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The Partial Sequence of Two Large Peptides from the *N*-Terminal Half of Heavy Chains from Normal Rabbit Immunoglobulin G

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The partial amino acid sequence of two large peptides is described. These were prepared from the *N*-terminal half of the heavy chain of immunoglobulin G from pooled normal rabbit serum by tryptic digestion after the ε-amino groups of the lysine residues had been blocked with *S*-ethyl trifluorothioacetate. These peptides are believed to account for about 145 residues of fragment C-1, the *N*-terminal section of rabbit immunoglobulin G heavy chain prepared by cyanogen bromide cleavage. The evidence from the present paper and the preceding paper (Cebra, Givol & Porter, 1968) suggests that it may be possible to deduce a predominant amino acid sequence for most, if not all, of this section of the molecule.

In the preceding paper (Cebra, Givol & Porter, 1968) the isolation of six peptides is described, which together appear to account for 85–90% of the amino acid sequence of fragment C-1, the *N*-terminal half of the heavy chain of rabbit IgG.§ When heavy chain was prepared by gentle reduction so that only 2·4 moles of disulphide bonds were split/2 moles of heavy chain and the resulting free thiol groups were blocked with iodo[^{14}C]acetate, all the label was present in fragment C-1 and, further, it was all present in peptides T2 and T3 of fragment C-1. Hence this label was a valuable marker and these peptides were chosen for the first detailed sequence studies. Peptide T2 consists of about 70 residues and contains homoserine. As fragment C-1 was prepared from the heavy chain by cyanogen bromide cleavage, giving a *C*-terminal homoserine residue, and the other five peptides, prepared from a tryptic digest of fragment C-1 in which the lysine residues had been blocked with *S*-ethyl trifluorothioacetate, had *C*-terminal arginine, it was concluded that peptide T2 was the *C*-terminal peptide. By comparison of the sequence of peptide T3 with that of the *N*-terminal half of the heavy chain of IgG from a pathological human serum (Piggot & Press, 1967; Press, 1967) it seems probable that peptide T3 immediately precedes peptide T2, but as no overlapping sequences have been obtained this alignment is uncertain.

The partial sequence of amino acids in peptides T2 and T3 is described in the present paper. Summaries of these results have been given by Cebra (1967) and Porter (1967*a*,*b*).

MATERIALS AND METHODS

Preparation of ^{14}C*-labelled fragment C-1, substituted with* [^{14}C]*carboxymethyl groups on easily reduced half-cystine residues.* Rabbit IgG (800mg.) was dissolved in 1·0mM-EDTA in 0·1M-tris–acetate buffer, pH 8·0 (35ml.), and dialysed against the same buffer. The protein solution was clarified by passage through a Millipore membrane filter (1·2μ) and the solution was made 5mM in dithiothreitol by the addition of the reagent in the 0·1M-tris buffer (about 5ml.). The final concentration of thiol groups was 10mM. After 1hr. incubation at 37°, iodoacetic acid dissolved in 1ml. of 0·1M-tris buffer, pH 8·0, was added in an amount equimolar to the thiol concentration. The alkylating agent contained 0·2mC of iodo[2-^{14}C]acetic acid (purchased from The Radiochemical Centre, Amersham, Bucks.). After 30min., during which time the pH was maintained strictly at 8·0, the reaction mixture was applied to a column of Sephadex G-25 (coarse grade) in 0·05N-NH_3 and freed of excess of reagents. The reduced protein was then dialysed directly against N-propionic acid to dissociate heavy and light chains, and these were then separated (Fleischman, Pain & Porter, 1962). The cyanogen bromide fragment C-1 was prepared from heavy chain (Givol & Porter, 1965) and, by analysis, contained 2·4 moles of *S*-carboxymethylcysteine/mole of fragment C-1 (based on 17 leucine residues/mol.). At least 95% of the radioactivity on the heavy chain was localized in fragment C-1.

The preparation of the trifluoroacetylated peptides T2 and T3 was carried out as described by Cebra *et al.* (1968). Tryptic digestion of fully reduced non-trifluoroacetylated fragment C-1 was carried out under the same conditions as

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§ Abbreviations: IgG, immunoglobulin G; Asx and Glx (in amino acid sequences), aspartic acid or asparagine and glutamic acid or glutamine respectively.

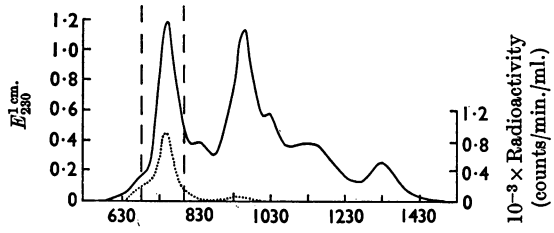

Fig. 1. Elution diagram of the acid-soluble peptides from a tryptic digest of fragment C-1 (labelled with iodo[^{14}C]-acetate) on a Sephadex G-50 column (190 cm. × 3·5 cm.) in 0·05 M-NH_4HCO_3. ——, E_{230}; ·····, radioactivity (counts/min./ml.of eluate). The broken vertical lines indicate the samples combined for further fractionation.

