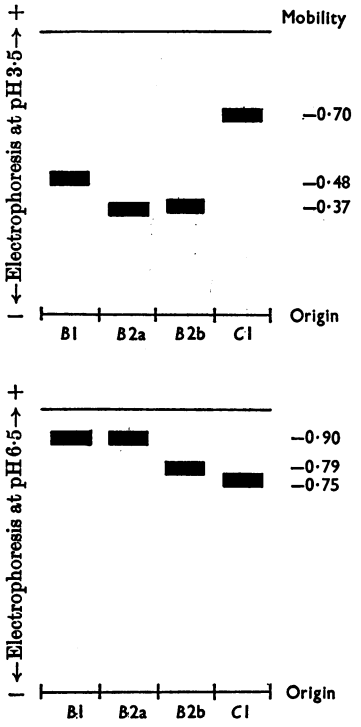

Fig. 1. Electrophoresis on paper of the *N*-terminal Pronase peptides from heavy chains of rabbit IgG of different allotypes at pH3·5 and 6·5. Mobilities are measured relative to PCA.

Ser. The mobilities of the four bands at pH6·5 and 3·5 are shown schematically in Fig. 1.

*Fraction B*1. This peptide was present in digests of Aa2 and Aa3, but not Aa1, heavy chain. It contained only glutamic acid on analysis and corresponded to the peptide previously designated PCA-Gln. As more material was available from the single-allotype preparations than from pooled heavy chain, a more detailed study of its sequence was possible. This was also facilitated by the isolation of the peptic peptide P3a from the Aa3 *N*-terminal cyanogen bromide fragment (see below) whose composition was Glu_3,Leu. Hydrazinolysis of peptide P3a gave *C*-terminal leucine, and digestion with Pronase gave fraction *B*1, showing the sequence of peptide P3a to be PCA-(Glx_2)-Leu. No *C*-terminal amino acid could be isolated from fraction *B*1 by hydrazinolysis, suggesting that glutamine was *C*-terminal. This was confirmed by redigestion of fraction *B*1 with Pronase for a further 5hr., which gave rise, on electrophoresis at pH3·5, to two ninhydrin-negative hypochlorite–starch–iodide-positive bands, one of which was unchanged fraction *B*1 and the other of which had a mobility of −0·65 relative to PCA, and a neutral ninhydrin-positive band, which was shown to be free glutamine by paper chromatography with butan-1-ol–acetic acid–water (12:3:5, by vol.) as solvent. Hydrazinolysis of the more negatively charged ninhydrin-negative band showed glutamic acid to be *C*-terminal. Thus the sequence of fraction *B*1 must be PCA-Glu-Gln.

*Fraction B*2a. This fraction was also present only in digests of Aa2 and Aa3, but not Aa1, heavy chain. The composition of the fraction was $Glu_{3\cdot3}$,$Ser_{0\cdot7}$,-$Leu_{1\cdot0}$. Further digestion with Pronase gave both PCA-Ser and PCA-Glu-Gln and also a ninhydrin-positive band with a mobility of −1·1 relative to aspartic acid, which contained only glutamic acid on analysis and which was in all probability Glu-Glu, but insufficient material was available for further characterization. It is clear from the isolation of the two *N*-terminal peptides that fraction *B*2a is a mixture of two peptides, which from their identical mobilities at pH3·5 and 6·5 must possess the same charge and be the same size. Thus, taking into account the sequence of the Aa3 *N*-terminal fragment (Fig. 4), fraction *B*2a is probably a mixture of PCA-Ser-Leu-Glu-Glu and PCA-Glu-Gln-Leu-Glu.

*Fraction B*2b. This fraction was present in digests of all three allotype heavy chains. The composition varied, however, being Glu_2,Ser,$Val_{0\cdot8}$,$Leu_{0\cdot2}$ for allotype Aa1, Glu_2,Ser,$Val_{0\cdot4}$,$Leu_{0\cdot6}$ for allotype Aa2 and Glu_2,Ser,Leu for allotype Aa3. All the fractions gave PCA-Ser on further digestion with Pronase and also a negatively charged ninhydrin-positive band that was shown to be either Val-Glu or Leu-Glu or a mixture of the two. Thus fraction *B*2b corresponds to the tetrapeptides isolated by Wilkinson *et al.* (1966) from pooled heavy chain and is a mixture of PCA-Ser-Val-Glu and PCA-Ser-Leu-Glu in proportions that vary according to the allotypic specificity of the heavy chain.

It is clear that all the *N*-terminal peptides in fractions *B* and *C* are related either to the tripeptide *B*1 or to one of the tetrapeptides of fraction *B*2b. In arriving at a quantitative estimate of the amount of each sequence present in any one allotype heavy chain it has been assumed that the PCA-Ser present in fraction *C* derives from the two tetrapeptides in the same proportion as they are present in fraction *B*2b. A further difficulty is the presence, in Aa2 and Aa3 heavy-chain digests, of the fraction *B*2a peptides; as these are present in rather small quantities and arise almost equally from the two *N*-terminal sequences, PCA-Ser-Leu-Glu and PCA-Glu-Gln, the further assumption has been made that all the amino acids present in fraction *B* are accounted for by the tri- and tetra-peptides.

The amounts of each peptide present in each allotype heavy chain, together with the total recoveries, are given in Table 2.

Table 2. *Distribution of N-terminal Pronase peptides from the heavy chain of IgG from rabbits of different allotype*

Peptide Allotype ...	Distribution (moles of peptide/mole of heavy chain) Aa1	Aa2	Aa3
PCA-Ser-Val-Glu	0·56	0·30	—
PCA-Ser-Leu-Glu	0·14	0·30	0·50
PCA-Glu-Gln	—	0·07	0·26
Total	0·70	0·67	0·76

N-*Terminal cyanogen bromide fragments from heavy chain*

The *N*-terminal cyanogen bromide fragment was initially prepared from pooled heavy chain by cleavage of the Fd fragment with cyanogen bromide followed by reduction and alkylation (Press *et al.* 1966*a*). In subsequent preparations and in the preparation of the fragments from the Aa1 and Aa3 allotype heavy chains, the cyanogen bromide fragment C-1 of heavy chain (Givol & Porter, 1965) was used, owing to its ease of preparation. In either case the *N*-terminal fragment was separated from other fragments after complete reduction and alkylation, by chromatography on a column of Sephadex G-50 (225cm. × 1·8cm.) in 0·05M-ammonia, a typical elution pattern being shown in Fig. 2. In most preparations iodoacetic acid was used as the alkylating agent. In some cases, however, ethyleneimine was used to provide an extra point of tryptic cleavage (Raftery & Cole, 1963); in these preparations it was necessary to separate the *N*-terminal fragment on a column of Sephadex G-100 (62cm. × 3·0cm.) in M-acetic acid before purification on Sephadex G-50 in 0·05M-ammonia, as the aminoethylated fraction C-1 was insoluble in 0·05M-ammonia.

