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Human Immunoglobulin Subclasses

PARTIAL AMINO ACID SEQUENCE OF THE CONSTANT REGION OF A γ4 CHAIN

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The heavy chain of a human myeloma protein (Vin) belonging to the γ4 subclass was subjected to tryptic digestion after reduction and carboxymethylation. Cyanogen bromide fragments were also prepared and all 19 tryptic peptides that account for one of them (the Fc-like fragment) were studied. Selected peptic peptides were isolated and provided evidence for the order of 15 of the tryptic peptides. In addition the sequence of two large peptic peptides derived from two sections of the molecule including all the interchain bridges is presented. Comparison with published data on other chains allows us to propose a sequence of γ4 chains that extends from just before the presumed starting point of the invariable region (at about residue 113) to the *C*-terminal end of the chain (approx. residue 446), except for a section of about 50 residues. The results of the comparison suggest that the immunoglobulin subclasses have a recent independent evolutionary origin in different species. Implications for complement fixation and for the evolutionary origin of antibody diversity are also discussed.

The heavy chains of human IgG‡ molecules belong to one of four subclasses, γ1, γ2, γ3 or γ4. The distinction between subclasses is made on the basis of antigenic (Grey & Kunkel, 1964; Terry & Fahey, 1964) or amino acid-sequence differences (Frangione, Milstein & Franklin, 1969*a*) in the heavy chains of these molecules. The commonest subclass is γ1 and the rarest is γ4 (Terry, Fahey & Steinberg, 1965). Different subclasses of IgG molecules have different biological properties: thus IgG4 molecules do not fix complement, whereas molecules of other subclasses do (Ishizaka, Ishizaka, Salmon & Fundenberg, 1967).

Amino acid-sequence studies (Milstein, Frangione & Pink, 1967; Prahl, 1967; Frangione, Milstein & Pink, 1969*b*) have demonstrated a large degree of homology between human IgG chains of different subclasses, at least in selected regions of the chains, and particularly around their intrachain disulphide bridges. The present paper presents sequence results on fragments of the heavy chains of a human IgG4 myeloma protein (protein Vin). One of them, of about 200 residues, is derived from the *C*-terminal half of the heavy chain and corresponds approximately to an Fc fragment. The results are compared with the sequences of Fc fragments from rabbit IgG (Hill, Lebovitz, Fellows & Delaney, 1967) and a human γ1 myeloma protein (protein Eu; Edelman *et al.* 1969) and the implications of the comparisons for the evolution of the subclasses are discussed.

Some additional results on sequences around the interchain bridges, which include the probable site of the switch from the constant to the variable regions of the heavy chain, are also included.

Earlier studies on cyanogen bromide fragments and selected sequences of protein Vin have been published (Pink & Milstein, 1967, 1968).

MATERIALS

Protein Vin (kindly supplied by Dr G. P. Clein) was normally purified by elution from a column (25 cm × 3 cm) of DEAE-cellulose (Whatman DE 52) with a linear sodium phosphate gradient (0.01–0.2 M; pH 7.9). One preparation, used in the cyanogen bromide cleavage experiments, was carried out at pH 6.9 to minimize possible disulphide exchange. The preparations were established as sufficiently pure for sequence work by serological typing (kindly carried out by Dr E. C. Franklin and Dr H. G. Kunkel), and by electrophoresis on paper in 0.05 M-veronal buffer, pH 8.2.

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‡ Abbreviations: IgG, immunoglobulin G; Ccm, carboxymethylcysteine; Cms, carboxymethylcysteine sulphone.

Reagents were generally of analytical reagent grade. Mβ-ercaptoethanol and reagents used in the Edman degradation cycle were redistilled and stored under N_2. Carrier iodoacetic acid solutions were extracted before use with a small amount of chloroform to remove iodine. Buffered 6.6M-guanidine chloride solutions were prepared by addition of conc. HCl to solid guanidine carbonate until the solution became slightly acid (pH4–5); solid tris was then added to give the desired concentration and the pH was adjusted to the required value with a small amount of conc. HCl.

Radioactive iodoacetic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Trypsin, pepsin and chymotrypsin were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Subtilisin was obtained from Novo Terapeutisk, Copenhagen, Denmark.

METHODS

Cyanogen bromide cleavage. Protein (150mg) in 7.5ml of aq. 70% formic acid was treated with 250mg of cyanogen bromide for 24h at room temperature, with stirring (Press, Piggot & Porter, 1966). The digest was diluted tenfold, freeze-dried and applied to an upward-flow Sephadex G-100 column (60cm × 3cm), run in 6M-urea–0.25M-formic acid. The extinction profile obtained is shown in Fig. 1. Fractions CB1 and CB2 were each dialysed against 0.5% formic acid before freeze-drying; the remaining fractions were desalted on a Sephadex G-25 (coarse grade) column run in 5% formic acid. Fragments were characterized by identification of their *N*-terminal residues and isolation of carboxymethylcysteine-containing peptides from each after total reduction and alkylation (Pink & Milstein, 1968).

Preparation of heavy chains. IgG interchain bridges were reduced by the addition of β-mercaptoethanol (final concn. 0.2M) to protein solutions (1% protein in 0.5M-tris–HCl buffer, pH8.2). After incubation for 90min under N_2 at 37°C, the solution was applied directly to a Sephadex G-100 column (80cm × 3cm), run in 5% formic acid. Yields (by weight) of the separated chains were within 10% of the theoretical values.

Total reduction and alkylation with iodo[^{14}C]acetic acid. The protein was totally reduced as follows. Solutions of protein (10mg/ml) in 6.6M-guanidine hydrochloride –0.1M-tris–HCl buffer, pH8.2, were treated with dithiothreitol (from a fresh 10mg/ml solution) at a final concentration of 2.8mM. This represents a two- to three-fold excess of reagent over protein disulphide bridges. Reduction was carried out for 90min at 37°C under N_2. Iodo-[^{14}C]acetic acid was then added to give a 9mM solution; the iodoacetic acid stock solution was 0.1M and 50μCi/ml. Blocking was carried out for 3h at room temperature and the solution was then dialysed extensively against 1% NH_4HCO_3 buffer, pH8.4, in the cold.

Selective reduction and radioactive labelling of interchain bridges. Protein Vin (40mg) was partially reduced and the interchain bridges were specifically labelled with iodo-[^{14}C]acetic acid (Frangione *et al.* 1969*a*). The chains were then further reduced and alkylated with non-radioactive iodoacetic acid after the addition of an equal volume of 7M-guanidine–0.1M-tris–HCl buffer, pH8.2. Reduction was carried out in 0.3M-mercaptoethanol for 90min at 37°C, and alkylation in 0.5M-iodoacetic acid for 2h at room temperature. The protein solution was dialysed against 5% formic acid in preparation for peptic digestion.

