Common Peptides from the *N*-Terminal Half of Heavy Chain of Immunoglobulin G from Normal Rabbit Serum and a Specific Antibody

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Fragment C-1, the *N*-terminal half of the heavy chain of rabbit immunoglobulin G, was prepared by cyanogen bromide cleavage from the heavy chain of immunoglobulin G obtained both from the pooled serum of normal rabbits and from specific anti-dinitrophenyl antibody. Tryptic digestion of fragment C-1 after the lysine residues had been allowed to react with *S*-ethyl trifluorothioacetate led to the isolation of six peptides from inert immunoglobulin G and specific antibody that appear to account for most of this section of the heavy chain. This approach should make possible comparative sequence studies of the Fd section of the heavy chain from different allotypes and from specific antibodies.

The antibody combining site of IgG§ molecules has been shown to be contained in Fab fragments (Porter, 1959). These are derived from papain proteolysis at approximately the middle of the heavy chains (Pain, 1963) with accompanying reduction of disulphide bonds (Cebra, Givol, Silman & Katchalski, 1961). The Fab fragment could be resolved into light chain and a fragment now called Fd, more or less homogeneous in size, representing the *N*-terminal half of the heavy chain (Fleischman, Porter & Press, 1963). Comparisons of tryptic peptides from the *C*-terminal fragment of heavy chain, fragment Fc, with those from the whole heavy chain, either by peptide 'mapping' (Frangione & Franklin, 1965; Small, Reisfeld & Dray, 1965; Merryman, Frangione, Franklin & Benacerraf, 1967) or by ion-exchange chromatography on Dowex 50 (Nelson, Noelken, Buckley, Tanford & Hill, 1965), have failed to reveal sufficient peptides to account for the Fd fragment. Thus the notion that Fd is markedly heterogeneous has arisen without direct evidence based on primary structural analysis (Cohen & Milstein, 1967). However, a few common structural features of Fd have been recognized: (1) a blocked *N*-terminal nonapeptide, with two replacements, present in 75% of the

molecules (Press, Givol, Piggot, Porter & Wilkinson, 1966a); (2) a half-cystine residue involved in inter-chain bonding with the light chain (Fleischman *et al.* 1963); (3) certain antigenic sites called allotypic markers (Kelus, 1963).

It therefore seemed desirable to attempt a direct assessment of the complexity of sequence in the *N*-terminal half of the heavy chain, the aim being to define and position the sections of relatively constant sequence. The difficulties of working with a peptide chain of over 200 residues in which many might be varying from molecule to molecule are obvious, as there is almost no limit to the number of small peptides that might be obtained on digestion with proteolytic enzymes. It was decided to work with fragment C-1, a fragment equivalent to, but slightly larger than, enzymic fragment Fd. This fragment is prepared by cyanogen bromide cleavage of the heavy chain (Givol & Porter, 1965), and it is easier to work with than fragment Fd, as all molecules have the same characteristic *C*-terminal residue, homoserine. When one is working with such a polypeptide, probably of mixed sequence, an ideal approach would be to break the chain into two approximately equal parts, where, even if the split occurred in a variable region, each part could be easily characterized by its distinctive *N*- or *C*-terminal end. Similarly, the two large sections would each be expected to behave in many systems as single components, as do the heavy and light chains themselves, even though neither is homogeneous. No technique to achieve a single split at about the mid-point of fragment C-1 could be devised, but hydrolysis at a small number of points

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§ Abbreviations: IgG, immunoglobulin G; TFA, trifluoroacetyl.

to give large sections was obtained by tryptic digestion at the arginine residues of substituted fragment C-1 in which the 12 lysine residues had been allowed to react with *S*-ethyl trifluorothioacetate (Goldberger & Anfinsen, 1962). The isolation and characterization of these large peptides from fragment C-1 prepared from both inert IgG and antibody to the DNP-hapten are described in the present paper. Sequence studies of two of these peptides are described in the following paper (Cebra, Steiner & Porter, 1968).