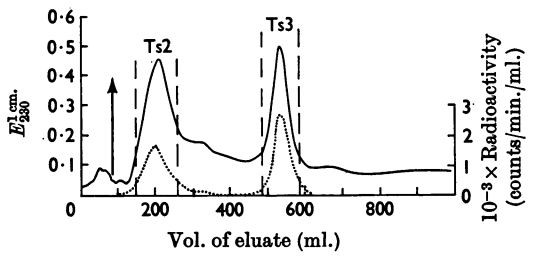

Fig. 2. Elution diagram of ^{14}C-labelled fraction from a tryptic digest of fragment C-1 (from fractionation on a column of Sephadex G-50, see Fig. 1) on a DEAE-Sephadex A25 column (20 cm. × 1·5 cm.). The column was equilibrated with 5 mM-NH_4HCO_3 and a gradient to 0·3 M-NH_4HCO_3 applied, with a 500 ml. mixing chamber, after approx. 100 ml., as indicated by the arrow. ——, E_{230}; ·····, radioactivity (counts/min./ml. of eluate).

the tryptic digestion of fragment C-1 after reaction with *S*-ethyl trifluorothioacetate; the principal labelled peptides were peptides Ts2 and Ts3 (see the Results section).

Direct isolation of peptides Ts2 and Ts3 from fragment C-1 or from whole heavy chain. The introduction of a specific label into the peptides Ts2 and Ts3 by reaction of the whole heavy chain, after gentle reduction with 5 mM-dithiothreitol, with iodo[^{14}C]acetate enabled these peptides to be easily identified and isolated from a tryptic digest of whole heavy chain or of the C-1 fraction prepared from it.

The tryptic digest (see above) was acidified (pH 4) by addition of acetic acid. About 45% of the material was precipitated but only 5% of the radioactivity. The clear supernatant was fractionated on a column of Sephadex G-50 in 0·05 M-NH_4HCO_3, giving the elution pattern shown in Fig. 1. Over 90% of the radioactivity was in a single peak appearing early. This fraction contained both peptides Ts2 and Ts3 and these could be separated by gradient elution on DEAE-Sephadex A-25 with NH_4HCO_3 buffers (Fig. 2). By analysis, peptide Ts3 appeared to be pure, but peptide Ts2 occasionally contained small amounts of other peptides that could be removed by rechromatography or electrophoresis on paper as listed in Table 1. The final yield of peptide Ts2 was 50–60% and that of peptide Ts3 was 50–85%, suggesting that most molecules of the IgG preparation contained these sequences.

Counting of ^{14}C-labelled peptides. A liquid-scintillation counter was used to determine radioactivity in fractions of column effluent. The solvent system of Kinard (1957) was used.

High-voltage electrophoresis of peptides on paper. This was carried out at pH 3·5 and 6·5 as described by Press, Piggot & Porter (1966*b*). A cooled-plate apparatus was used for analytical and preparative high-voltage electrophoresis at pH 9·25; a 0·1 M-NH_4HCO_3 buffer, pH 9·25, was employed.

Enzymic digestion of peptides. The enzymes used were those described by Press *et al.* (1966*b*), together with pepsin that had been purified by chromatography (Ryle & Porter, 1959), which was a gift from Dr A. P. Ryle. The conditions of digestion were varied to obtain maximum hydrolysis for the different peptides and were as follows: tryptic digestion in 10 mM-NH_4HCO_3 buffer, pH 8·3, at 37° for peptide T2 (300 μg. of trypsin/μmole of peptide for 12 hr.) and for peptide T3 (500 μg. of trypsin/μmole of peptide for 5 hr.); chymotryptic digestion in 10 mM-NH_4HCO_3 buffer, pH 8·0, at 37° for peptide T3 (500 μg. of chymotrypsin/μmole of peptide for 4 hr.), for peptide Ts2 (500 μg. of chymotrypsin/μmole of peptide for 4½ hr.), for peptide Ts3 (100 μg. of chymotrypsin/μmole of peptide for 3 hr.) and for peptide T-T2-LB (500 μg. of chymotrypsin/μmole of peptide for 7 hr.); pepsin digestion of peptide C-Ts3-A in dil. HCl, pH 2·0, at 37° (170 μg. of pepsin/μmole of peptide for 3½ hr.); papain digestion of peptide C-Ts2-A2 in 0·15 mM-dithiothreitol–0·15 mM-EDTA, pH 6·0, at 37° (400 μg. of papain/μmole of peptide for 16 hr.).

Determination of amino acid sequences. This was carried out by the 'dansyl'-Edman method of Gray (1967) as described briefly by Piggot & Press (1967). When *S*-carboxymethylcysteine was present in the peptide, samples were evacuated before hydrolysis. Difference analysis was carried out as required to confirm the identification of the 'dansyl' derivatives. *C*-Terminal residues were determined by hydrazinolysis (Bradbury, 1958) or by carboxypeptidase digestion as described by Press *et al.* (1966*b*).

Amino acid analyses. These were as described by Press *et al.* (1966*b*). Hydrolysis was for 24 hr. and no corrections for destruction were made.