The yield of the *N*-terminal fragment from pooled heavy chain by either method was about 30%, as reported by Press *et al.* (1966*a*). The yields obtained from the Aa1 and Aa3 allotype heavy chains, 42% and 20% respectively, were in agreement with those reported by Prahl & Porter (1968). As the yield from the Aa2 heavy chain was so small no attempt was made to prepare and investigate this fragment. The compositions of the fragments from the allotype heavy chains are given in Table 3; these are the means of several determinations and differ in a few instances from those given by Prahl & Porter (1968). On digestion of the Aa1 and Aa3 fragments with Pronase and isolation of the peptides containing no free α-amino group on a small column (9·0cm. × 0·5cm.) of Dowex 50 (X2; H^+ form), the peptides expected from the

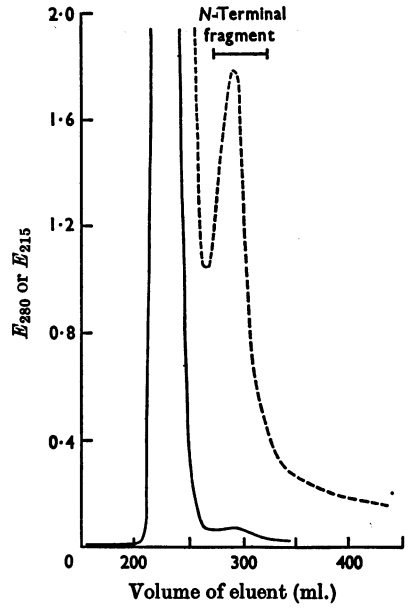

Fig. 2. Fractionation of the *N*-terminal fragment from a CNBr digest of the C-1 fragment from Aa1 or Aa3 heavy chain on a column (225cm. × 1·8cm.) of Sephadex G-50 in 0·05M-ammonia. ——, E_{280}; – – – –, E_{215}.

Table 3. *Amino acid composition of the N-terminal cyanogen bromide fragments isolated from Aa1 and Aa3 allotype heavy chains*

Amino acid Allotype ...	Composition (moles of amino acid/mole of fragment) Aa1	Aa3
Lys	0·0	1·0
Arg	1·0	0·2
Asp	0·7	1·6
Thr	5·1	2·9
Ser	5·1	4·9
Glu	3·0	3·9
Pro	2·4	1·4
Gly	4·6	4·8
Ala	0·9	1·7
Val	2·3	1·6
Ile	0·3	0·3
Leu	4·5	3·8
Tyr	1·0	1·4
Phe	1·0	1·6
Cmc	0·9	0·8
Hsr	1·1	0·9
Total	33·9	32·8

Pronase digest of the whole Aa1 and Aa3 heavy chains were isolated; these peptides were present in similar relative amounts to those from whole heavy chain.

Initial sequence work was carried out with the fragment obtained from pooled heavy chain, but after isolation of the fragments from Aa1 and Aa3 heavy chains it became obvious that this contained predominantly the Aa1 fragment with the addition of a small amount of Aa3 material. This was to be expected in the light of the higher yield of this fragment from allotype Aa1 than from Aa3 obtained by Prahl & Porter (1968) and the relative amounts of allotype heavy chains in the pooled serum used, which were determined to be 56%, 10% and 34% for Aa1, Aa2 and Aa3 respectively (J. Oudin, personal communication).

Thus the sequence of the Aa1 fragment was determined mainly from peptides isolated from the pooled fragment. This was confirmed by the isolation of these peptides from the Aa1 fragment, particular note being made of the composition of those peptides, T2a, C2a and P3b, in which less than molar amounts of certain amino acids were found. In these cases the same amounts were found in peptides isolated from the Aa1 fragment as in those from the pooled fragment.

The sequences of the two fragments were determined by studying the peptides produced by digestion of the aminoethylated fragments with trypsin and the carboxymethylated fragments with chymotrypsin and pepsin. In general digests were made with about 5mg. of *N*-terminal fragment and an enzyme/substrate ratio 1:50 (by wt.). Tryptic and chymotryptic digests were performed at pH 8·4 at 37° for 4hr., the pH being adjusted with M-ammonia. Peptic digests were performed at pH 2·0 at 37° for 3hr., the pH being adjusted with M-hydrochloric acid. All digests were fractionated initially on a column of Sephadex G-50 (142cm. × 1·7cm.), in 0·05M-ammonia followed by electrophoresis at pH 6·5 or 3·5.

Sequence of the Aa1 N-terminal fragment

The compositions of the peptides from which the sequence was deduced are shown in Tables 4, 5 and 6, and the sequence results on them are given below. The sequence is shown in Fig. 3.

Trypsin. (*a*) Peptide T2a. This peptide was ninhydrin-negative but stained with the hypochlorite–starch–iodide reagent. Its composition was $Ser_2,Glu_3,Gly_2,Val_{0\cdot8},Leu_{0\cdot2},Arg$, and it was identical with the *N*-terminal tryptic peptide isolated by Wilkinson *et al.* (1966), except that it contained non-integral amounts of valine and leucine, these being in the same ratio as in the *N*-terminal Pronase tetrapeptide *B*2b isolated from Aa1 heavy chain. Thus the sequence is PCA-Ser-$\frac{Val}{Leu}$-Glu-Glu-Ser-Gly-Gly-Arg.

(*b*) Peptide T2c. The composition was Thr_4,Pro_2,-Gly,Val,Leu_3,Aec). Digestion with chymotrypsin gave two positively charged peptides on electrophoresis at pH 6·5 with the compositions Thr_2,Leu,Aec and Thr,Aec. 'Dansylation' of both peptides gave *N*-terminal threonine and, with the assumption that aminoethylcysteine is *C*-terminal, the *C*-terminal sequence of the peptide must be -Thr-Leu-Thr-Aec. The *N*-terminal sequence was determined by five steps of the 'dansyl'–Edman technique and shown to be Leu-Val-Thr-Pro-Thr-. Thus the partial sequence of the peptide is Leu-Val-Thr-Pro-Thr-(Pro,Gly,Leu)-Thr-Leu-Thr-Aec.