MATERIALS

Normal rabbit IgG and heavy chain. The IgG was prepared from pooled rabbit serum by Na_2SO_4 precipitation, followed by chromatography on DEAE-Sephadex A-50 as described by Wilkinson, Press & Porter (1966). The heavy chain was obtained from the IgG by reduction with 0.4M-mercaptoethanol followed by alkylation with iodoacetic acid and dissociation and separation of chains on a column of Sephadex G-75 in *n*-propionic acid (Fleischman, Pain & Porter, 1962). Labelling of the easily reduced disulphide bonds in the heavy chain was done by reduction in 5mM-dithiothreitol in 0.1M-tris-HCl buffer, pH 8, and reaction with 1.5 molar excess of iodo[^{14}C]acetate relative to moles of SH.

Antibody to the DNP-hapten. This was isolated by methods based on those of Eisen (1964). Outbred rabbits were immunized with DNP-(bovine γ -globulin) in complete Freund's adjuvant, as described for other protein antigens (Porter, 1955). Antibody specific for the DNP group was precipitated from 215 ml. of pooled serum (containing about 1.5 mg. of specific antibody/ml.) by addition of the optimum amount of DNP-(human serum albumin). The washed precipitate was suspended in 150 ml. of 0.1M-potassium phosphate buffer, pH 7.0, dissolved by the addition of 140 mg. of ϵ -DNP-lysine to give a concentration of 0.4 mM, and stirred at 2° for 48 hr. After centrifugation the clear supernatant was applied to a column of DEAE-Sephadex A-50 in 0.1M-potassium phosphate buffer, pH 7.0. The anti-DNP IgG was unretarded and was concentrated by pressure dialysis. A sample of antibody (350 mg.; 2.3 μ moles), still containing 1.6 μ mole of ϵ -DNP-lysine/ μ mole of antibody, was reduced and alkylated as described for normal IgG. The chains were then dissociated and separated after dialysis against 5M-guanidine hydrochloride on an upward-flowing column of Sephadex G-200 in 5M-guanidine (Small & Lamm, 1966). In this way 140 mg. of heavy chain was prepared.

Other materials. Dithiothreitol (A grade; Calbiochem, Los Angeles, Calif., U.S.A.) and *S*-ethyl trifluorothioacetate and CNBr (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) were used as supplied. Trypsin (twice crystallized; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was treated with chloromethyl *L*-(2-phenyl-1-toluene-*p*-sulphonamido)ethyl ketone (Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.) (Kostka & Carpenter, 1964). Guanidine hydrochloride was purified by filtration of a 7M solution through activated charcoal-Celite (Small & Lamm, 1966). Piperidine (Hopkin and Williams Ltd., Chadwell Heath, Essex) was redistilled over KOH. Iodoacetic acid was recrystallized from light petroleum. Iodo[^{2-14}C]acetic acid (The Radiochemical Centre, Amersham, Bucks.) was

obtained with a specific radioactivity of 5–7 mc/m-mole and added in the appropriate amount to unlabelled recrystallized iodoacetic acid.

METHODS

Cyanogen bromide cleavage. Heavy chain (1g.) was dissolved in 98–100% formic acid (35 ml.) and then diluted with water (15 ml.) to a protein concentration of 20 mg./ml. in 70% formic acid (Steers, Craven, Anfinsen & Bethune, 1965). Quantitative cleavage of the heavy chain at methionine residues (Givol & Porter, 1965) was achieved by adding solid CNBr (5g.) and allowing the mixture to stand at 5° in a stoppered flask for 24 hr. The reaction mixture was then diluted with 5–10 vol. of water and freeze-dried.

Fractionation of cyanogen bromide digest mixture. The freeze-dried peptides (1g.) were dissolved in 5M-guanidine hydrochloride (10 ml.) and then diluted with *n*-acetic acid (40 ml.). The components in the digest were separated by gel filtration through Sephadex G-100 in *n*-acetic acid as described previously (Givol & Porter, 1965; Press *et al.* 1966a). A 100 cm. $\times$ 7.5 cm. column was used for 1g. of material and a 140 cm. $\times$ 3.5 cm. column for 100–300 mg. samples (8 ml.). The first component eluted was fragment C-1 (Givol & Porter, 1965; Press *et al.* 1966a), which served as the starting material for the present study.