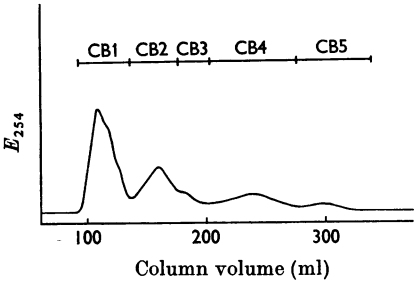

Fig. 1. Separation of the products of cyanogen bromide cleavage of protein Vin on a Sephadex G-100 column. The solvent was 6M-urea–0.2M-formic acid.

Preparation and fractionation of enzyme digests. Totally carboxymethylated heavy chain (300mg in 35ml of 1% NH_4HCO_3) was digested with trypsin at pH8.5 in a pH-stat at room temperature for 90min. The enzyme/protein ratio was 1:100 (w/w). The digestion was stopped by addition of di-isopropyl phosphorofluoridate [5μl of solution diluted 1/100 (v/v)], and after 1h applied to a column (40cm × 2cm) of Dowex 1 (X2) resin. Fractionation (as shown in Fig. 2*a*) was accomplished with the nine-chamber Varigrad system of Funatsu (1964).

A second tryptic digest of heavy chains (80mg) was prepared in the same way, but fractionated (as shown in Fig. 2*b*) on a column of Sephadex G-50 run in 1% NH_4HCO_3.

A mild peptic digestion of protein with selectively radioactively labelled interchain bridges (prepared as described above) was carried out at room temperature for 16h at enzyme:protein ratio 1:100. Other peptic digestions of protein and cyanogen bromide fragments were carried out in 5% formic acid for 16h at 37°C. The protein concentration was 1% and the enzyme:protein ratio was 1:40. Digestions of peptides were carried out for 2–6h at 37°C on 20–200nmol of peptide, with 5–50μg of enzyme. The solvents used (20–200μl) were 1% NH_4HCO_3 (for trypsin, chymotrypsin and subtilisin) and 0.01M-HCl (for pepsin).

Electrophoresis and chromatography. High-voltage electrophoresis of peptides was carried out essentially as described by Ambler (1963) or Milstein (1966). Electrophoreses were carried out for 40–100min unless otherwise stated. Mobilities (*m*) (Ambler, 1963; Offord, 1966) at pH6.5 are expressed as fractions of the distance between ϵ-DNP-lysine and aspartic acid. A butanol–acetic acid–water–pyridine (15:3:12:10, by vol.) system (BAWP) (Waley & Watson, 1953) was used for chromatographic separations. Peptides were eluted with water, 0.5% acetic acid or 0.5% ammonia. Ammonia was avoided for peptides containing carboxymethylcysteine sulphone. Detection of material on paper, acid hydrolysis and amino acid analysis were carried out as described by Milstein (1966). The HCl used contained phenol (1mg/ml) to prevent destruction of tyrosine (Sanger & Thompson, 1963).

N-*Terminus and sequence determination.* The *N*-termini of proteins and cyanogen bromide fragments

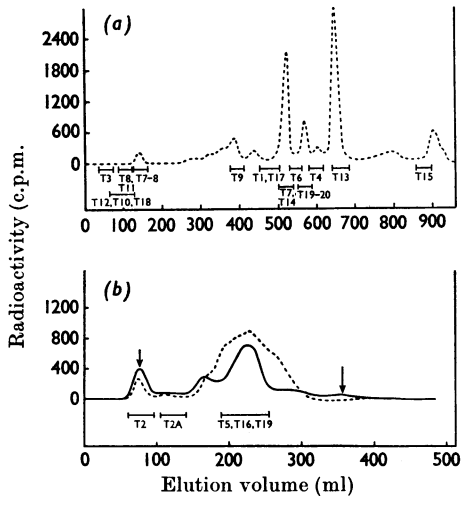

Fig. 2. Fractionation of peptides resulting from tryptic digestion of Vin γ4 chains. The elution position of the different peptides is shown with horizontal bars. (*a*) On a column (40 cm × 2 cm) of Dowex 1 (X2) resin. The nine-chamber Varigrad pyridine–collidine acetate system of Funatsu (1964) was used for fractionation. (*b*) On a column (60 cm × 2 cm) of Sephadex G-50 in 1% NH_4HCO_3. Arrows mark the breakthrough and hold-up volumes. Peptide T2A was not purified from heavy chains, but from a similar fractionation of tryptic peptides from cyanogen bromide fragment CB2. ——, E_{254}; ----, c.p.m. (10 μl samples).

were determined by 'dansylation' of samples (2 mg) in 8 M-urea–0.1 M-Na_2CO_3 (Gray, 1967). After reaction at room temperature overnight the protein was precipitated by adding 1 vol. of 10% (v/v) acetic acid to it, keeping it for 1 h at 4°C and centrifuging it. The precipitated protein was washed by dissolving it in conc. acetic acid and reprecipitating it with 4 vol. of acetone. The protein precipitate was then dried and hydrolysed.

N-Terminal residues of peptides were made to react with DNS chloride (Gray, 1967) and the derivatives were identified by two-dimensional t.l.c. (Woods & Wang, 1967). A third solvent (solvent IV of Crowshaw, Jessup & Ramwell, 1967) was used to separate glutamic acid and aspartic acid, and threonine and serine; this chromatography was carried out in the second dimension.

The results of the 'dansyl'–Edman procedure are shown in the figures, with arrows over the peptide on which the experiments were performed. The double arrow is used to indicate that the DNS-amino acid was obtained without the acid hydrolysis, establishing the release of a free amino acid in the *C*-terminal position.

Amide residues were assigned on the basis of electrophoresis mobilities of peptides at pH 6.5 (Offord, 1966).

RESULTS

The results are described in three sections. The isolation of and sequence studies on 19 unique tryptic peptides from totally carboxymethylated Vin heavy chains are presented in section (*a*). These peptides are present in cyanogen bromide fragment CB2. Overlaps between 15 of them were established by isolation of the peptic peptides from fragment CB2 described in section (*b*). A partial sequence for fragment CB2 was deduced from these results. The sequences of two peptic peptides from regions including the interchain bridges of the molecules are described in section (*c*).

(*a*) *Tryptic peptides from Vin heavy chain.* Tryptic peptides isolated from carboxymethylated heavy chains are numbered and presented in their final sequence order. A summary of the isolation procedure and analysis of each are given in Table 1 and their complete or partial sequences in Fig. 3. The sequences of some peptides were established by the 'dansyl'–Edman procedure, and these and some dipeptides are not discussed further (peptides T1, T3, T6, T7, T8, T9, T10, T11, T12, T17 and T18). The other peptides are discussed below.