RESULTS

Partial amino acid sequence of peptide T2

Tryptic peptides. After removal of the trifluoroacetyl groups from lysyl residues, peptide T2 was digested with trypsin (300 μg. of trypsin/μmole of peptide T2 at 37° for 12 hr.). Three lysyl bonds were hydrolysed rapidly, a fourth slowly and the others not at all. The digest was fractionated on a column of Sephadex G-50 in 0·05 N-ammonia, giving three principal fractions, which were each purified further by electrophoresis on paper at either pH 6·5 or pH 3·5, as shown in Table 1. Four main peptides were isolated, two large, peptides Ts2 and T-T2-LB, and two small, peptides T-T2-SN

Table 1. *Isolation procedures for component peptides of peptide T2*

Peptide	*N*-Terminal residue	Sephadex-gel filtration: Type	Sephadex-gel filtration: Solvent	Sephadex-gel filtration: Elution volume*	Paper electrophoresis: pH	Paper electrophoresis: Mobility†
Tryptic peptides from peptide T2						
Ts2	Thr	G-50	0·05 N-NH_3	1·5	3·5	+0·20
T-T2-SA	Asp	G-50	0·05 N-NH_3	2·4	6·5	−0·48
T-T2-SN	Val	G-50	0·05 N-NH_3	2·4	3·5	+0·46
T-T2-LB	Ser	G-50	0·05 N-NH_3	1·5	3·5	+0·24
T-T2-LB2	Ser	G-50	0·05 N-NH_3	1·7	3·5	+0·33
Chymotryptic peptide from peptide T2						
C-T2-A	Thr	G-50	0·05 M-NH_4HCO_3	1·6	3·5	+0·18
Chymotryptic peptides from peptide Ts2						
C-Ts2-A1	Thr	G-50	0·05 N-NH_3	1·4	6·5	−0·39
C-Ts2-A2	Thr	G-50	0·05 N-NH_3	1·4	6·5	−0·31
C-Ts2-N	Gly	G-50	0·05 N-NH_3	2·4	3·5	+0·16
C-Ts2-B	Ile	G-50	0·05 N-NH_3	2·2	6·5	+0·69
C-Ts2-C	Thr	G-50	0·05 N-NH_3	2·4	3·5	+0·24
Papain peptides from peptide C-Ts2-A2						
Pa-C-Ts2-A2-C	Thr	G-25	0·05 N-NH_3	1·1	6·5	−0·34
Pa-C-Ts2-A2-B	Ser	G-25	0·05 N-NH_3	1·6	3·5	+0·65
Pa-C-Ts2-A2-M	CyS·CH_2·CO_2H	G-25	0·05 N-NH_3	1·1	6·5	−0·65
Pronase peptides from fragment C-1						
P-A1	Ser	G-25	0·05 M-NH_4HCO_3	1·1	3·5	+0·19
P-A2	CyS·CH_2·CO_2H	G-25	0·05 M-NH_4HCO_3	1·1	3·5	−0·03
					6·5	−0·35
Chymotryptic peptides from peptide T-T2-LB						
C-T-T2-LB-B	Thr	G-50	0·05 N-NH_3	1·8	3·5	+0·79
C-T-T2-LB-A	Not estimated	G-50	0·05 N-NH_3	1·1	3·5	−0·40
C-T-T2-LB-H	Not estimated	—	—	—	3·5	+0·46

* Given relative to exclusion volume = 1·0.
† Mobility given relative to lysine = +1·0, aspartic acid = −1·0, neutral amino acids = 0.

Table 2. *Analysis of tryptic peptides from peptide T2*

	Composition (moles of amino acid residue/mole of peptide): Tryptic peptides of peptide T2: Peptide Ts2	Peptide T-T2-SA	Peptide T-T2-SN	Peptide T-T2-LB	Sum of peptides	Peptide T2	Peptide T-T2-LB2*
Lys	2·9	—	0·99	1·0	4·9	5·2	1·1
His	—	—	—	0·94	0·94	1·0	0·96
Asp	—	0·98	1·0	2·0	4·0	4·0	2·0
Thr	2·9	0·93	—	3·7	7·5	7·8	2·7
Ser	3·1	—	—	4·8	7·9	9·8	2·9
Glu	0·98	—	—	1·1	2·1	2·9	1·0
Pro	9·8	—	—	3·1	13	12	2·1
Gly	2·0	—	—	—	2·0	4·2	—
Ala	1·1	—	—	2·0	3·1	3·1	2·0
Val	2·3	—	1·0	4·0	7·3	7·9	2·0
Ile	0·91	—	—	—	0·91	1·1	—
Leu	2·0	1·1	—	—	3·1	5·0	—
Tyr	—	—	—	—	—	0·9	—
Phe	1·9	—	—	—	1·9	2·0	—
CyS·CH_2·CO_2H	1·6	—	—	0·73	2·3	2·3	0·78
Homoserine	—	1·0	—	—	1·0	1·0	—

* Peptide T-T2-LB2 is a partial hydrolysis product of peptide T-T2-LB. For details see the text.

and T-T2-SA, together with a fifth, peptide T-T2-LB2, present only in small yield. The analyses of these peptides are given in Table 2 and the sum of the four main peptides is compared with that of the whole of peptide T2. The agreement for many of the residues is good, but there is clearly a peptide missing that contains the single tyrosine residue and perhaps nine or ten additional residues. A tyrosine-containing peptide was identified during fractionation by paper electrophoresis, but it appeared to be adsorbed strongly and elution in satisfactory yield was not achieved. The alignment of the other four peptides suggests that this tyrosine peptide is *N*-terminal in peptide T2. The sequences of the two small peptides, T-T2-SA and T-T2-SN, were established directly by the 'dansyl'-Edman technique: that of peptide T-T2-SN was found to be Val-Asp-Lys, and that of peptide T-T2-SA was Asp-Thr-Leu-homoserine. The peptide Val-Asp-Lys was found by Hill, Delaney, Lebovitz & Fellows (1966) in a tryptic digest of the whole heavy chain, but not in a tryptic digest of fragment Fc. The presence of the homoserine established Asp-Thr-Leu-homoserine as the *C*-terminal peptide of peptide T2 and therefore of the whole fragment C-1. This peptide was isolated by Hill *et al.* (1966) from a tryptic digest of fragment Fc.