Total reduction and alkylation of fragment C-1. The freeze-dried fragment C-1 (400 mg.) was dissolved in 7M-guanidine hydrochloride in 0.1M-tris-acetate buffer, pH 8.0 (20 ml.). Then solid dithiothreitol (154 mg.) was added to give a concentration of 50 mM and the reaction mixture was left at room temperature for 2 hr. A neutral solution of iodoacetic acid (560 mg. in 2.5 ml. of 2.5M-tris) was added to the reaction mixture to give a 1.5-fold molar excess of alkylating agent relative to thiol concentration. The pH was maintained strictly at 8.0 for 30 min. and then the mixture was dialysed in $\frac{3}{8}$ in. tubing against 2 l. of 30 mM-tris-acetate buffer, pH 8.0. After four changes of outer fluid over 24 hr. the alkylated fragment C-1 was dialysed against water for 2–3 hr. and then freeze-dried. In some instances, the half-cystine residues not reduced during the initial separation of the heavy chain, presumably those involved in the more stable intrachain bonding, were labelled by incorporating 0.1 mc of iodo[^{2-14}C]acetic acid in the solution of the alkylating agent before adding it to the reduced fragment C-1.

Trifluoroacetylation of reduced carboxymethylated fragment C-1. The freeze-dried product (400 mg.) was dissolved in unbuffered 5M-guanidine hydrochloride (40 ml.) and brought to pH 10 by the addition of *n*-NaOH. Reaction with *S*-ethyl trifluorothioacetate (2 ml. in three portions over the first $\frac{1}{2}$ hr.) was carried out as described by Goldberger & Anfinsen (1962) and after 1 hr. of vigorous stirring at pH 9.8–10.2 the reaction mixture was dialysed in $\frac{3}{8}$ in. tubing at 5° against 10 mM-tris-acetate buffer, pH 8.0 (2 l.). After five changes of buffer over 48 hr. the substituted fragment C-1 was dialysed against distilled water (2 l.) for 24 hr. before digestion.

Tryptic digestion of trifluoroacetylated fragment C-1. The substituted fragment C-1 (400 mg.) in clear solution (40 ml.) after dialysis against water was brought to pH 8.3 and 37° in an autotitrator. Trypsin (8 mg.) was added in four portions at 30 min. intervals while the pH was maintained at 8.3 with 0.1N-NaOH. Digestion was complete in 4 hr.,

when the clear solution was either applied immediately to the gel-filtration column or was stored at 0° and run on the column later.

Gel filtration of tryptic digest. A portion of the digestion mixture (20 ml., 200 mg. of peptides) was applied to a column of Sephadex G-50 (190 cm. × 3.5 cm.) in 0.05 M-NH₄HCO₃. Pools of effluent were made as described in the Results section and freeze-dried to remove salt.

Removal of TFA groups. The blocking groups were removed from lysine residues (either before or after chromatography on DEAE-Sephadex; see the Results section) at 0° in *m*-piperidine, as described by Goldberger & Anfinsen (1962), except that the peptides (4 mg./ml.) were kept at 0° for 24 hr. before neutralization of the piperidine with acetic acid, and were then freed of reagents by passage through a column of Sephadex G-25 in 0.05 N-NH₃.

Amino acid analyses. These were carried out as described by Press, Piggot & Porter (1966b) after 24 hr. hydrolysis.

RESULTS

Incubation of the heavy chain of rabbit IgG with cyanogen bromide results in its cleavage at methionine residues into a number of fragments (Givol & Porter, 1965; Press *et al.* 1966a). These fragments can be separated partially by filtration of the reaction mixture through a column of Sephadex G-100 in *N*-acetic acid. Fig. 1 shows an elution diagram obtained from the cyanogen bromide digest of heavy chain derived from purified anti-DNP antibody. This pattern was very similar to that recorded after separation of the components of a cyanogen bromide digest of heavy chain from pooled normal IgG (Press *et al.* 1966a). The size and quantities of the fragments formed on cleavage of heavy chains from a specific antibody and from pooled IgG were about the same, suggesting a similar distribution of methionine residues in all heavy chains.