(i) Peptide T2 was isolated by gel filtration (Fig. 2*b*) followed by electrophoresis at pH 8.9; the electrophoresis was run at 20 V/cm. Better purification was achieved by isolation of the peptide from a tryptic digest of totally carboxymethylated fragment CB2; the digest was prepared and then fractionated on a Sephadex G-50 column in 1% ammonium carbonate as described for heavy hydrogen chains. From this digest were isolated peptide T2 and a related peptide T2A. Both peptides had *N*-terminal threonine. Their compositions are shown in Table 1. Both peptides were digested with pepsin and the isolated products were analysed (Table 2) and subjected to the 'dansyl'–Edman procedure (Fig. 4). Amide residues were assigned as follows. The mobilities of the peptic peptides T2P1, T2AP2, T2P4, and T2P5 indicate that they contain glutamic acid, aspartic acid, asparagine and aspartic acid respectively. The mobility of peptide T2AP4 indicates that it contains two net negative charges. After three Edman-degradation steps the peptide had been converted into an acidic dipeptide (*m* 1.05) still containing two net negative charges. The sequence of peptide T2AP4 is therefore Val-Ser-Gln-Glu-Asp. Peptide T2P3 contains three net negative charges (two already assigned, since peptide T2P3 includes peptide T2AP4).

How peptide T2A was formed is not clear since a split by trypsin at an aspartic acid–proline bond is most unlikely. The peptide was isolated from the cyanogen bromide fragment CB2 and could have originated as a secondary split due to the acid lability of the bond.

(ii) Peptide T4 was purified by gel filtration on Sephadex G-50 in 0.1 M-ammonia followed by electrophoresis at pH 6.5 and chromatography with BAWP. It had *N*-terminal glutamic acid. Chymotryptic digestion gave three products (Table 3 and

Table 1. *Tryptic peptides from Vin heavy chain that account for the sequence of cyanogen bromide fragment CB2 (see section b)*

Peptides are presented in their correct sequence order, and fragment CB2 extends between the methionine residues in peptides T1 and T19. The procedure for the isolation of each peptide involved Dowex or Sephadex columns (Fig. 2), indicated as D or S, and paper chromatography (BAWP) and electrophoresis at the indicated pH. Ins., insoluble; n.d., not determined; *m*, mobility at pH 6.5.

Peptide	T1	T2	T2A	T3	T4	T5	T6	T7	T8	T7–8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	T19	T19–20
Lys		0.9		1.0		0.8	1.1	1.0	1.1	1.7	1.1	1.2	1.0		1.0	1.1	1.3		1.2		+	1.0
His		0.8				0.8															+++	2.2
Arg	1.0			0.9	1.0									1.1				+		1.0		
Trp		+				+											+				+	+
Amino sugar					+																	
Ccm		0.7	0.6					0.6		0.7						+					0.6	+
Asp	0.8	4.6*	1.9		1.0	1.9			1.0	1.1						1.0	3.8	2.0	1.1		2.0	2.1
Thr	1.0	1.8	1.6	0.8	1.0	1.0						0.7			1.9	0.9		1.7	0.9		0.9	1.1
Ser	1.1	1.2	1.1		1.1	1.3			1.0	1.3	1.9	0.9			1.3	1.2	1.7	2.7		1.0	2.0	4.7
Glu		6.0*	2.9		3.0	1.2	1.0				1.0			1.0	4.8	1.1	3.9				3.5	3.8
Pro		1.7	1.0	1.1							1.0			1.0	3.0		2.2	1.7				
Gly		1.0				1.3					0.9			1.0			1.8	1.2			1.1	2.1
Ala		1.0											1.0				1.2				1.1	1.2
Val		8.3*	4.5*			2.4			1.0	1.0					0.9	1.8	1.1	1.1	0.8		1.6	2.0
Met	0.8														1.0						0.6	0.9
Ile	1.0										0.9	0.8					1.0					
Leu	1.2					2.5					1.1				1.1	1.9		1.9	1.1		0.9	3.8
Tyr		0.9			0.8		1.0								0.9		1.9	0.8			0.7	1.1
Phe		0.9			1.1												1.1	1.6			0.7	1.0
m	0	Ins.	n.d.	−0.83	0.20	Ins.	0	0.05	−0.50	−0.18	0	−0.58	−0.8	−0.5	0.42	0.03	0.40	Ins.	0	−0.60	0.45	n.d.
Isolation methods	D	S	S†	D	D	S	D	D	D	D	D	D	D	D	D	D	D	S	D	D	S	D
	BAWP	8.9		6.5	S	6.5	BAWP	6.5	6.5	6.5	3.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	BAWP	6.5	BAWP	S†
	3.5			8.9	6.5	BAWP		8.9				3.5	8.9			8.9		BAWP	3.5	8.9	3.5	8.9
					BAWP	3.5								8.9		BAWP		3.5				

* Value after 72 h hydrolysis.
† See the text.

Peptide	Sequence	
T1	Asp-Thr-Leu-Met-Ile-Ser-Arg	
T2	Thr-Pro-Glu-Val-Thr-Ccm-Val-Val-Val-Asp-Val-Ser-Gln-Glu-Asp-Pro-Glx-(Glx,Val,Phe)-Asn-Trp-Tyr-Val-Asp-Gly-Val-Glx-Val-His-Asx-Ala-Lys	(Fig. 4)
T3	Thr-Lys-Pro-Arg	
T4	Glu-Glu-Gln-Phe-Asx-Ser-Thr-Tyr-Arg; ←T4C1→←T4C2→	
T5	Val-Val-(Ser,Val,Leu)-(His, Asx_2,Thr,Glx,Gly,Val_{0-1},Leu_{1-2},Trp)-Lys	
T6	Glu-Tyr-Lys	
T7	Ccm-Lys	
T8	Val-Ser-Asn-Lys	
T9	Gly-Leu-Pro-Ser-Ser-Ile-Glu-Lys	
T10	Thr-Ile-Ser-Lys	
T11	Ala-Lys	
T12	Gly-Gln-Pro-Arg	
T13	Glu-Pro-Gln-Val-Tyr-Thr-Leu-Pro-Pro-Ser-Gln-Glu-Glu-Met-Thr-Lys; ←T13P1→←T13P2→←T13P3→	
T14	Asn-Gln-Val-Ser-Leu-Thr-Ccm-Leu-Val-Ly. ←T14P1→←T14P2→←T14P3→	
T15	Gly-Phe-Tyr-Pro-Ser-Asp-Ile-Ala-Val-Glu-Trp-Glx-Ser-(Asx_3,Glx_2, Pro,Gly,Tyr)-Lys	(Fig. 5)
T16	Thr-Thr-Pro-Pro-(Val,Leu)-Asp-Ser-Asp-Gly-Ser-Phe-(Phe)-Leu-Tyr-Ser-Arg	(Fig. 6)
T17	Leu-Thr-Val-Asp-Lys	
T18	Ser-Arg	
T19-2	(Trp,Asx,Glx_2,Gly,Val,Phe)-(His,Ccm,Ser_2,Glx,Ala,Val,Met,Leu)-(His_2,Asx,Tyr)-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser-Leu-Gly	(Fig. 7)

Fig. 3. Tryptic peptides from Vin heavy chain. Suggested sequences for the regions in parentheses are discussed in the text. The sequences of longer peptides are further described in the figures indicated. →, Residue shown by 'dansyl'–Edman method; ⇒, residue shown to be *C*-terminal by 'dansyl'–Edman method.