Amino acid sequence of peptide Ts2. The *N*-terminal sequence was determined directly for four residues. Peptide Ts2 was then digested with chymotrypsin (as shown in Fig. 3) and after fractionation of the digest, as outlined in Table 1, the peptides whose composition is given in Table 3 were isolated. Peptide C-Ts2-B contained two of the three lysine residues of peptide Ts2, and the sequence by the 'dansyl'-Edman technique showed that one was *C*-terminal and hence that this was the *C*-terminal peptide of peptide Ts2. A partial digest product was obtained whose composition equalled the sum of peptides C-Ts2-N and C-Ts2-B, placing peptide C-Ts2-N immediately before peptide C-Ts2-B. Both peptides were subjected to sequence analysis as shown in Fig. 3 to give the order of the *C*-terminal 13 residues of peptide Ts2. The peptides C-Ts2-A1 and C-Ts2-A2 had *N*-terminal threonine as did the whole of peptide Ts2, and hence either or both could be *N*-terminal. However, peptides C-Ts2-C and C-Ts2-A1 together had a composition equal to that of peptide C-Ts2-A2, and peptide C-Ts2-C contained the *N*-terminal tetrapeptide of peptide Ts2 together with serine. It is clear that peptide C-Ts2-C and therefore peptide C-Ts2-A2 are *N*-terminal, as shown in Fig. 3. Confirmation of this was obtained by showing the *N*-terminal sequence of peptide C-Ts2-A1 to be Thr-Cys and by the isolation, after papain digestion of peptide C-Ts2-A2, of the peptide Pa-C-Ts2-A2-C, which confirmed the overlap of peptides C-Ts2-C and

Table 3. *Analysis of component peptides of peptide Ts2*

Composition (moles of amino acid residue/mole of peptide)

	Peptide Ts2	Chymotryptic peptides of peptide Ts2					Pronase peptides of fragment C-1		Papain peptides of peptide C-Ts2-A2		
		Peptide C-Ts2-A1	Peptide C-Ts2-A2	Peptide C-Ts2-N	Peptide C-Ts2-B	Peptide C-Ts2-C	Peptide P-A1	Peptide P-A2	Peptide Pa-C-Ts2-A2-C	Peptide Pa-C-Ts2-A2-B	Peptide Pa-C-Ts2-A2-M
Lys	2·9	1·0	1·0	—	2·1	—	1·1	1·0	—	1·0	—
Thr	2·9	1·8	2·4	—	—	0·90	0·93	0·98	1·6	0·80	—
Ser	3·1	1·0	2·0	0·91	—	1·0	0·87	0·93	1·1	0·99	—
Glu	0·98	1·0	1·0	—	—	—	1·0	1·0	—	—	1·0
Pro	9·8	3·9	4·8	1·2	3·5	0·92	4·2	3·9	1·1	0·85	3·1
Gly	2·0	—	—	1·9	—	—	—	—	—	—	—
Ala	1·1	—	1·0	—	—	1·0	—	—	1·0	—	—
Val	2·3	—	0·96	1·0	—	1·0	—	—	1·0	—	—
Ile	0·91	—	—	—	0·95	—	—	—	—	—	—
Leu	2·0	2·0	2·0	—	—	—	0·55*	—	—	—	1·9
Phe	1·9	—	—	0·99	1·0	—	—	—	—	—	—
$CyS{\cdot}CH_2CO_2H$	1·6	0·81	0·84	—	—	—	0·84	1·3	0·61	—	0·43

* Mixture of two peptides, one with leucine and the other without.

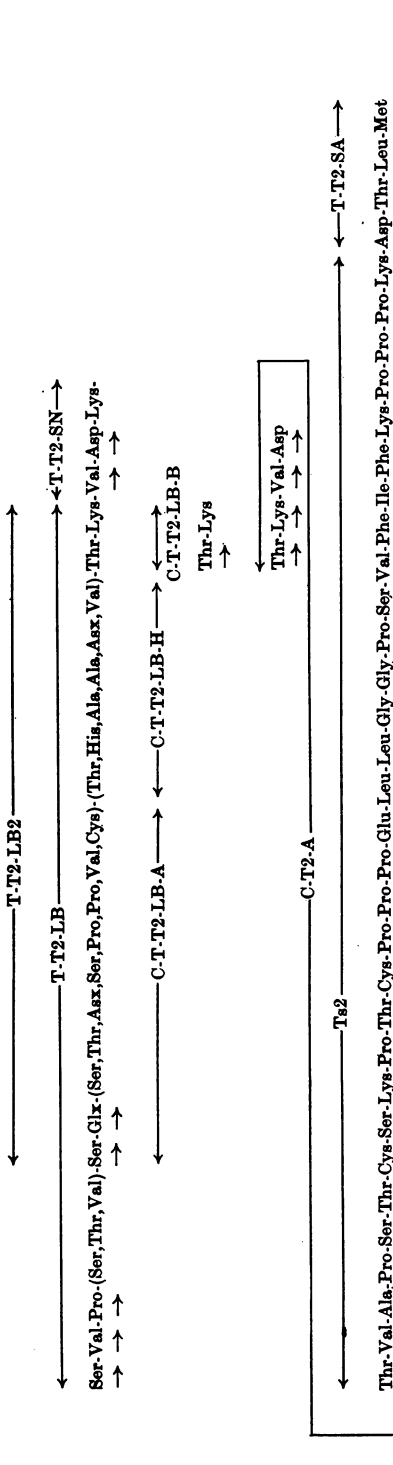

Fig. 3. Partial amino acid sequence of tryptic peptide T2 showing the peptides derived from it. Sequence determination from the *N*-terminal end was by the 'dansyl'-Edman technique, shown by →, and from the *C*-terminal end was by hydrazinolysis or hydrolysis with carboxypeptidase A, shown by ←.