Table 4. *Amino acid composition of the tryptic peptides from the Aa1 N-terminal fragment*

Mobility was measured relative to aspartic acid = −1 or lysine = +1.

Amino acid Peptide ...	Composition (moles of amino acid/mole of peptide) T2a	T2c
Arg	0·9	—
Thr	—	3·5
Ser	2·1	—
Glu	2·8	—
Pro	—	2·2
Gly	2·2	1·1
Val	0·8	1·0
Leu	0·2	2·9
Aec	—	0·5
Mobility at pH 6·5	−0·55	+0·30
Ninhydrin colour	None	Blue

Table 5. *Amino acid composition of the major chymotryptic peptides from the Aa1 N-terminal fragment*

Mobility was measured relative to aspartic acid = −1; N, neutral.

Amino acid Peptide ...	Composition (moles of amino acid/mole of peptide) C1b	C2a	C4a	C4b	C4d
Arg	0·9	—	—	—	—
Asp	—	—	1·0	—	—
Thr	2·0	2·7	—	—	—
Ser	2·1	1·0	—	2·8	—
Glu	2·9	—	—	—	—
Pro	2·3	—	—	—	—
Gly	3·1	1·0	—	—	—
Ala	—	0·4	—	—	1·0
Val	1·8	0·6	—	—	—
Leu	2·1	1·0	—	1·0	—
Tyr	—	—	—	1·0	—
Phe	—	—	—	—	—
Cmc	—	0·8	—	—	—
Hsr	—	—	1·0	—	1·0
Mobility at pH 6·5	−0·36	−0·30	0·71	N	N
Ninhydrin colour	None	Yellow	Blue	Blue	Blue

Table 6. *Amino acid composition of the major peptic peptides from the Aa1 N-terminal fragment*

Mobility was measured relative to aspartic acid = −1; N, neutral.

Amino acid Peptide ...	Composition (moles of amino acid/mole of peptide) P3b	P5d	P5f
Thr	1·5	—	—
Ser	1·2	3·1	—
Gly	1·1	—	—
Ala	0·2	—	1·0
Val	0·8	—	—
Leu	—	0·9	—
Tyr	—	1·0	—
Phe	—	1·0	—
Cmc	0·7	—	—
Hsr	—	—	0·9
Mobility at pH 6·5	−0·38	N	N
Ninhydrin colour	Yellow	Blue	Blue

Chymotrypsin. (*a*) Peptide C1b. The composition was $Arg,Thr_2,Ser_2,Glu_3,Pro_2,Gly_3,Val_2,Leu_2$. Digestion with trypsin gave rise to the *N*-terminal peptide, T2a, and hydrazinolysis of peptide C1b showed leucine to be *C*-terminal. On digestion with carboxypeptidase A for 16 hr. at pH 8 at 37° and isolation of the residual peptide by electrophoresis at pH 6·5, it was shown that only leucine had been removed, and hydrazinolysis of this peptide showed glycine to be the penultimate residue at the *C*-terminus of peptide C1b. As peptides C1b and T2c contain the only two proline residues in the fragment, peptide C1b must overlap peptides T2a and T2c, which must be adjacent, and its sequence must be PCA-Ser-$\frac{Val}{Leu}$-Glu-Glu-Ser-Gly-Gly-Arg-Leu-Val-Thr-Pro-Thr-Pro-Gly-Leu.

(*b*) Peptide C2a. The composition was Thr_3,-Ser,Gly,$Ala_{0\cdot4}$,$Val_{0\cdot6}$,Leu,Phe,Cmc. 'Dansylation' showed threonine to be *N*-terminal and hydrazinolysis gave *C*-terminal phenylalanine. Thus the partial sequence is Thr-(Leu,Thr,Cmc,Thr,$\frac{Val}{Ala}$,Ser,-Gly)-Phe.

(*c*) Peptide C4b. The composition was Ser_3,Leu,-Tyr. It was neutral at pH 6·5 and was purified by electrophoresis at pH 3·5. It stained positive with the 1-nitroso-2-naphthol reagent for tyrosine. The *N*-terminal sequence was shown to be Ser-Leu- by the 'dansyl'–Edman technique and the *C*-terminal amino acid was shown to be tyrosine by hydrazinolysis. Analysis of the peptide after the second Edman step gave a composition of Ser_2,Tyr. Thus the sequence is Ser-Leu-Ser-Ser-Tyr.

(*d*) Peptides C4a and C4d. The compositions were

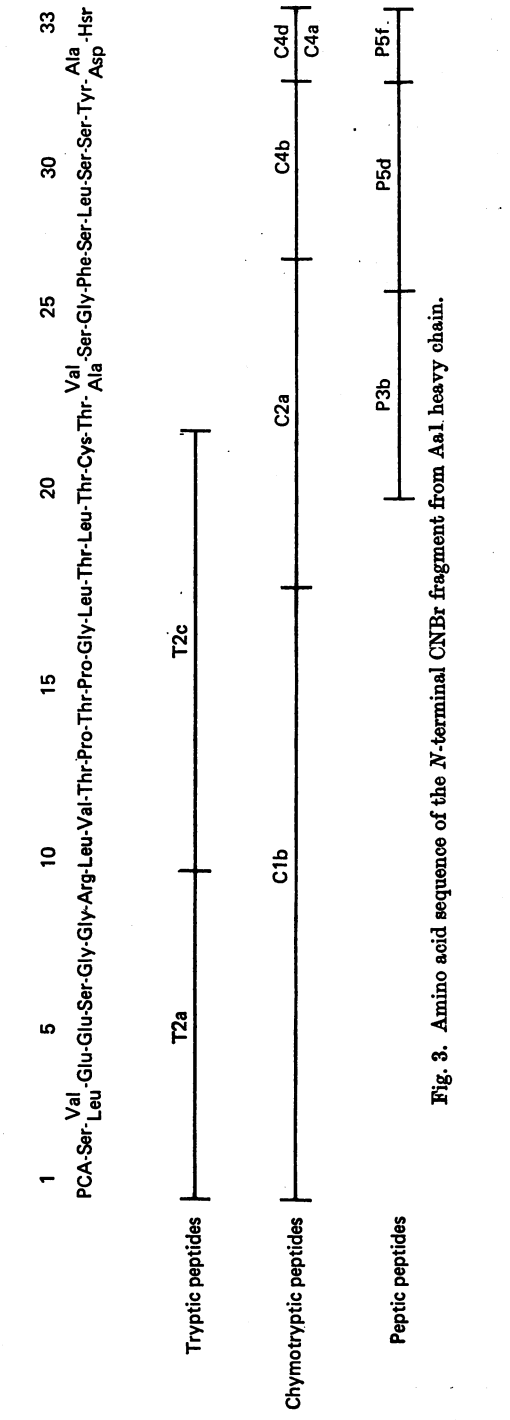

Fig. 3. Amino acid sequence of the *N*-terminal CNBr fragment from Aa1 heavy chain.