A major component in the cyanogen bromide digest was fragment C-1 (Fig. 1), which accounted for 40–50% of the original weight of heavy chain. Fragment C-1 appears to represent the *N*-terminal half of the heavy chain, having a blocked *N*-terminal end, a *C*-terminal homoserine residue and an amino acid composition similar to but not identical with that of the enzymic fragment Fd (Press *et al.* 1966a). Table 1 presents the amino acid compositions obtained for three preparations of fragment C-1 from normal IgG. The data for Preparation 1 have been given by Press *et al.* (1966a), but are here recalculated assuming 17 residues of leucine/mol. of fragment C-1. It is shown in the following paper (Cebra *et al.* 1968) that fragment C-1 extends about 24 residues beyond the *C*-terminus of fragment Fd. Since there are 14 leucine residues in fragment Fd and 3 additional ones in the *C*-terminal extension, a total of 17 was assumed for fragment C-1.

Table 1 also presents the composition of the fragment C-1 derived from anti-DNP antibody.

Fig. 1. Fractionation of a cyanogen bromide digest of the heavy chain of rabbit anti-DNP antibody. A sample (140 mg.; 2.8 μ moles) of digest was run on a Sephadex G-100 column (140 cm. × 3.5 cm.) in *N*-acetic acid.

The similarity between the amino acid composition of fragment C-1 from antibody and the average composition for fragment C-1 from pooled IgG suggests a similar basic primary structure for fragment C-1 from all IgG antibodies.

Fragment C-1 was totally reduced, carboxymethylated to block cysteine residues and then treated with *S*-ethyl trifluorothioacetate to block lysine residues reversibly (Goldberger & Anfinsen, 1962). Tryptic cleavage of this product would be expected to occur only at the arginine residues, of which there are about 6 in fragment C-1. When a tryptic digest of the substituted fragment C-1 was subjected to gel filtration on a column of Sephadex G-50, an elution diagram such as the one shown in Fig. 2 was obtained. The major fractions were numbered T1–T7 and the eluate was pooled as marked. The fractionation shown in Fig. 2 is of a preparation of fragment C-1 that was completely reduced and alkylated with iodo[¹⁴C]acetic acid before digestion. Since the fragment C-1 was made from heavy chain prepared by mild reduction (Fleischman *et al.* 1962) and alkylation with unlabelled iodoacetic acid, only the half-cystine residues involved in the more stable, presumably intrachain, disulphide bonding were labelled. The major labelled fractions were T1, T2, T3 and T4. Fractionation of a tryptic digest of fragment C-1 alkylated with iodo[¹⁴C]acetic acid after mild reduction showed only fractions T2 and T3 to be labelled significantly.

None of the fractions obtained from the Sephadex G-50 column was sufficiently homogeneous and each had to be subjected to an additional purification step. Fractions T2 and T3 were pooled, treated with piperidine to remove the TFA groups

Table 1. *Amino acid composition of cyanogen bromide fragment C-1 from several samples of normal IgG and specific anti-DNP antibody*

Amino acid content is expressed as residues/mol., assuming 17 leucine residues/mol. of fragment C-1.

Amino acid	Composition of fragment C-1 (moles of amino acid residue/mole of fragment C-1)				
	Normal IgG				Anti-DNP antibody
	Prep. 1	Prep. 2	Prep. 3	Average values	
Lys	12	11	12	12	11
His	1.7	1.1	2.1	1.6	1.8
Arg	6.5	5.1	7.4	6.3	6.0
Asp	14	19	17	17	17
Thr	30	32	29	30	32
Ser	29	27	27	28	26
Glu	14	15	17	15	18
Pro	19	22	24	22	18
Gly	20	23	20	21	23
Ala	13	15	14	14	16
Val	22	24	23	23	24
Ile	6.7	7.2	7.6	7.2	6.8
Leu	17	17	17	17	17
Tyr	9.2	9.7	9.3	9.4	9.0
Phe	7.6	8.0	8.0	7.9	7.7
Cys·CH ₂ ·CO ₂ H	7.4	7.1	7.6	7.4	8.0
Homoserine	1.8	1.3	2.2	1.8	1.9

Fig. 2. Fractionation of a tryptic digest of fragment C-1 that had been fully reduced and blocked with iodo[¹⁴C]acetate and had then been treated with *S*-ethyl trifluorothioacetate to block the ϵ -amino groups of lysine. A sample (350 mg.) of digest was put on a Sephadex G-50 column (190 cm. $\times$ 3.5 cm.) in 0.05 M-NH₄HCO₃. ----, E_{230} ; —, E_{240} ; ·····, radioactivity (counts/min./ml. of eluate).