Table 4. *Analysis of chymotryptic peptides of peptides T-T2-LB and T2*

	Composition (moles of amino acid residue/mole of peptide)			
	Chymotryptic peptides of peptide T-T2-LB			Chymotryptic peptide of peptide T2: peptide C-T2-A
	Peptide C-T-T2-LB-B	Peptide C-T-T2-LB-A	Peptide C-T-T2-LB-H	
Lys	1·0	—	—	3·0
His	—	—	0·86	—
Asp	—	1·0	0·95	1·1
Thr	0·83	1·0	0·75	3·6
Ser	—	2·6	—	1·9
Glu	—	0·98	—	1·0
Pro	—	0·98	—	5·3
Gly	—	—	—	—
Ala	—	—	2·1	1·1
Val	—	1·2	1·0	2·0
Ile	—	—	—	—
Leu	—	—	—	2·0
Tyr	—	—	—	—
Phe	—	—	—	—
$CyS{\cdot}CH_2{\cdot}CO_2H$	—	0·4	—	0·91
Homoserine	—	—	—	—

C-Ts2-A1. Another papain-digest peptide, Pa-C-Ts2-A2-M, accounted for the *C*-terminal end of peptide C-Ts2-A2 as the full sequence by the 'dansyl'-Edman technique overlapped those of the peptides P-A1 and P-A2 isolated from a Pronase digest of the whole fragment C-1. These Pronase peptides could be identified by their radioactive markers and were isolated as shown in Table 1. The sequence of peptide P-A1 and the partial sequence of peptide P-A2 were determined by the 'dansyl'-Edman method and gave the sequence of the rather difficult section between the two half-cystine residues, as shown in Fig. 3.

Partial sequence of peptide T-T2-LB. This was derived from direct determination of the *N*-terminal sequences both on the whole peptide and on the partial digest product peptide T-T2-LB2 derived from it. This was extended by chymotryptic digestion of peptide T-T2-LB and isolation of three peptides (Table 1). The compositions of peptides C-T-T2-LB-A, -H and -B are given in Table 4. Peptide C-T-T2-LB-B was the *C*-terminal dipeptide Thr-Lys. Peptides C-T-T2-LB-A and C-T-T2-LB-H accounted for the remainder of peptide T-T2-LB2 except for a discrepancy of one proline residue, arising probably from an analytical error.

Alignment of the tryptic peptides. The alignment of peptides T-T2-LB2, T-T2-SN and Ts2 was given by the isolation of a large peptide, C-T2-A, from a chymotryptic digest of the whole peptide T2. The method of isolation is given in Table 1 and the analysis in Table 4. It contained three lysine residues and tryptic digestion gave two small peptides, Thr-Lys, as obtained from the chymotryptic digest of peptide T-T2-LB, and Val-Asp-Lys, identical with peptide T-T2-SN. Hence this placed the tryptic peptides in the order shown in Fig. 3. This order was confirmed by direct determination of the *N*-terminal sequence of peptide C-T2-A. Carboxypeptidase digestion of peptide C-T2-A released two leucine residues quantitatively and hydrazinolysis of the residual peptide gave glutamic acid as the *C*-terminal residue. This placed the *C*-terminal end of peptide C-T2-A in the sequence in agreement with its composition. The point of chymotryptic cleavage that released this peptide from the whole peptide T2 molecule was in agreement with the chymotryptic splits observed in the constituent peptides.

The positioning of peptide T-T2-SA immediately after peptide Ts2 is taken from the partial sequence obtained by Hill *et al.* (1966) and the results reported here for the *C*-terminal part of peptide T2 are in agreement with that work.

From this alignment it follows that the missing peptide containing a tyrosine residue must be from the *N*-terminal end of peptide T2.

Partial amino acid sequence of peptide T3

Tryptic peptides. Tryptic digestion of peptide T3 after removal of the trifluoroacetyl groups gave rapid splitting at the three lysyl residues, and three of the four tryptic peptides were isolated as shown in Table 5. Peptide Ts3 came off a column of Sephadex G-50 in 0·05N-ammonia cleanly and could be purified further if necessary by electro-