Asp,Hsr and Ala,Hsr respectively. Peptide C4a was negatively charged at pH 6·5 and thus must contain aspartic acid. These, together with peptide P5f, were the only peptides isolated that contained homoserine, and must therefore both be *C*-terminal, alanine and aspartic acid being variants at position 32 in the Aa1 sequence. The *C*-terminal sequence of the fragment is therefore -$\frac{\text{Ala}}{\text{Asp}}$-Hsr.

Pepsin. (*a*) Peptide P3b. The composition was Thr_2,Ser,Gly,$Ala_{0\cdot2}$,$Val_{0\cdot8}$,Cmc. Hydrazinolysis gave *C*-terminal glycine and 'dansylation' showed threonine to be *N*-terminal, but after one step of the Edman degradation no new *N*-terminal amino acid was found by 'dansylation'. Consequently an analysis was made of the peptide after two Edman steps, giving the composition (Thr,$Val_{0\cdot8}$,$Ala_{0\cdot2}$,Ser,-Gly); 'dansylation' of this peptide gave threonine, and after a third Edman step, valine, with a small amount of alanine in addition, was obtained. The sequence of the peptide must be thus Thr-Cmc-Thr-$\frac{\text{Val}}{\text{Ala}}$-Ser-Gly. As this peptide contained a residue of *S*-carboxymethylcysteine it must overlap peptide T2c and also be part of peptide C2a.

(*b*) Peptide P5d. The composition was Ser_3,Leu,-Tyr,Phe. 'Dansylation' showed phenylalanine to be *N*-terminal and hydrazinolysis gave *C*-terminal tyrosine. Thus the partial sequence is Phe-(Ser,Leu,Ser,Ser)-Tyr. This peptide overlaps peptides C2a and C4b and completes the sequence to residue 31.

It has not proved possible to isolate a peptide overlapping peptides C4b, C4a and C4d. A search was made for the presumed *C*-terminal tryptic peptide of the aminoethylated fragment, but without success. However, as the sequence proposed in Fig. 3 accounts for all the amino acids found by analysis, it seems most likely that the *C*-terminal sequence is -Tyr-$\frac{\text{Ala}}{\text{Asp}}$-Hsr.

Sequence of the Aa3 N-terminal fragment

The composition of the peptides from which the sequence was deduced are shown in Tables 7, 8 and 9, and the sequence results on them are given below. The sequence is shown in Fig. 4.

Trypsin. (*a*) Peptide T2a. The composition was $Asp_{0\cdot7}$,Thr_2,Ser_3,Glu_5, Pro,Gly_4,$Val_{1\cdot7}$, Leu_4,Aec. The peptide was faintly positive with ninhydrin but stained heavily with the hypochlorite–starch–iodide reagent. Digestion with chymotrypsin gave a ninhydrin-negative peptide on electrophoresis at pH 6·5, identical with peptide C1a, and a positively charged peptide identical with the peptide Thr-Leu-Thr-Aec isolated by chymotryptic digestion of peptide T2b. Digestion of peptide T2a with pepsin gave peptides P2a, P3a and P4a after electrophoresis at pH 6·5; peptide P3a, however, was present in greater amounts than peptide P4a and only a trace of peptide P2a was present. The peptide Leu-Thr-Leu-Thr-Aec, isolated by peptic digestion of peptide T2b, was also isolated. Thus peptide T2a is *N*-terminal and extends to the half-cystine residue; it is very similar to peptide T2b, the main differences

Table 7. *Amino acid composition of the major tryptic peptides from the Aa3 N-terminal fragment*

Mobility was measured relative to aspartic acid = −1; N, neutral.

Amino acid	Peptide ...	Composition (moles of amino acid/mole of peptide) T2a	T2b	T5
Lys		—	0·9	—
Asp		0·7	0·9	1·0
Thr		2·0	2·2	1·1
Ser		2·8	3·0	3·1
Glu		5·1	4·2	—
Pro		1·1	0·9	—
Gly		4·1	4·0	1·7
Ala		—	0·9	1·3
Val		1·7	1·2	—
Leu		4·0	4·0	—
Tyr		—	—	1·3
Phe		—	—	1·7
Aec		0·5	0·8	—
Hsr		—	—	1·0
Mobility at pH 6·5		−0·48	−0·34	N
Ninhydrin colour		Blue	Blue	Yellow

Table 8. *Amino acid composition of the major chymotryptic peptides from the Aa3 N-terminal fragment*

Mobility was measured relative to aspartic acid = −1; N, neutral.

Amino acid	Peptide ...	Composition (moles of amino acid/mole of peptide) C1a	C1b	C3a	C5a
Lys		—	1·0	—	—
Asp		—	1·0	—	—
Thr		—	—	1·6	—
Ser		1·4	2·8	1·0	2·0
Glu		3·2	3·5	—	—
Pro		—	1·1	—	—
Gly		2·5	3·4	1·1	—
Ala		—	0·9	1·0	—
Val		0·9	1·1	—	—
Leu		1·9	3·0	—	—
Tyr		—	—	—	1·1
Phe		—	—	1·0	0·8
Cmc		—	—	0·9	—
Mobility at pH 6·5		−0·66	−0·58	−0·46	N
Ninhydrin colour		None	Blue	Yellow	Blue

Table 9. *Amino acid composition of the major peptic peptides from the Aa3 N-terminal fragment*

Mobility was measured relative to aspartic acid = −1 or lysine = +1; N, neutral.

Amino acid	Peptide ...	Composition (moles of amino acid/mole of peptide) P2a	P2c	P3a	P4a	P4b	P4Na	P4Nb	P5b
Lys		—	1·0	—	—	1·0	—	—	—
Asp		1·0	0·9	—	—	—	—	—	—
Thr		—	—	—	—	—	—	1·0	—
Ser		1·1	2·1	—	1·1	1·1	1·8	—	—
Glu		2·0	2·1	3·1	1·0	—	—	—	—
Pro		—	1·0	—	—	0·9	—	—	—
Gly		2·2	3·2	—	—	1·3	—	—	—
Ala		—	1·0	—	—	1·0	—	—	—
Val		—	1·1	—	—	1·0	—	—	—
Leu		—	1·2	1·0	0·9	1·2	—	2·0	—
Tyr		—	—	—	—	—	0·4	—	1·0
Phe		—	—	—	—	—	0·6	—	—
Hsr		—	—	—	—	—	—	—	1·0
Mobility at pH 6·5		−1·0	−0·48	−0·86	−0·65	+0·45	N	N	N
Ninhydrin colour		Blue	Blue	None	None	Blue	Blue	Blue	Blue

being the higher proportion of PCA-Glu-Gln at the *N*-terminal end and valine at position 10, and the lack of lysine and alanine. It was only possible to isolate this peptide in small amounts, and insufficient was available to identify the replacements for the lysine and alanine residues in peptide T2b.