(Goldberger & Anfinsen, 1962) and then refractionated on a column of DEAE-Sephadex A-25, as shown in Fig. 3, to obtain peptides T2 and T3. Alternatively, peptide T2 could be obtained in a

slightly purer form by taking fraction T2 from the gel-filtration step and chromatographing it on DEAE-Sephadex A-25 before removal of the TFA groups, as shown in Fig. 4. The shoulder on the

Fig. 3. Chromatography of fractions T2 and T3 (from the Sephadex G-50 column), after removal of TFA groups, on a DEAE-Sephadex A-25 column (20 cm. $\times$ 1.5 cm.). The starting buffer was 5 mM- NH_4HCO_3 , pH 8.5, and the first linear gradient was to 0.2 M- NH_4HCO_3 , pH 8.5, with 350 ml. in each open chamber; the second linear gradient was from 0.2 M- NH_4HCO_3 , pH 8.5, to 0.4 M- NH_4HCO_3 , pH 8.5, with 350 ml. in each open chamber. The arrows show the commencement of the gradients.

Fig. 4. Chromatography of fraction T2 (from the Sephadex G-50 column), before the removal of TFA groups, on a DEAE-Sephadex A-25 column (20 cm. $\times$ 1.5 cm.). The starting buffer was 5 mM- NH_4HCO_3 , pH 8.5, with a gradient to 0.2 M- NH_4HCO_3 , pH 8.5, with a constant-volume mixing chamber of 500 ml. capacity. The arrow shows the commencement of the gradient. —, E_{215} ;, radioactivity (counts/min./ml. of eluate).

leading edge of T2 had a very similar amino acid composition to that of the main peak, and it may have arisen from partial removal of the TFA groups during handling. The accompanying fraction T3 from the gel-filtration step was treated to remove TFA groups and chromatographed separately under the conditions described in Fig. 3.

Fig. 5. Chromatography of fraction T4 (from the Sephadex G-50 column), after removal of TFA groups, on a DEAE-Sephadex A-25 column (20 cm. $\times$ 1.5 cm.). The starting buffer was 5 mM- NH_4HCO_3 , pH 8.5, with a linear gradient to 0.45 M- NH_4HCO_3 , pH 8.5, with 350 ml. in each open chamber. The arrow shows the commencement of the gradient.

Fraction T4 contained a very acidic peptide as a major component. Ion-exchange chromatography of this fraction, after removal of TFA groups, on DEAE-Sephadex A-25 with a steeper linear salt gradient than that employed for elution of fractions T2 and T3, yielded peptide T4 as shown in Fig. 5.

Fraction T1 from the gel-filtration step on Sephadex G-50 contained peptide T1 as a main component along with other peptides absorbed to peptide T1. The elution volume for peptide T1 was the exclusion volume for the column, suggesting a large molecular weight for this component. However, it appears that peptide T1 is aggregated in both 0.05 M-ammonium hydrogen carbonate and 0.05 N-ammonia, and this results in its elution at the breakthrough volume; the extinction of its fractions is high owing to light-scattering at 240 mμ and 230 mμ. To purify fraction T1 it was only necessary to refractonate it (10–20 μmoles) on a column of Sephadex G-75 (145 cm. $\times$ 3.5 cm.) in 0.05 N-ammonia, when peptide T1 was eluted at the exclusion volume (comprising about 30% of the total weight of fraction T1), although it was a peptide of only 37 residues. The other fractions from the Sephadex G-75 column contained a mixture of peptides, all in low yield.

In this manner, 5–25 μmoles of peptides T1, T2, T3 and T4 could be purified. Rechromatography of peptides T2, T3 and T4 on DEAE-Sephadex, under the original conditions, was sometimes necessary when large amounts (15–25 μmoles) were being prepared.

Fraction T5 was fractionated by electrophoresis

Table 2. *Amino acid composition of the principal tryptic peptides of trifluoroacetylated fragment C-1*

The amino acid composition is calculated assuming 17 residues of leucine/mol. of fragment C-1 and one residue of arginine or homoserine/mol. of peptide.