Table 5. *Isolation procedures for component peptides of peptide T3*

Peptide	*N*-Terminal residue	Sephadex-gel filtration: Type	Sephadex-gel filtration: Solvent	Sephadex-gel filtration: Elution volume*	Paper electrophoresis: pH	Paper electrophoresis: Mobility†
Tryptic peptides from peptide T3						
Ts3	Ala	G-50	0·05 N-NH_3	1·2	6·5	−0·32
T-T3-LY	Gly	(*a*) G-50	0·05 N-NH_3	1·6	9·25	−0·26
		(*b*) G-50	0·05 N-Acetic acid	1·1		
T-T3-LB	Asx	(*a*) G-50	0·05 N-NH_3	1·6	9·25	−0·47
		(*b*) G-50	0·05 N-Acetic acid	1·1		
Chymotryptic peptide from peptide T3						
C-T3-H	Val	G-50	0·05 N-NH_3	1·2	6·5	−0·21
Chymotryptic peptides from peptide Ts3						
C-Ts3-B	Gly	G-25	0·05 N-NH_3	1·3	—	—
C-Ts3-A	Ala	G-25	0·05 N-NH_3	1·0	—	—
Peptic peptides from peptide Ts3						
Pe-C-Ts3-Aa	Val	G-50	0·05 N-NH_3	2·4	3·5	+0·29
Pe-C-Ts3-Ad	Ala	G-50	0·05 N-NH_3	2·4	3·5	+0·18
Pe-C-Ts3-AA	Pro	G-50	0·05 N-NH_3	1·6	—	—
Pronase peptide from fragment C-1						
P3	Ala	G-25	0·05 M-NH_4HCO_3	1·1	3·5	−0·60
Papain peptides from peptide P3						
Pa-P3-2	Ala	—	—	—	3·5	−0·79
Pa-P3-4	Asp	—	—	—	3·5	−0·53
Chymotryptic peptide from peptide C-T-T3-LB						
C-T-T3-LB-B	Val	—	—	—	3·5	Weakly basic

* Given relative to exclusion volume = 1·0.

† Mobility given relative to lysine = +1·0 (except at pH 9·25, given relative to arginine = +1·0), aspartic acid = −1·0, neutral amino acids = 0.

Table 6. *Analysis of component peptides of peptide T3*

	Composition (moles of amino acid residue/mole of peptide): Tryptic peptides of peptide T3: Peptide Ts3	Tryptic peptides of peptide T3: Peptide T-T3-LY	Tryptic peptides of peptide T3: Peptide T-T3-LB	Tryptic peptides of peptide T3: Sum of peptides	Peptide T3	Chymotryptic peptide of peptide T3: peptide C-T3-H	Chymotryptic peptide of peptide T-T3-LB: peptide C-T-T3-LB-B
Lys	1·0	—	1·0	2·0	3·0	1·0	1·0
Arg	—	0·83	—	0·83	1·0	—	—
Asp	1·0	2·1	1·0	4·1	6·2	0·98	—
Thr	3·0	3·7	1·9	8·6	9·3	3·2	0·9
Ser	2·8	1·2	2·0	6·0	7·2	4·2	1·9
Glu	—	1·1	1·3	2·4	4·4	1·2	1·0
Pro	4·1	2·0	1·4	7·5	7·7	5·5	1·0
Gly	2·2	3·1	3·1	8·4	9·0	2·0	1·2
Ala	2·0	—	—	2·0	3·9	2·1	—
Val	2·8	3·0	1·9	7·7	8·2	2·9	1·2
Ile	—	—	—	—	1·0	—	—
Leu	3·0	2·0	1·8	6·8	7·3	1·9	—
Tyr	—	0·87	—	0·87	2·8	—	—
Phe	1·0	—	—	1·0	2·1	0·9	—
$CyS{\cdot}CH_2{\cdot}CO_2H$	3·0	—	—	3·0	3·1	1·4	—

phoresis on paper at pH 6·5. Peptides T-T3-LY and T-T3-LB were not separated on Sephadex columns in either ammonia or acetic acid solutions, but they could be resolved from each other by electrophoresis on paper at pH 9·25. Analysis (Table 6) suggested that peptides Ts3, T-T3-LY and

Table 7. *Analysis of component peptides of peptide Ts3*

	Composition (moles of amino acid residue/mole of peptide)								
		Chymotryptic peptides of peptide Ts3		Peptic peptides of peptide C-Ts3-A			Pronase peptide of fragment C-1: peptide P3	Papain peptides of peptide P3	
	Peptide Ts3	Peptide C-Ts3-B	Peptide C-Ts3-A	Peptide Pe-C-Ts3-Ad	Peptide Pe-C-Ts3-Aa	Peptide Pe-C-Ts3-AA		Peptide Pa-P3-2	Peptide Pa-P3-4
Lys	1·0	1·0	—	—	—	—	—	—	—
Asp	1·0	—	1·0	—	—	1·0	1·0	—	1·0
Thr	3·0	—	2·9	—	0·88	2·0	1·0	—	0·98
Ser	2·8	—	2·8	0·98	—	2·0	1·0	—	1·0
Pro	4·1	—	4·7	1·2	—	3·4	1·9	0·87	0·96
Gly	2·2	1·0	1·3	—	—	1·1	1·0	1·0	—
Ala	2·0	—	2·1	0·99	—	0·95	0·96	0·68	—
Val	2·8	1·1	2·0	1·0	0·99	—	—	—	—
Leu	3·0	1·0	2·0	—	1·0	0·98	—	—	—
Phe	1·0	—	1·0	0·98	—	—	—	—	—
$CyS{\cdot}CH_2{\cdot}CO_2H$	3·0	0·5	2·0	—	—	1·7	1·8	2·0	—

T-T3-LB were pure, but they accounted for only 61 of the estimated 76 residues in peptide T3, and it is clear that there is a fourth peptide that has not yet been isolated. This missing peptide, which terminates in lysine, contains two tyrosine and one phenylalanine residues and probably 15 residues in all.