Table 2. *Peptides obtained after peptic digestion of peptides T2 and T2A*

m, Mobility at pH 6.5.

Peptide	Relative yield	*m*	Purification (electrophoresis pH)	Composition (residues/mol)
T2P1	+++	0.70	6.5,3.5	Ccm positive, Thr 1.8, Glu 1.0, Pro 0.9, Val 1.0
T2P2	++	0.74	6.5,3.5	Asp 1.1, Ser 0.8, Glu 1.7, Val 1.0
T2P3	+	0.66	6.5,3.5	Asp 1.1, Ser 0.9, Glu 4.2, Pro 0.8, Val 2.2, Phe 0.8
T2P4	++	0.05	6.5,3.5	Asp positive, Trp positive
T2P5	+	0.44	6.5,BAWP,3.5	Asp 0.9, Gly 1.1, Val 0.7, Tyr 0.6
T2P6	+	0	6.5,3.5	Lys 1.0, His 1.0, Asp 2.2, Glu 1.2, Gly 1.1, Ala 1.0, Val 2.5, Tyr 0.5
T2P7	+++	−0.28	6.5	Lys 0.7, His 0.7, Asp 1.2, Glu 1.0, Ala 1.0, Val 1.5
T2AP1	+++	0.66	6.5,3.5	Ccm +, Thr 1.9, Glu 1.2, Pro 0.8, Val 2.1*
T2AP2	++	0.50	6.5	Asp 1.0, Val 1.5
T2AP3	+	0.60	6.5	Asp positive, Val positive
T2AP4	++	0.70	6.5,3.5	Asp 1.0, Ser 0.9, Glu 2.1, Val 1.0

* 72 h hydrolysis.

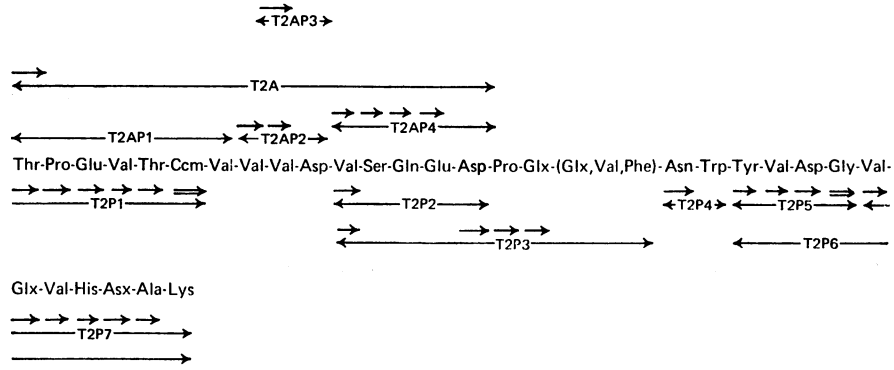

Fig. 4. Sequence of peptides T2 and T2A, deduced from the products of peptic digestion of each. Peptic peptides were isolated as shown in Table 2, which also gives their amino acid compositions.

Table 3. *Peptides obtained after chymotryptic digestion of peptide T4, and peptic digestions of peptides T13 and T14*

m, Mobility at pH 6.5.

Peptide	Relative yield	*m*	Purification (electrophoresis pH)	Composition
T4C1	+	0.80	6.5,3.5	Glu 3.0, Phe 0.9
T4C2*	+	0.06	6.5	Asp positive, Thr positive, Ser positive, Tyr positive, amino sugar positive
T4C3	++	−0.85	6.5	Arg
T13P1	++	0.49	6.5	Glu 1.9, Pro 1.1, Val 0.9
T13P2	++	0.26	6.5	Thr 0.9, Ser 1.0, Glu 2.0, Pro 2.3, Leu 0.9, Tyr 1.0
T13P3	+	0	6.5,2.1	Lys 0.9, Thr 1.0, Glu 0.9, Met 1.0
T14P1	++	0.05	6.5,3.5	Asp 0.9, Ser 1.1, Glu 1.1, Val 1.0, Leu 0.8
T14P2	+	0.55	6.5	Ccm 0.3, Thr 0.9, Leu 1.2
T14P3*	++	−0.65	6.5	Val positive, Lys positive

* Amino acid analysis by paper electrophoresis at pH 2.0.

Fig. 3). Amide residues in peptide T4C1 were assigned by determining the mobilities at pH 6.5 of its Edman-degradation products (Table 4). The *C*-terminal phenylalanine in peptide T4C1 and tyrosine in peptide T4C2 are tentatively assigned on the basis of chymotrypsin specificity. The carbohydrate in peptide T4 was found in peptide T4C2 and is probably attached to the aspartyl residue, as in other γ-chains.

(iii) Peptide T5 was obtained only in very small yields and was difficult to purify. It had *N*-terminal valine. A DNS derivative with mobilities corresponding to DNS-Val-Val was also detected. (After 20 h hydrolysis the sequence Val-Val is incompletely hydrolysed.)

Chymotryptic digestion gave two products. Only one, peptide T5C1, was isolated (by electrophoresis at pH 3.5) in amounts sufficient for analysis. Its composition was valine 2.2, serine 1.0, leucine 1.0, residues/mol. The low yields of valine are probably due to the presence of acid-stable peptide bonds.

(iv) Peptides T7 and T8 were shown to be adjacent in sequence by the isolation of an overlap peptide, T7–8. Peptides T7 and T7–8 both had *N*-terminal carboxymethylcysteine, and peptide T7–8 gave rise to T7 and T8 on tryptic digestion.

(v) Peptide T13 had *N*-terminal glutamic acid. Peptic digestion gave rise to three peptides (Table 3), whose sequences were established as shown in Fig. 3. Amide residues were established by determining the mobilities of Edman-degradation products of each peptide. Peptide T13P3 was neutral and therefore contained glutamic acid. Other mobilities are shown in Table 5.

(vi) Peptide T14 had *N*-terminal aspartic acid or asparagine, and on peptic digestion gave rise to three products (Table 3) whose sequences are shown in Fig. 3.

(vii) Peptide T15 had *N*-terminal glycine. Subtilisin and chymotryptic digestions gave products whose analyses are given in Table 6 and sequences in Fig. 5. The mobility of peptide T15S2 indicates that both aspartic acid and glutamic acid are present. The mobility of peptide T15C2 suggests that the amide assignments in this peptide may be identical with those in the homologous region of human γ1 molecules (Edelman *et al.* 1969).