(*b*) Peptide T2b. The composition was Lys,Asp,-Thr_2,Ser_3,Glu_4,Pro,Gly_4,Ala,Val,Leu_4,Aec. Digestion with chymotrypsin followed by electrophoresis at pH 6·5 gave a negatively charged peptide identical with peptide C1b and two positively charged peptides whose compositions were Thr_2,Leu,Aec and Thr,Aec: 'dansylation' showed threonine to be *N*-terminal in both peptides, and leucine was shown to be the second amino acid from the *N*-terminus in the former peptide by 'dansylation' after one step of the Edman degradation. Thus the *C*-terminal sequence must be -Thr-Leu-Thr-Aec. Digestion of peptide T2b with pepsin gave rise to peptide bands, on electrophoresis at pH 6·5, corresponding to peptides P2a, P2c, P3a, P4a and P4b, peptide P4a being present in relatively greater amounts than peptide P3a. Thus peptide T2b represents the major part of the sequence of the *N*-terminal fragment of Aa3 heavy chain up to and including the half-cystine residue.

(*c*) Peptide T5. The composition was Asp,Thr,-Ser_3,$Gly_{1\cdot7}$,$Ala_{1\cdot3}$,$Tyr_{1\cdot3}$,$Phe_{1\cdot7}$,Hsr. This peptide was isolated in an essentially pure form after chromatography on Sephadex G-50. It was neutral on electrophoresis at pH 6·5 and hence the aspartic acid must be present as asparagine. As it contains homoserine it must be the *C*-terminal peptide of the fragment and together with peptides T2a and T2b accounts for all the amino acids found by analysis. The *N*-terminal sequence was shown to be Thr-Ala-by the 'dansyl'–Edman technique. After five steps of the Edman degradation, asparagine was shown to be *N*-terminal by 'dansylation', but further degradation was blocked, possibly by an α-aspartyl–β-aspartyl rearrangement (Naughton, Sanger, Hartley & Shaw, 1960). Analysis of the residual peptide gave the composition $Asp_{0\cdot8}$,Ser_2,$Gly_{0\cdot7}$,-$Ala_{0\cdot4}$,$Tyr_{1\cdot2}$,$Phe_{0\cdot8}$,Hsr; thus the partial sequence must be Thr-Ala-(Ser,Gly,Phe)-Asn-$\left(\begin{matrix}Gly\\Ala\end{matrix}\right)$,Ser,Ser,-$\left(\begin{matrix}Phe\\Tyr\end{matrix}\right)$,Tyr)-Hsr.

. *Chymotrypsin.* (*a*) Peptide C1a. The composition was $Ser_{1\cdot4}$,$Glu_{3\cdot2}$,$Gly_{2\cdot5}$,Val,Leu_2. This peptide was ninhydrin-negative but stained with the hypochlorite–starch–iodide reagent. No *C*-terminal amino acid was found by hydrazinolysis, but after digestion for 3 hr. with carboxypeptidase A leucine was released in good yield with a small amount of valine in addition; thus the *C*-terminal sequence is probably -Val-Leu. Digestion of peptide C1a with pepsin gave rise, as expected, to peptides P3a and P4a on electrophoresis at pH 6·5, and also to a negatively charged ninhydrin-positive band whose composition on analysis was Ser,Glu_2,Gly_2,Val,Leu; there was, however, insufficient material remaining to investigate it further.

(*b*) Peptide C1b. The composition was Lys,Asp,-Ser_3,$Glu_{3\cdot5}$,Pro,Gly_3,Ala,Val,Leu_3. Digestion with carboxypeptidase A for 3 hr. released only leucine. Digestion with pepsin followed by electrophoresis at pH 6·5 gave the expected bands corresponding to peptides P2a, P3a, P4a, P4b and P2c.

(*c*) Peptide C3a. The composition was Thr_2,Ser,-Gly,Ala,Phe,Cmc. Hydrazinolysis gave *C*-terminal phenylalanine, and 'dansylation' showed threonine