Amino acid sequence of peptide Ts3. Preparative amounts of this peptide were obtained directly by tryptic digestion of reduced carboxymethylated fragment C-1 that had not been trifluoroacetylated (see the Materials and Methods section). Chymotryptic digestion of peptide Ts3 gave two peptides, C-Ts3-A and C-Ts3-B, which together accounted for the whole of peptide Ts3 (Table 7). The sequence of the smaller peptide, C-Ts3-B, was determined by the 'dansyl'-Edman technique and was Gly-Cys-Leu-Val-Lys; hence this peptide was assumed to be *C*-terminal (Fig. 4). Peptide C-Ts3-A had *N*-terminal alanine, as did whole peptide Ts3, and the 'dansyl'-Edman technique gave the sequence of the first eight *N*-terminal residues and carboxypeptidase followed by hydrazinolysis gave the first three *C*-terminal residues. Peptide digestion of peptide C-Ts3-A gave three peptides, Pe-C-Ts3-Ad, Pe-C-Ts3-AA and Pe-C-Ts3-Aa (Tables 5 and 7). The sequences of peptides Pe-C-Ts3-Ad and Pe-C-Ts3-Aa showed these to be the *N*-terminal pentapeptide and *C*-terminal tripeptide respectively of peptide C-Ts3-A. The large peptide, Pe-C-Ts3-AA, gave the *N*-terminal sequence Pro-Leu-Ala by the 'dansyl'-Edman technique, and carboxypeptidase followed by hydrazinolysis gave the *C*-terminal tripeptide sequences Ser-Ser-Thr. The sequence was completed by isolating a nonapeptide, peptide P3, from a Pronase digest of fragment C-1 (Tables 5 and 7). The *N*-terminal sequence of peptide P3 by the 'dansyl'-Edman technique gave Ala-Pro-Cys, but could not be continued. However, hydrolysis of peptide P3 with papain at pH 4·3 split it into a pentapeptide [Pa-P3-2 (*S*-carboxymethyl-Cys_2,Pro,Gly,Ala)] and a tetrapeptide [Pa-P3-4-(Asp,Thr,Ser,Pro)] (Tables 5 and 7). Hydrazinolysis gave glycine as the *C*-terminal residue of the pentapeptide Pa-P3-2 and hence its sequence must be Ala-Pro-Cys-Cys-Gly. The sequence of the tetrapeptide Pa-P3-4 by the 'dansyl'-Edman technique and hydrazinolysis was found to be Asp-Thr-Pro-Ser. Hence the full sequence of peptide Ts3 was as given in Fig. 4.

Partial sequence of peptide T-T3-LB. The 'dansyl'-Edman technique gave the *N*-terminal sequence Asx-Leu and chymotryptic digestion gave a peptide, C-T-T3-LB-B, with valine *N*-terminal. As peptide C-T-T3-LB-B contained the only lysine residue it was presumed to be from the *C*-terminal section, as shown in Fig. 4.

Partial sequence of peptide T-T3-LY. As this peptide contains the only arginine residue it is assumed to be the *C*-terminal peptide of peptide T3. No sequence studies of peptide T-T3-LY were carried out, except to identify glycine as the *N*-terminal residue.

Alignment of the tryptic peptides. The alignment of the three peptides was derived from a chymotryptic digest of peptide T3, which led to the isolation of the peptide C-T3-H. The method of isolation of the peptide is given in Table 5 and its composition in Table 6. Peptide C-T3-H was found on analysis to contain 29 residues including both the *N*-terminal 21 residues of peptide Ts3, which constitute the chymotryptic peptide C-Ts3-A, and an additional eight residues including lysine. The peptide C-T3-H must therefore be an *N*-terminal extension of peptide Ts3. The 'dansyl'-Edman technique gave Val-Gly-Glx-Pro. Tryptic digestion of peptide C-T3-H gave a basic peptide T-C-T3-H-a with the same composition as the chymotryptic peptide C-T-T3-LB-B from the *C*-terminal end of peptide T-T3-LB. Hence peptide T-T3-LB must precede peptide Ts3.