(viii) Peptide T16 had *N*-terminal threonine. Edman degradation of the whole peptide gave the sequence Thr-Thr-Pro-Pro. The products of tryptic and chymotryptic digestion of T16 are described in Table 7 and Fig. 6. The results do not definitely position a phenylalanine residue (in parentheses in Fig. 6).

(ix) Peptide T19 was obtained from the Sephadex fractionation. From the Dowex fractionation a larger peptide T19–20 was isolated by gel filtration on Sephadex G-50 in 0.1M-ammonia and electrophoresis at pH 8.9. Neither peptide T19 nor peptide T19–20 gave a positive *N*-terminal residue. The products of chymotryptic digestion of peptide T19–20 are shown in Table 8 and Fig. 7. No further work was done on them. The yields of the tryptic peptides were rather low. A probable sequence can be derived from further results described below and by comparison with homologous sequences (see below).

All the non-methionine-containing tryptic peptides discussed were shown to be present in fragment CB2, either by isolation from a tryptic digest of carboxymethylated fragment CB2 (peptides T2, T4, T5, T7, T8, T10, T11, T14, T15 and T16) or by isolation of peptic peptides (see below) overlapping or including the tryptic peptides (peptides T3, T6, T9, T12, T17 and T18). In addition we have presented three methionine-containing peptides (T1, T13 and T19). Fragment CB2 extends between the methionine residues in peptides T1 and T19 since the *C*-terminal sequence of peptide T1 and the *N*-terminal sequence of peptide T19 are present in fragment CB2; they are included in the peptic peptides R1 and P4 respectively (see below and Fig. 8). Although peptide T13 contains methionine, it is not split with good yields by cyanogen bromide; a peptide corresponding in amino acid analysis to complete T13, but giving rise to homoserine instead of methionine on acid hydrolysis, was isolated from the tryptic digest of carboxymethylated fragment CB2. It appears that conversion of methionine into

Table 4. *Mobilities at* pH 6.5 *of peptides derived from Edman degradation of peptides T4C1*

m, Mobility at pH 6.5.

Peptide	Edman steps completed	Assumed sequence	*m*
T4C1	0	Glu-Glu-Gln-Phe	0.80
T4C1A1	1	Glu-Gln-Phe	0.50
T4C1A2	2	Gln-Phe	0

Table 5. *Mobilities at* pH 6.5 *of Edman degradation products of peptides T13P1 and T13P2*

m, Mobility at pH 6.5

Peptide	Edman steps completed	Assumed sequence	*m*
T13P1	0	Glu-Pro-Gln-Val	0.49
T13P1A2	2	Gln-Val	0
T13P2	0	Tyr-Thr-Leu-Pro-Pro-Ser-Gln-Glu	0.26
T13P2A7	7	Glu	0.95

Table 6. *Peptides obtained after chymotryptic and subtilisin digestions of peptide T15*

n.d. Not determined; *m*, mobility at pH 6.5.

Peptide	Relative yield	*m*	Purification (electrophoresis pH)	Composition
T15C1	++	0.40	6.5,3.5	Trp positive, Asp 0.9, Ser 1.1, Glu 0.9, Pro 1.0, Gly 1.1, Ala 0.9, Val 0.9, Ile 0.7, Tyr 0.9, Phe 0.7
T15C2	+	0.20	6.5,3.5	Lys 0.9, Asp 2.9, Ser 1.3, Glu 2.8, Pro 1.2, Gly 1.1, Tyr 0.8
T15S1	++	0	6.5,3.5	Ser 1.3, Pro 1.1, Gly 1.1, Tyr 0.8, Phe 0.8
T15S2	++	0.75	6.5	Asp 1.0, Glu 1.0, Ala 1.0, Val 0.9, Ile 0.8
T15S3	+	0.50	6.5	Lys n.d., Trp positive, Asp 2.9, Ser 1.3, Glu 3.0, Pro 0.9, Gly 1.1, Tyr 0.9

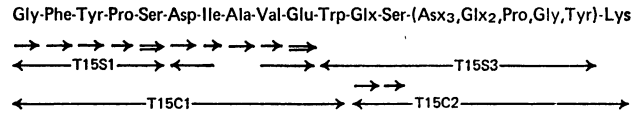

Fig. 5. Partial sequence of peptide T15 deduced from the products of chymotryptic and subtilisin digestions described in Table 6.

Table 7. *Peptides obtained from chymotryptic and peptic digests of peptide T16*

m, Mobility at pH 6.5.

Peptide	Relative yield	*m*	Purification (electrophoresis pH)	Composition
T16C1	++	0.46	6.5	Asp 2.0, Thr 1.9, Ser 2.1, Pro 2.1, Gly 1.2, Val 0.8, Leu 0.9, Phe 1.8
T16C2	+	0	6.5,3.5	Leu 1.0, Tyr 1.0
T16C3	++	−0.55	6.5	Arg 1.0, Ser 0.8
T16P1	++	0	6.5,3.5	Thr 1.9, Pro 2.2, Val 1.0, Leu 1.0
T16P2	++	0.76	6.5	Asp 1.9, Ser 1.9, Gly 1.1, Phe 1.0
T16P3	+	0	6.5,3.5	Phe
T16P4	++	−0.43	6.5	Arg 1.0, Ser 1.0, Tyr 0.9

Fig. 6. Sequence of peptide T16 deduced from the products of peptic and chymotryptic digestions described in Table 7.

a derivative, identified as homoserine after acid hydrolysis, may take place without concomitant splitting.

(*b*) *Peptic peptides from cyanogen bromide fragment CB2.* The order of the tryptic peptides was mostly obvious by a comparison with the Fc fragment of rabbit IgG (Hill *et al.* 1967) and of the IgG1 myeloma protein Eu (Edelman *et al.* 1969). However, some small peptides could not be placed with sufficient confidence and confirmation of most overlaps was considered desirable. This was achieved by isolating selected peptides from a peptic digest of the cyanogen bromide fragment CB2. The isolation of the peptic peptides described below permitted the assignment of 15 overlaps out of a total of 19.

(i) Radioactive peptides. Four radioactive peptides, R1–R4, were purified from a peptic digest of carboxymethylated fragment CB2. Purification was by electrophoresis at pH 6.5 and 3.5, followed by oxidation and a second electrophoresis at pH 3.5. Analyses of the peptides are shown in Table 9.

As shown in Fig. 8, peptide R1 overlaps the tryptic peptides T1 and T2; peptide R2 overlaps peptides T6, T7, T8 and T9; and peptides R3 and R4 are derived from peptides T14 and T19 respectively. These peptides have not been studied further because of their strong homology with the corresponding cysteine peptides obtained from the other three subclasses of heavy chains (Frangione *et al.* 1969*b*). Peptide R4 was isolated as an acidic peptide, but a neutral peptide with identical composition (presumably containing *C*-terminal homoserine in the lactone form) was also isolated in lower yields.