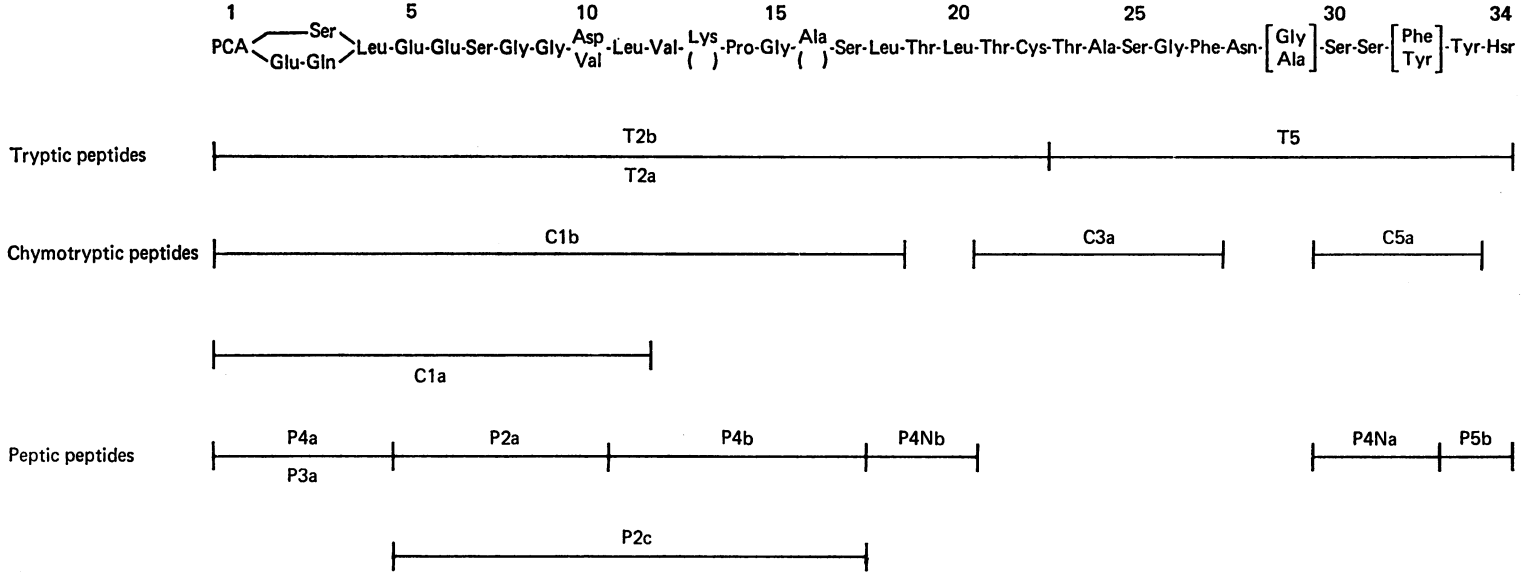

Fig. 4. Amino acid sequence of the *N*-terminal CNBr fragment from Aa3 heavy chain. The peptides whose symbols appear below the lines are those that contain the bottom one of the two variants at any variable position in the sequence. The bar at position 2 indicates a presumed deletion. The empty parentheses at position 13 and 16 indicate unidentified variants, and the square brackets at positions 29 and 32 indicate some uncertainty in the variants at these positions (see the text).

to be *N*-terminal. Analysis of the residual peptide after two Edman steps gave the composition Thr,Ser,Gly,Ala,Phe, showing the *N*-terminal sequence to be Thr-Cmc-. Three further steps of the 'dansyl'–Edman procedure showed the sequence to be Thr-Cmc-Thr-Ala-Ser-Gly-Phe. This peptide thus provides an overlap between peptides T2b and T5.

(*d*) Peptide C5a. The composition was Ser_2,-$Tyr_{1\cdot1}$,$Phe_{0\cdot8}$. 'Dansylation' gave *N*-terminal serine, and hydrazinolysis showed tyrosine to be *C*-terminal. Thus the partial sequence is Ser-(Ser,$^{Phe}_{Tyr}$)-Tyr.

Pepsin. (*a*) Peptide P2a. The composition was Asp,Ser,Glu_2,Gly_2. Aspartic acid was found to be *C*-terminal by hydrazinolysis. The *N*-terminal sequence was shown to be Glx-Glx-Ser- by three steps of the 'dansyl'–Edman procedure. After the first Edman degradation the mobility of the peptide was −0·85 and after the second step −0·62; thus all the acidic amino acid side chains must have free carboxyl groups and the sequence is Glu-Glu-Ser-Gly-Gly-Asp.

(*b*) Peptide P2c. The composition was Lys,Asp,-Ser_2,Glu_2,Pro,Gly_3,Ala,Val,Leu. Eight steps of the 'dansyl'–Edman procedure showed the *N*-terminal sequence to be Glx-Glx-Ser-Gly-Gly-Asx-Leu-Val-. Peptide P2a is clearly part of this peptide. The sequence is Glu-Glu-Ser-Gly-Gly-Asp-Leu-Val-(Lys,Pro,Gly,Ala,Ser).

(*c*) Peptide P3a. The composition was Glu_3,Leu. The sequence of this peptide has been discussed above and is PCA-Glu-Gln-Leu.

(*d*) Peptide P4a. The composition was Ser,Glu,-Leu; the peptide was ninhydrin-negative. Hydrazinolysis showed leucine to be *C*-terminal. Digestion with Pronase followed by electrophoresis at pH 6·5 gave PCA-Ser and a neutral ninhydrin-positive band. Thus the sequence must be PCA-Ser-Leu. Thus both peptide P3a and peptide P4a must be *N*-terminal in the Aa3 fragment, giving a mixed *N*-terminal sequence, as found in the whole Aa3 heavy chain.

(*e*) Peptide P4b. The composition was Lys,Ser,-Pro,Gly,Ala,Val,Leu. Hydrazinolysis showed serine to be *C*-terminal. Five steps of the 'dansyl'–Edman procedure showed the *N*-terminal sequence to be Leu-Val-Lys-Pro-Gly-. Thus the sequence is Leu-Val-Lys-Pro-Gly-Ala-Ser. This must be the *C*-terminal sequence of peptide P2c.

(*f*) Peptide P4Na. The composition was Ser_2,$Tyr_{0\cdot4}$,$Phe_{0\cdot6}$. 'Dansylation' showed serine to be *N*-terminal and hydrazinolysis gave both phenylalanine and tyrosine in the same ratio as they were found in the original peptide. Thus the sequence is Ser-Ser-$^{Phe}_{Tyr}$.

(*g*) Peptide P4Nb. The composition was Thr,Leu_2. Two steps of the 'dansyl'–Edman procedure showed the sequence to be Leu-Thr-Leu.

(*h*) Peptide P5b. The composition was Tyr,Hsr. The peptide was isolated by electrophoresis at pH 3·5 after treatment with anhydrous trifluoroacetic acid for 1 hr. at room temperature, under which conditions it was positively charged. As it contains homoserine it must be *C*-terminal in the fragment and have the sequence Tyr-Hsr. It must thus be adjacent to peptide P4Na, these two being overlapped by peptide C5a.

Tryptic digestion of heavy chains of different allotype

Heavy chain of all three allotypes was digested with trypsin to determine whether the *N*-terminal peptide PCA-Ser-$^{Val}_{Leu}$-Glu-Glu-Ser-Gly-Gly-Arg was present or not. About 50 mg. of heavy chain was fully reduced and alkylated with ethyleneimine, to give a soluble preparation, and the ϵ-amino groups of lysine and aminoethylcysteine were blocked with maleic anhydride (Butler, Harris, Hartley & Leberman, 1967) to minimize the number of small tryptic peptides produced. The heavy chains were digested for 4 hr. at pH 8·2 at 37° with 2% (by wt.) of trypsin. The digest, which was completely soluble, was chromatographed on a column (142 cm. × 1·7 cm.) of Sephadex G-50 in 0·05 M-ammonia. The fraction known to contain the *N*-terminal nonapeptide was evaporated to dryness *in vacuo* and the maleyl groups were removed by incubation with pyridine–acetate buffer, pH 3·5, for 16 hr. at 60°. The amount of *N*-terminal peptide present was then assessed by electrophoresis at pH 6·5. The peptide was obtained in good yield from Aa1 heavy chain, the yield from Aa2 heavy chain was about half that from Aa1, and it was absent from the digest of Aa3 heavy chain.