Amino acid	Composition (moles of amino acid residue/mole of peptide)							
	Peptide T1	Peptide T2	Peptide T3	Peptide T4	Peptide T5	Peptide T7	Sum of peptides	Fragment C-1 (average value)
Lys	2.0	5.2	3.0	0	0	0	10	12
His	0	1.0	0	0	0	0	1.0	1.6
Arg	1.0	0	1.0	1.0	1.0	1.0	5.0	6.3
Asp	1.7	4.0	6.2	5.5	0	0	17	17
Thr	5.3	7.8	9.3	3.3	0	1.0	27	30
Ser	4.2	9.8	7.2	1.2	1.8	1.0	25	28
Glu	2.0	2.9	4.4	6.4	3.0	0	19	15
Pro	2.5	12	7.7	2.3	0	0.9	25	22
Gly	4.0	4.2	9.0	0	2.2	0	19	21
Ala	3.0	3.1	3.9	0	0	0	10	14
Val	2.0	7.9	8.2	6.8	1.1	0.7	27	23
Ile	2.0	1.1	1.0	1.1	0	0	5.2	7.2
Leu	2.6	5.0	7.3	0	0	0	15	17
Tyr	2.1	0.9	2.8	1.1	0	0	6.9	9.4
Phe	1.4	2.0	2.1	1.1	0	0.8	7.4	7.9
CyS·CH ₂ ·CO ₂ H	0.9	2.3	3.1	1.0	0	0	7.3	7.4
Homoserine	0	1.0	0	0	0	0	1.0	1.8
Trp	0	0	0	2.0	0	0	2.0	4.0
Total residues	37	70	76	33	9.1	5.4	230	244

on paper at pH 3.5 and the arginine-containing peptide was identified as the *N*-terminal nonapeptide reported by Wilkinson *et al.* (1966). Fraction T6 contained only minor peptides common to fraction T5, but fraction T7 had a major arginine peptide, peptide T7, which could be isolated by electrophoresis at pH 3.5, when it was the main basic peptide. A variety of other peptides were present in fractions T5, T6 and T7, and some were shown by the Sakaguchi reaction to contain arginine, but all appeared to be present in low yields.

The amino acid compositions of peptides T1, T2, T3, T4, T5 and T7 are given in Table 2 and are based on analyses of three or four preparations of each peptide. Peptides T1, T4, T5 and T7 were peptides containing arginine and contained about 37, 33, nine and six amino acid residues respectively. Peptide T1 contained two lysine residues that had reacted with *S*-ethyl trifluorothioacetate, whereas peptides T4, T5 and T7 had arginine as the sole basic residue. Peptide T7 had the same analysis and sequence as a hexapeptide isolated in good yield by Hill, Delaney, Lebovitz & Fellows (1966) from a tryptic digest of whole heavy chain but not from a tryptic digest of fragment Fc. This confirms its presence as a part of the sequence of fragment Fd. Peptide T4 was the only tryptophan-containing peptide isolated and it was unusual in its high content of acidic amino acids, containing about five

residues of aspartic acid and about six of glutamic acid out of about 33 total residues. Peptide T4 also lacked glycine, alanine and leucine, residues occurring frequently in fragment C-1. Peptide T3 contained about 76 residues, with arginine *C*-terminal and three lysine residues.

Peptide T2 contained no arginine but did have one residue of homoserine. It represented the *C*-terminal end of fragment C-1 and was comprised of about 70 residues. This peptide was also distinguished by its single histidine residue and 12 residues of proline.

The *N*-terminal residue of peptide T2 was found to be glutamic acid by dinitrophenylation and threonine was found at the *N*-terminal end of peptide T4 by the 'dansylation' technique. No *N*-terminal residues could be identified for peptides T1 and T3 by either procedure.

In Table 2 the sum of the content of each amino acid residue in peptides T1, T2, T3, T4, T5 and T7 is given and compared with the average composition of fragment C-1 itself. The comparison is only meaningful if the assumption that peptides T1, T2, T3, T4, T5 and T7 occur together in the same molecule of fragment C-1 is true. However, the sum of the analyses of all the peptides does approximate to that of fragment C-1 reasonably well. A conspicuous difference is that only five of the six arginine residues of fragment C-1 are accounted for in the peptides. In connexion with this comparison

it should again be pointed out that the absolute numbers for each residue in fragment C-1 are based on the assumption of 17 leucine residues/mol.