(ii) Non-radioactive peptides. The non-radioactive peptides P1–P4 had the analyses given in Table 9 and gave tryptic digestion products whose analyses are presented in Table 10. As shown in Fig. 8, peptide P1 overlaps peptides T2, T3 and T4; peptide P2 contains a lysine residue probably derived from peptide T9, and overlaps peptides T10, T11, T12 and T13; peptide P3 overlaps peptide T14 and T15; and peptide P4 overlaps peptides T17, T18 and T19.

A subtilisin digest of peptide P1 was carried out to assign amide groups in the sequence (from peptide T2):

-Val-Glx-Val-His-Asx-Ala-Lys-

Two peptides derived from this sequence were identified. One had the composition (His 1.0, Glx 1.0, Val 1.7) and mobility +0.05 at pH 6.0; this defines the Glx as a glutamic acid residue. A second peptide contained only Asx and alanine and had mobility 0.05 at pH 6.5, indicating the presence of asparagine.

(*c*) *Sequences around interchain bridges.* Two radioactive peptides, B1 and B2, were obtained on

Table 8. *Peptides obtained from chymotryptic digestion of peptide T19–20*

m, Mobility at pH 6.5.

Peptide	Relative yield	*m*	Purification (electrophoresis pH)	Composition
T19–20C1	+	0.35	6.5	Trp, Asp, Glu, Gly, Val, Phe (qualitative only)
T19–20C2	++	0.42	6.5,3.5	His 0.8, Ccm positive, Ser 1.8, Glu 1.1, Ala 1.2, Val 1.1, Met 0.3, Leu 1.0
T19–20C3	+	0	6.5,3.5	His 2.6, Ccm positive, Asp 1.0, Ser 1.9, Glu 1.1, Ala 1.0, Val 1.0, Met 0.8, Leu 1.0, Tyr 1.0
T19–20C4	+++	−0.50	6.5	Lys 1.0, Thr 0.9, Glu 1.0
T19–20C5	+++	0.05	6.5,3.5	Ser 1.0, Leu 1.0
T19–20C6	+++	0.05	6.5,3.5	Ser 0.9, Gly 1.0, Leu 1.0

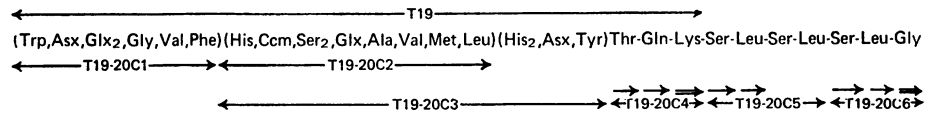

Fig. 7. Partial sequence of peptides T19–20 deduced from the products of chymotryptic digestion described in Table 8. Peptide T19 has been isolated separately (see the text).

Table 9. *Radioactive (R1–4) and non-radioactive (P1–4) peptides isolated from peptic digests of fragment CB2*

m, Mobility at pH 6.5; Hsr, homoserine.

Peptide	Purification (electrophoresis pH)	*m*	Composition
R1	See the text	0.30	Arg 0.8, Cms 0.7, Thr 2.0, Ser 1.0, Glu 1.0, Pro 0.9, Val 1.0, Ile 0.9
R2	See the text	−0.25	Lys 3.0, Cms 0.5, Asp 1.0, Ser 2.9, Glu 1.0, Pro 0.9, Gly 1.1, Val 1.1, Ile 1.0, Leu 1.1, Tyr 0.9
R3	See the text	0.55	Cms 0.6, Thr 1.0, Leu 1.0
R4	See the text	0.50	Cms 0.3, Ser 2.0, Hsr 0.6, Val 1.0
P1	6.5,3.5	−0.1	Lys 1.7, His 0.7, Arg 0.7, Asp 2.0, Thr 1.8, Ser 1.0, Glu 4.2, Pro 1.1, Ala 1.0, Val 1.7, Tyr 1.0, Phe 1.0, amino sugar positive
P2	6.5,2.1,3.5	−0.58	Lys 2.8, Arg 1.0, Thr 0.9, Ser 1.1, Glu 3.1, Pro 1.9, Gly 1.1, Ala 1.0, Val 1.0, Ile 0.9
P3	6.5,BAWP,3.5	0	Lys 0.9, Asp 1.2, Ser 1.2, Pro 0.9, Gly 1.2, Val 0.9, Tyr 0.9, Phe 0.9
P4	6.5,BAWP,3.5	0	Lys 1.2, Arg 0.8, Trp positive, Asp 1.9, Thr 1.0, Ser 1.1, Glu 1.7, Gly 1.0, Val 1.9, Phe 0.9

partial reduction and limited peptic digestion of protein Vin as described under 'Selective reduction and radioactive labelling' in the Methods section. The two peptides were purified by paper electrophoresis at pH 6.5 and 3.5 followed by performic acid oxidation, and then chromatography in BAWP with peptide B2 and further electrophoresis at pH 3.5. Their analyses are given in Table 11 together with the products of the tryptic digestion of each. These products were purified by paper electrophoresis at pH 6.5 (products of peptide B1) and at pH 3.5 (products of peptide B2).

The *N*-terminal residues of peptides B1 and B2 were valine and tyrosine respectively. The order of digestion products in peptide B1 is therefore established unequivocally, but in peptide B2 the order of digestion products B2T2 and B2T3 is indicated by a comparison with the homologous sequence of a human γ1 chain (Steiner & Porter, 1967). The sequences deduced for peptides B1 and B2 are shown in Fig. 9.

DISCUSSION

Like light chains, heavy chains seem to consist of two regions: one is made up of a sequence common to all the members of each subclass and allotype (*C*-region); the other includes sequences that are specific to the products of individual cell lines (*N*-region) (Frangione *et al.* 1969*b*; Edelman *et al.*