DISCUSSION

The sequences of the *N*-terminal Pronase peptides from heavy chains from IgG of rabbits homozygous for one of the alleles at the *a* locus have been determined, as have the sequences of the *N*-terminal cyanogen bromide fragments from Aa1 and Aa3 heavy chains. Originally Wilkinson *et al.* (1966) concluded, from investigation of the *N*-terminal sequence of pooled and Aa1 heavy chain, that the *N*-terminal sequence was not allotype-related. It is now clear that this result was due to the fact that the Aa1 allotype is the major component of the pooled heavy chain used for the original experiments, and hence no difference from the Aa1 sequence could be found. The *N*-terminal sequence is indeed allotype-related, although not

in any simple way. This allotype-related sequence difference has been shown to be present also in the sequence of the *N*-terminal 34 residues of the Aa1 and Aa3 heavy chain.

Press *et al.* (1966*a*) reported that the *N*-terminal fragment was obtained from only about 30% of the molecules in a pooled heavy-chain preparation, and this was confirmed by Prahl & Porter (1968), who found that the fragment was obtained in different and less than molar amounts from each allotype heavy chain. Koshland (1967) reported that the *N*-terminal fragments were obtained in molar yields from some preparations of Aa1 and Aa3 heavy chains; the reason for this difference in results is not known, but it may be a characteristic of the different populations of rabbits used.

The determination of the *N*-terminal sequence of only a part of the heavy-chain population raises the question whether this is representative of the whole. The finding of the *N*-terminal Pronase peptides in approximately the same yield from both the heavy chain and the *N*-terminal cyanogen bromide fragments and the fact that the *N*-terminal tryptic nonapeptide PCA-Ser-$\frac{\text{Val}}{\text{Leu}}$-Glu-Glu-Ser-Gly-Gly-Arg is present in good yield from Aa1 heavy chain but absent from Aa3 heavy chain suggests that this is so, but final confirmation must await the isolation of peptides containing the replacement for methionine at position 34.

In Fig. 5 the two rabbit sequences are compared with one another; it is necessary to postulate a deletion at position 2 in the Aa1 sequence and in one of the variants of the Aa3 sequence to obtain the maximum homology. There are 13 differences between the two sequences and at ten of these there are subvariants in one or the other; these are at positions 4, 24 and 33 in the Aa1 sequence and at positions 2, 3, 10, 29, 32 and probably also at 13 and 16 in the Aa3 sequence. The presence of these variable positions led to considerable difficulties in the elucidation of the sequences; thus if the two amino acids at one position differ in charge or susceptibility to enzyme digestion they will not be isolated together and the minor variant may be missed; indeed, Porter (1967) pointed out that fractional residues present in less than 20% of the molecules are unlikely to be detected by the techniques used. In the Aa1 heavy chain it was possible to show the presence of two amino acids at a single position with all three variants; but in the Aa3 sequence the assignment of variants to positions 29 and 33 depends mainly on the analysis of the peptides isolated, and at positions 13 and 16 it has not been possible to locate the replacements owing to the very low yields of peptide T2a.

The ratios of the amounts of the variant amino acids within each sequence vary from 4:1 to 1:1,

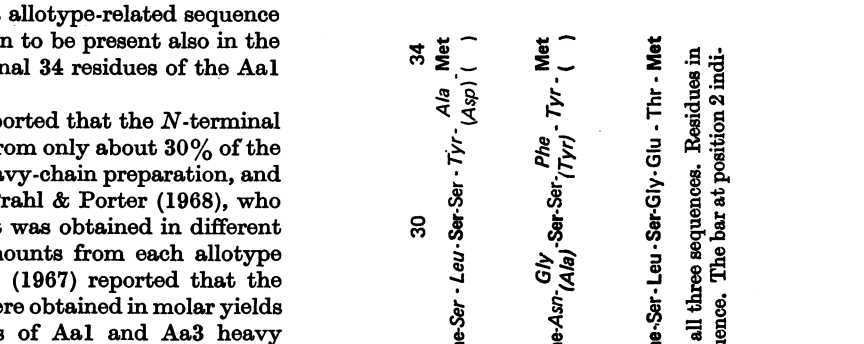

Fig. 5. *N*-Terminal sequences of heavy chains from rabbit and man. Amino acid residues shown in **bold type** are common to all three sequences. Residues in *italics* are those that vary between the rabbit Aa1 and Aa3 sequences, and those in parentheses are minor variants in each sequence. The bar at position 2 indicates a presumed deletion. The human γ1 (Daw) sequence is taken from Piggot & Press (1967).

which argues against the possibility of their being due to the presence of the 10–20% of heavy-chain molecules with no detectable allotypic marker (Dray, Young & Nisonoff, 1963; Stemke, 1965). The blank molecules may be due to a small content of a minor subclass, but if Aa1, Aa2 and Aa3 are true alleles it is unlikely that the differences observed between the sequences of the Aa1 and Aa3 heavy chains are related to the presence of different subclasses in these preparations of IgG. It is unlikely also that the variations are due to differences between individual rabbits, as the initial preparations of Prahl & Porter (1968) were from sera from single rabbits, and no differences were found between these and later preparations from pooled rabbit serum.

All the substitutions within the Aa1 and Aa3 sequences, with the exception of position 3 in Aa3, can be accounted for by single base changes in the DNA, and of the 13 differences between the two sequences only four require more than one base change.

It would be very difficult to decide whether there was any form of linkage between the replacements in the sequences; there was some evidence in the Aa3 fragment that the PCA-Ser-Leu- sequence was linked to lysine at position 13 and that the PCA-Glu-Gln- sequence to non-lysine, but this was by no means clear cut. As the amounts of the variant amino acids differ from position to position, it is clear that more than two fundamental sequences are involved.

In Fig. 5 the two rabbit sqeuences are also compared with the *N*-terminal sequence of the human γ1 heavy chain (Daw) obtained by Piggot & Press (1967). The extent of homology between the rabbit sequences is about 60%, which is of the same order as that between either one and the human sequence. Comparison with the only other complete heavy-chain *N*-terminal sequence available, the human γ4, Vin (Pink & Milstein, 1968), shows there to be again about 60% homology with the Aa3 sequence but only about 35% with the Aa1. The most striking similarity is the sequence from position 19 to position 27 around the half-cystine at position 22; this is identical, except for one position, with that of Daw but is rather different from that of Vin (Pink & Milstein, 1967) or the γ1 heavy chain Dee (Frangione & Milstein, 1967). It is clearly too early to draw any conclusions from these homologies until rather more is known about the *N*-terminal sequence of human heavy chain.