Evidence that peptides T1, T2, T3 and T4 are major components of fragment C-1 and that at least peptides T1, T2 and T3 can occur together in the same molecule was obtained by measuring the yields of these peptides. In Table 3 the actual uncorrected yields obtained from one typical preparative isolation of the peptides from fragment C-1 of normal IgG are tabulated. Yields of peptides T1, T2 and T3 of 55%, 59% and 72% respectively

were estimated after gel-filtration steps. Final purification of peptides T2, T3 and T4 by chromatography on DEAE-Sephadex lowered the final absolute yields to 31%, 27% and 18% respectively. The final yield of peptide T7 after preparative paper electrophoresis was about 30%. The yields of the five major arginine-containing peptides together with peptide T2, the homoserine-containing peptide, suggest that there is a basic common sequence in the fragment C-1, although clearly some heterogeneity is superimposed on it.

Investigation of fragment C-1 prepared from the

Table 3. Yields of tryptic peptides from trifluoroacetylated fragment C-1 derived from either normal IgG (23.4 μ moles) or anti-DNP antibody (1.52 μ moles)

	Recovery							
	Peptide T1		Peptide T2		Peptide T3		Peptide T4	
	(μ moles)	(%)	(μ moles)	(%)	(μ moles)	(%)	(μ moles)	(%)
Normal IgG								
Crude TFA-peptides after G-50	—	—	13.8†	59	16.8‡	72	—	—
TFA-peptides after A-25 chromatography or G-75 filtration*	12.5	55	7.2	31	6.2	27	4.1	18
Anti-DNP antibody								
Crude TFA-peptides after G-50	—	—	0.85†	56	0.86‡	57	—	—
TFA-peptides after A-25 chromatography or G-75 filtration*	0.8	53	0.26	17	0.21	14	0.19	13

* Yields given represent absolute recoveries uncorrected for losses during isolation process.

† Yields based on histidine and homoserine.

‡ Yields based on arginine.

Table 4. Comparison of compositions of tryptic peptides derived from fragment C-1 of normal IgG and of anti-DNP antibody

	Composition (moles of amino acid residue/mole of peptide)							
	Peptide T1		Peptide T2		Peptide T3		Peptide T4	
	Normal IgG	Anti-DNP	Normal IgG	Anti-DNP	Normal IgG	Anti-DNP	Normal IgG	Anti-DNP
Lys	2.0	1.8	5.2	5.2	3.0	3.0	0	0
His	0	0	1.0	0.8	0	0	0	0
Arg	1.0	1.0	0	0	1.0	0.9	1.0	0.9
Asp	1.7	1.6	4.0	4.0	6.2	6.5	5.5	4.6
Thr	5.3	4.3	7.8	7.7	9.3	11.0	3.3	2.9
Ser	4.2	3.5	9.8	9.7	7.2	10	1.2	2.0
Glu	2.0	2.6	2.9	2.7	4.4	4.3	6.4	5.4
Pro	2.5	2.0	12	12	7.7	9.8	2.3	2.1
Gly	4.0	4.2	4.2	4.0	9.0	11	0	1.5
Ala	3.0	3.2	3.1	3.0	3.9	5.3	0	0
Val	2.0	2.2	7.9	8.1	8.2	10	6.8	5.8
Ile	2.0	1.6	1.1	1.0	1.0	0.7	1.1	1.1
Leu	2.6	2.6	5.0	4.8	7.3	8.8	0	0
Tyr	2.1	1.7	0.9	0.9	2.8	1.4	1.1	0.7
Phe	1.4	1.4	2.0	2.1	2.1	2.4	1.1	1.2
CyS·CH ₂ ·CO ₂ H	0.9	1.0	2.3	2.0	3.1	2.2	1.0	0.4
Homoserine	0	0	1.0	1.1	0	0	0	0

heavy chain of anti-DNP antibody in a similar manner showed the same main peptides to be present in similar yields (Table 3). As much less material was available from the anti-DNP antibody, the yields were rather lower after chromatography on the DEAE-Sephadex. The similarity of analysis of the main peptides is striking (Table 4) and such differences as are apparent are of uncertain significance, as the values for peptides from fragment C-1 of normal rabbit IgG are averages of several samples, whereas those for the anti-DNP peptides are for a single preparation.