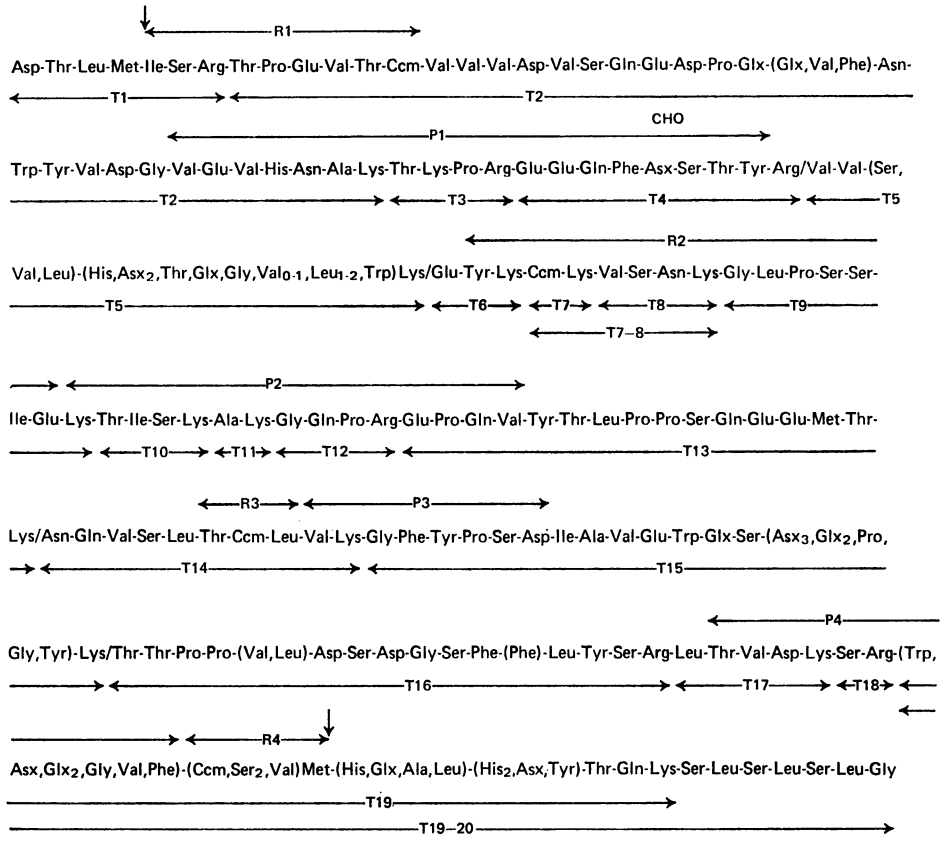

Fig. 8. Partial sequence of cyanogen bromide fragment CB2 deduced from results on tryptic peptides (T), radioactive peptic peptides (R) and non-radioactive peptic peptides (P). Overlaps assigned only by homology with other γ-chain sequences (see Fig. 10) are marked /. The points of cyanogen bromide cleavage that give rise to fragment CB2 are marked by arrows.

Table 10. *Tryptic digestion products from peptic peptides P1–4*

Compositions in which no values are given are qualitative only. *m*, Mobility at pH 6.5.

Peptide	*m*	Purification	Composition
P1T1	−0.3	6.5	Lys+, His+, Asp+, Glu+, Ala+, Val+
P1T2	−0.8	6.5	Lys 1.1, Arg 0.8, Thr 0.9, Pro 1.0
P1T3	0.35	6.5	Asp+, Thr+, Ser+, Glu+++, Tyr+, Phe+, amino sugar++
P2T1	−0.9	6.5	Lys
P2T2	−0.5	6.5,BAWP	Not determined; mobilities identical with those of tryptic peptide T10 (Thr-Ile-Ser-Lys)
P2T3	−0.7	6.5,BAWP	Lys+, Ala+
P2T4	−0.6	6.5,BAWP	Arg 0.9, Glu 1.2, Pro 0.9, Gly 1.0
P2T5	0.5	6.5,BAWP	Glu 2.0, Pro 1.1, Val 0.9
P3T1	−0.6	6.5	Val+, Lys+
P3T2	0.4	6.5	Asp+, Ser+, Pro+, Gly+, Tyr+, Phe+
P4T1	−0.55	6.5,BAWP	Arg+, Ser+
P4T2	0	6.5,BAWP	Lys+, Asp+, Thr+, Val+
P4T3	0.38	6.5,BAWP	Trp+, Asp 1.1, Glu 1.7, Gly 1.2, Val 0.8, Phe 1.0

Table 11. *Radioactive peptides, B1 and B2, obtained from a limited peptic digest of partially reduced ^{14}C-carboxymethylated Vin heavy chain*

Before digestion the protein was further completely reduced and carboxymethylated with non-radioactive iodoacetate. The products of their digestion with trypsin are also included. The asterisk indicates radioactive peptides. *m*, mobility at pH6.5. The low value of tyrosine in some peptides is probably due to conversion into chlorotyrosine during the performic acid oxidation.

		Products of tryptic digestion				Products of tryptic digestion			
	*B1	B1T1	*B1T2	B1T3	*B2	B2T1	B2T2	B2T3	*B2T4
Lys	1.0	+			4.1	1.7	1.1	0.8	
His					0.9	0.7			
Arg	1.2		+		1.0			1.0	
Cms	0.7		0.4		1.7	+			0.6
Asp					3.9	2.7		1.0	
Thr	3.6	1.9		1.8	1.9	1.4			
Ser	7.9	3.2	1.9	2.9	2.9	1.1	0.9		1.0
Glu	1.1			1.0	2.2		1.0		1.0
Pro	3.2		2.9		5.6	1.2			5.0
Gly	1.2		1.0		1.3				1.2
Ala	2.3	1.1	1.1		1.1				1.1
Val	2.8	1.9	1.0		2.8	1.1	1.1	1.0	
Ile									
Leu	1.1		1.0						
Tyr					0.8	0.5			0.7
Phe	1.0		0.9		0.9				0.9
m	0	−0.33	0	0.44	0.1				

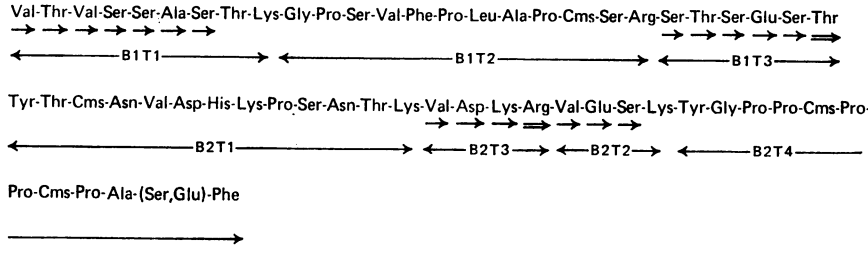

Fig. 9. Sequences of peptic peptides B1 and B2, deduced from the products of tryptic digestion described in Table 11. Peptides B1T2, B2T1 and B2T4 are of known sequence (Pink & Milstein, 1967).

1969; Press & Hogg, 1969). The *C*-region of the four γ-chain subclasses involves three disulphide-bridged loops (Frangione *et al.* 1969*b*): its total sequence in a chain of the γ1 subclass has been established (Edelman *et al.* 1969). With the results in the present paper we define a partial sequence of the *C*-region of a γ4 chain, extending from residue 113, in the 'switch peptide', to the *C*-terminus at residue 446 (Eu numbering), but a section of probably 50 residues located within the first disulphide bridge loop of the *C*-region (the 'invariant Fd loop') has not been studied. The two sequences are compared in Fig. 10, which also includes the partial sequence of the Fc fragment of rabbit IgG (Hill *et al.* 1967). The sequences of fragments of the *N*-terminal quarter and the distribution of disulphide bridges of the same γ-chain of Vin have been published (Pink & Milstein, 1967, 1968).