Sequence variations that correlate with changes in allotypic specificity have been reported for the Inv locus of human κ-chains (Hilschmann & Craig, 1965; Milstein, 1966; Baglioni, Alescio Zonta, Cioli & Carbonara, 1966) and for some of the Gm loci of human heavy chains (Thorpe & Deutsch, 1966; Prahl, 1967); these, however, involve only one or two amino acids and not the multiple variations found here. Multiple differences were reported between the amino acid compositions at Ab4 and Ab5 light chains from the rabbit (Reisfeld, Dray & Nisonoff, 1965), but as no sequence results have been published it is not possible to say whether this is a similar phenomenon to that reported here. Publication of differences in the amino acid composition of Fd fragments (Inman & Reisfeld, 1968) and heavy chains (Koshland, Reisfeld & Dray, 1968) from rabbit IgG that differed in allotype at the *a* locus confirms the present finding that differences in allotype are related to multiple amino acid differences. It may be that this greater heterogeneity is associated with their presence in the variable part of the heavy chain.

Oudin (1966) noted that the rabbit allotypes appear to be families of specificities rather than the products of a single locus, and the variations found in the *N*-terminal sequences of the Aa1 and Aa3 heavy chains may be a reflection of this. This could be explained by their being the products of two or more closely linked genes.

In view of the remarkable observation by Todd (1963) that immunoglobulin M carries the Aa1, Aa2 and Aa3 allotypes and subsequent observation that this is true also of immunoglobulin A (Feinstein, 1963; Pernis, Torrigiani, Amante, Kelus & Cebra, 1968) it will be of interest to find if there is any identity of sequence in the *N*-terminal end of the heavy chain in contrast with the *C*-terminal end, which is known to differ between the γ-, α- and μ-chains.

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REFERENCES

Ambler, R. P. (1965). *Biochem. J.* **96**, 32P.
Baglioni, C., Alescio Zonta, L., Cioli, D. & Carbonara, A. (1966). *Science*, **152**, 1517.
Bradbury, J. H. (1956). *Nature, Lond.*, **178**, 912.
Butler, P. J. G., Harris, J. I., Hartley, B. S. & Leberman, R. (1967). *Biochem. J.* **103**, 78P.
Cohen, S. & Milstein, C. (1967). *Advanc. Immunol.* **7**, 1.
Crumpton, M. J. & Wilkinson, J. M. (1965). *Biochem. J.* **94**, 545.
Dray, S., Dubiski, S., Kelus, A. S., Lennox, E. S. & Oudin, J. (1962). *Nature, Lond.*, **195**, 785.

Dray, S., Young, G. O. & Nisonoff, A. (1963). *Nature, Lond.*, **199**, 52.
Feinstein, A. (1963). *Nature, Lond.*, **199**, 1197.
Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys.* Suppl. 1, p. 174.
Frangione, B. & Milstein, C. (1967). *Nature, Lond.*, **216**, 939.
Givol, D. & Porter, R. R. (1965). *Biochem. J.* **97**, 32c.
Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 469. Ed. by Hirs, C. H. W. New York: Academic Press Inc.
Haber, E. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 1099.
Hilschmann, N. & Craig, L. C. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1403.
Inman, J. K. & Reisfeld, R. A. (1968). *Immunochemistry*, **5**, 415.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Koshland, M. E. (1967). *Cold Spr. Harb. Symp. quant. Biol.* **32**, 119.
Koshland, M. E., Reisfeld, R. A. & Dray, S. (1968). *Immunochemistry*, **5**, 471.
Kusama, K. (1957). *J. Biochem., Tokyo*, **44**, 375.
Milstein, C. (1966). *Nature, Lond.*, **205**, 1171.
Morse, D. & Horecker, B. L. (1966). *Analyt. Biochem.* **14**, 429.
Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C. (1960). *Biochem. J.* **77**, 149.
Oudin, J. (1966). *J. Cell Physiol.* **67**, Suppl. 1, 77.
Pan, S. C. & Dutcher, J. D. (1956). *Analyt. Chem.* **28**, 836.
Pernis, B., Torrigiani, G., Amante, L., Kelus, A. S. & Cebra, J. J. (1968). *Immunology*, **14**, 445.
Piggot, P. J. & Press, E. M. (1967). *Biochem. J.* **104**, 616.
Pink, J. R. L. & Milstein, C. (1967). *Nature, Lond.*, **216**, 941.
Pink, J. R. L. & Milstein, C. (1968). *Proc. 5th Meet. Fed. Europ. biochem. Soc., Prague.* London: Academic Press (Inc.) Ltd. (in the Press).
Porter, R. R. (1967). *Biochem. J.* **105**, 417.
Porter, R. R. & Weir, R. C. (1966). *J. Cell Physiol.* **67**, Suppl. 1, 51.
Prahl, J. W. (1967). *Biochem. J.* **105**, 1019.
Prahl, J. W. & Porter, R. R. (1968). *Biochem. J.* **107**, 753.
Press, E. M., Givol, D., Piggot, P. J., Porter, R. R. & Wilkinson, J. M. (1966*a*). *Proc. Roy. Soc.* B, **166**, 150.
Press, E. M., Piggot, P. J. & Porter, R. R. (1966*b*). *Biochem. J.* **99**, 356.
Raftery, M. A. & Cole, R. D. (1963). *Biochem. biophys. Res. Commun.* **10**, 467.
Reisfeld, R. A., Dray, S. & Nisonoff, A. (1965). *Immunochemistry*, **2**, 155.
Sanger, F. & Thompson, E. O. P. (1963). *Biochim. biophys. Acta*, **71**, 468.
Smith, I. (1960). In *Chromatographic and Electrophoretic Techniques*, vol. 1, pp. 96, 97. Ed. by Smith, I. London: William Heinemann (Medical Books) Ltd.
Steers, E., Craven, G. R., Anfinsen, C. B. & Bethune, J. L. (1965). *J. biol. Chem.* **240**, 2478.
Stemke, G. W. (1965). *Immunochemistry*, **2**, 359.
Thorpe, N. O. & Deutsch, H. F. (1966). *Immunochemistry*, **3**, 329.
Todd, C. W. (1963). *Biochem. biophys. Res. Commun.* **11**, 170.
Whitney, P. L. & Tanford, C. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 524.
Wilkinson, J. M. (1968). *Proc. 5th Meet. Fed. Europ. biochem. Soc., Prague.* London: Academic Press (Inc.) Ltd. (in the Press).
Wilkinson, J. M., Press, E. M. & Porter, R. R. (1966). *Biochem. J.* **100**, 303.