DISCUSSION

The evidence that fragment C-1 is derived from the *N*-terminal half of the heavy chain is based on the presence of a main *N*-terminal sequence that is the same as that of the whole chain (Cebra *et al.* 1968). Further sequence studies show that the *C*-terminal sequence extends beyond that of fragment Fd and agrees with the partial *N*-terminal sequence of fragment Fc as described by Hill *et al.* (1966), and yields of both sequences suggest that this is true for most of the molecules. However, there is significantly more than the 1 mole of homoserine/mole of fragment C-1 that would be expected if fragment C-1 were homogeneous in this respect. A fractional residue of methionine has been identified at position 35 from the *N*-terminal end of the heavy chain (Press *et al.* 1966a; J. M. Wilkinson, unpublished work); this accounts for about 0.3 residue of homoserine. The *N*-terminal peptide remains bound to fragment C-1 by a disulphide bond, but is removed on total reduction before tryptic digestion. Another fractional homoserine residue has been placed near the *C*-terminal end (J. W. Prahl, unpublished work), but the corresponding peptide does not remain bound to fragment C-1 before total reduction. It is possible that there is a third fractional residue near the centre of fragment C-1 that, if it exists, would not be expected to release any peptide without reduction. It appears therefore that fragment C-1 does represent the whole of the *N*-terminal half of the heavy chain but that on total reduction 30% of the *N*-terminal sequence and perhaps a similar amount of the *C*-terminal sequence is freed and partially lost on dialysis. If any further split is caused by reaction with cyanogen bromide, for instance near the centre of fragment C-1, it does not lead to further loss, possibly because the two presumably large polypeptides remain associated with the main fraction owing to aggregation or adsorption. Hence the peptides whose isolation is described here were derived from the whole of fragment C-1 except for possibly lacking 10–20% of short *N*- and *C*-terminal sequences.

The significance of the results described depends on whether there is sufficient evidence to justify the conclusion that the one homoserine-containing peptide and the five arginine-containing peptides together account for the major part of a single main amino acid sequence. The yields of the isolated peptides are rather low, 20–30%, but no lower than would be expected if one was isolating peptides of similar size and by similar methods from a protein known to have a unique sequence. When estimations of yields are made of some of the peptides in an earlier stage of fractionation, based on the content of a unique amino acid such as histidine or homoserine, the yields are correspondingly higher. There seems good ground therefore to believe that these peptides are not derived from variant sequences of overlapping sections of the peptide chain, but do represent distinct sections and hence must form 80–90% of the whole peptide chain. Only the *C*-terminal peptide T2 and *N*-terminal peptide T5 come from known positions, but comparison of the compositions and sequences of the peptides with the *N*-terminal sequence of a pathological human IgG heavy chain (Piggot & Press, 1967; Press, 1967) suggests the order T5, T1, T4, T3, T2, with T7 not placed. Proof that the six peptides do constitute the major portion of the sequence must await the isolation of overlapping peptides to give their definite alignment. One arginine residue has not been accounted for; this, if the argument above is correct, must be associated with a relatively small peptide. Failure to isolate it may be technical or may be due to its occurrence as a fractional residue in several positions, giving rise to the peptides staining for arginine that were observed in low yield in fractions T5, T6 and T7.

Some heterogeneity of sequence is apparent such as in the methionine residues and also in the *N*-terminal sequence (Wilkinson *et al.* 1966). This arises in part from the presence of the three allotypic forms, as the IgG was prepared from pooled serum. A correlation between allotype and sequence in fragment C-1 is being sought. Further, subclasses of IgG have been shown to exist in both human and mouse serum (see Cohen & Milstein, 1967). Their detection was facilitated by the use of myeloma proteins, each of which belongs to only one subclass. Myelomatosis has not been found in rabbits and IgG subclasses have not been demonstrated, but it is likely that they exist and are responsible for some of the heterogeneity observed. If the basic sequences represented by these peptides can be established, the prospect of detailed correlation with allotypic variation and IgG subclass will be considerably improved. Isolation of the same peptides in similar yield from fragment C-1 prepared from anti-DNP antibody suggests that, as might be expected, both specific antibody and inert IgG have

similar amino acid sequences throughout this section of the molecule. The likelihood that antibody specificity may be related to the presence of certain unique sequences remains, and the results reported here suggest that a direct correlation of specificity and sequence may be feasible.

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