As the alignment of peptide T-T3-LY with peptide

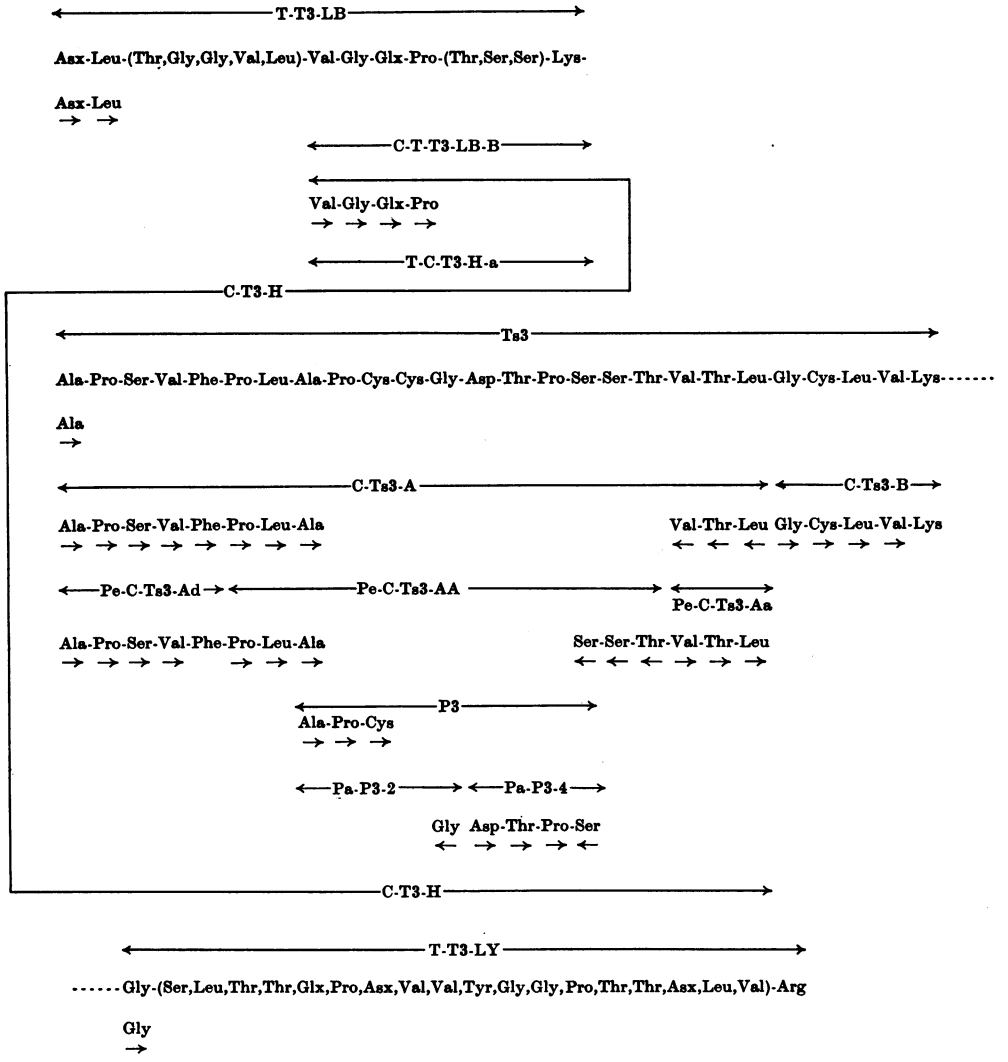

Fig. 4. Partial amino acid sequence of the tryptic peptide T3 showing the peptides derived from it. Sequence determination from the *N*-terminal end was by the 'dansyl'-Edman technique, shown by →, and from the *C*-terminal end by hydrazinolysis or hydrolysis with carboxypeptidase A, shown by ←. The broken lines (------) indicate the most probable position of the missing peptide.

Ts3 has not been achieved, it leaves the question open whether the missing peptide is *N*-terminal or comes between peptides Ts3 and T-T3-LY. As peptide T-T3-LB has *N*-terminal aspartic acid, and no *N*-terminal acid could be detected in the whole of peptide T3, it is most likely that the missing peptide is *N*-terminal, but further study will be necessary to establish this point.

DISCUSSION

A partial sequence has been described for two large peptides that together account for about 145 residues of the 240 believed to be present in the *N*-terminal section of the rabbit IgG heavy chain split off by cyanogen bromide cleavage and named fragment C-1. Though incomplete, these sequences prove that peptides T2 and T3 are from distinct sections of the heavy chain, and it is likely that this is true also for the other peptides (T1, T4, T5 and T7) from fragment C-1 (Cebra *et al.* 1968). The higher yields (about 50–85%) of peptides Ts2 and Ts3 by direct isolation from a tryptic digest of fragment C-1 suggest that the larger peptides T2 and T3 are also present in most of the molecules and that the lower recoveries of these are due to the difficulties of

fractionation. The agreement of the *C*-terminal sequence of peptide T2 with the partial *N*-terminal sequence of fragment Fc given by Hill *et al.* (1966) places peptide T2 as *C*-terminal. Indirect evidence (Cebra *et al.* 1968) suggests that peptide T3 immediately precedes peptide T2, but this cannot be established until overlapping peptides have been isolated. It seems probable that a comprehensible sequence may be obtainable for most, and perhaps all, of the Fd section of the heavy chain of rabbit IgG from pooled serum. The isolation of the same fractions in about the same yield from purified anti-DNP antibody (Cebra *et al.* 1968) suggests that a similar sequence will be found also in the Fd section of purified antibodies. Clearly, many possibilities of variation remain. Two methionine residues in fragment C-1 are known to be present in only a fraction of the molecules (J. W. Prahl, unpublished work; Press, Givol, Piggot, Porter & Wilkinson, 1966*a*) and other variants have been reported in the *N*-terminal sequence (Wilkinson, Press & Porter, 1966). There is no doubt that others will be found, but none of those identified so far correlates with antibody specificity. If the specificity is dependent on sequence, then the variable section may well be in a relatively short section. In IgG from pooled serum of normal animals, which is believed to be a mixture of many thousands of antibodies, such a section would be so complex as to yield no detectable peptides. Until a continuous sequence for the whole of the fragment Fd has been established this possibility remains. The results reported here suggest that such a section, if it exists, will be less than, say, 25 residues, as 85–90% of the fragment Fd appears to be accounted for. There could, however, be a larger variable section controlling antibody specificity if only, say, 10% of the changing residues were altered in any one antibody. If such was the case, then in a large pool of antibodies the residues would be constant in 90% of the molecules and detection of the 10% that were different would not be possible by the methods used. A coherent sequence would then be obtained and such variations as were found would be due to allotypic or subclass changes. However, if a purified antibody were studied the differences in 10% of the variable positions should be apparent. If the work described here can be taken to completion to give a full sequence of the Fd section of the heavy chain both from pooled IgG and from a purified antibody, it should lead to the identification of residues concerned in the control of antibody specificity, whatever mechanism is involved.

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