The existence of subclasses of IgG has now been established (or is strongly suspected) in all carefully studied mammals. The subclasses have sometimes been shown to possess different electrophoretic mobilities or biological properties. Of the four known subclasses of human IgG, the electrophoretically faster moving IgG4 molecules do not fix complement whereas IgG1 molecules do (Ishizaka *et al.* 1967). The fixation of complement is a property associated with the Fc fragment, and Utsumi (1969) suggested that residues approximately between 233 and 280 are involved in

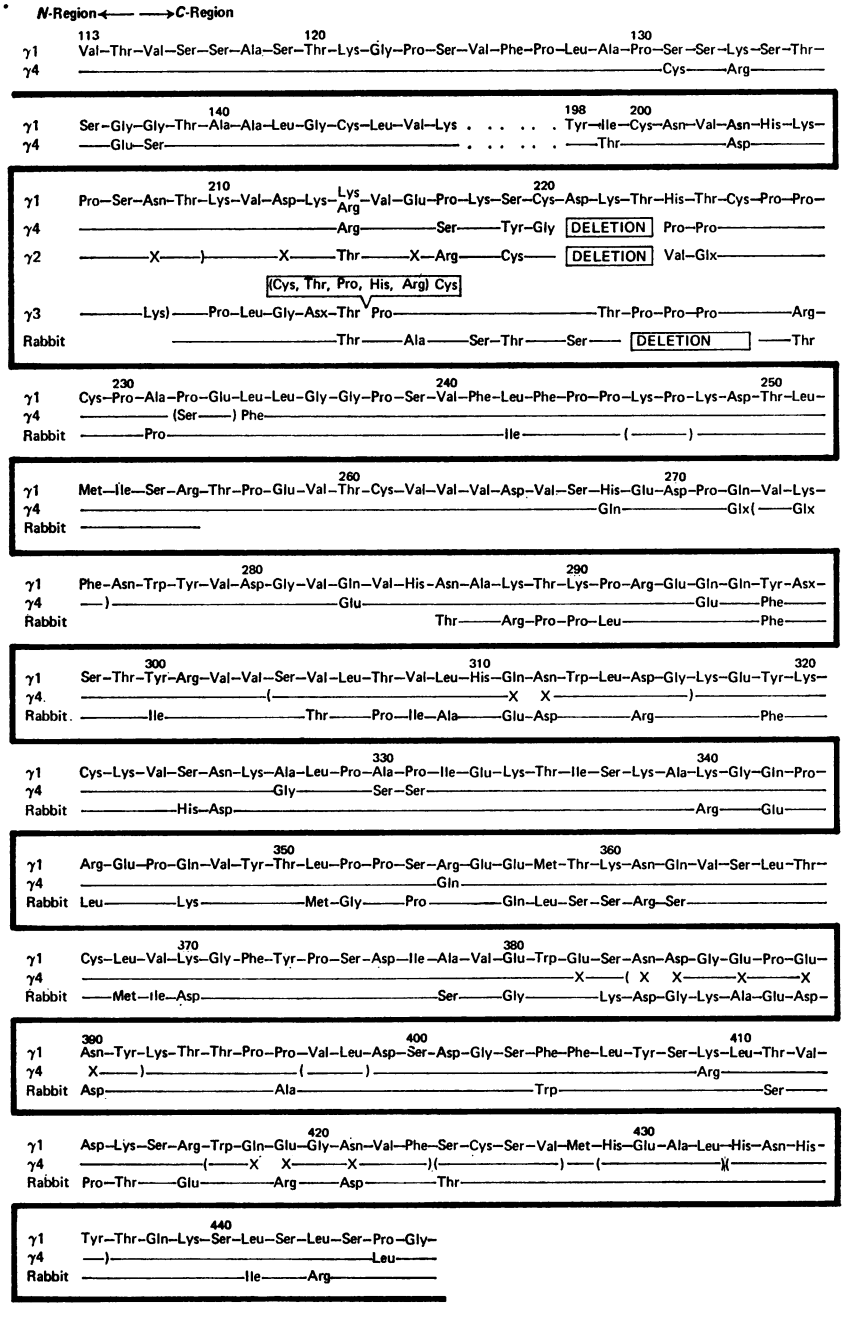

Fig. 10. Comparison of $\gamma 1$ and $\gamma 4$ *C*-regions. The $\gamma 4$ sequence is from protein Vin. Horizontal lines indicate identity with $\gamma 1$. X is used when amide groups have not been established and parentheses to show that the identity is based on amino acid composition only. The arrow indicates the possible beginning of the *C*-region. The sequence around the inter-heavy chain bridges of $\gamma 2$, $\gamma 3$ and rabbit and the partial sequence of rabbit Fc fragment are also included for comparison. Data are taken as follows. $\gamma 1$: Edelman *et al.*, 1969; Press & Hogg, 1969. Rabbit: Hill *et al.* 1967; Cebra *et al.* 1968. $\gamma 2$: Frangione *et al.* 1969*b*. $\gamma 3$: Frangione & Milstein, 1969. Vin ($\gamma 4$): the present paper and Pink & Milstein, 1967.

Table 12. *'Evolutionary rates' of different proteins (expressed as number of amino acid differences out of the total number of residues compared)*

Data are from Dayhoff & Eck (1967–8) and from the present paper.

Proteins compared	Amino acids (differences/total)
κ-Chain common region: mouse/human	43/110
Haemoglobin α-chain: mouse/human	17/141
Haemoglobin β-chain: mouse/human	23/145
γ-Chain Fc region: rabbit/human γ4	60/190
Haemoglobin α-chain: rabbit/human	25/141
Haemoglobin β-chain: rabbit/human	14/145
Cytochrome *c*: rabbit/human	9/104

complement fixation. Kehoe & Fougereau (1969) in fact isolated a 60-residue fragment from the homologous region of a mouse myeloma protein that had complement-fixing activity. In the stretch between residues 230 and 255, where the rabbit IgG sequence is known, the only difference between γ1 and rabbit is a leucine-for-isoleucine interchange, but the variation in the remainder of the Fc section is considerably higher. The comparison (Fig. 10) between γ1 and γ4 sequences in this stretch is thus specially notable, and this shows possibly four differences between them, including leucine for phenylalanine at residue 234, histidine for glutamine at residue 268 and lysine for glutamic acid (or glutamine) at residue 274 substitutions. It is possible that some or all of these residues are directly involved in the complement-binding reaction.

Several species possess IgG subclasses that appear analogous to human IgG4 molecules in their fast electrophoretic mobility and absence or weakness of complement-fixing properties; these include mouse, guinea pig and horse (see Cohen & Milstein, 1967). Frangione, Franklin, Fudenberg & Koshland (1966) suggested that these IgG subclasses have a common evolutionary origin. We argue here that this is not the case and that, in general, immunoglobulin subclasses in different species have recent independent evolutionary origins.

Strong evidence for the recent origin of the human γ-chain subclasses is provided by the comparison of the sequences of γ1, γ4 and rabbit Fc as shown in Fig. 10. From residue 230 to residue 446 there are only 13 established differences between γ1 and γ4 chains, whereas both of them differ in more than 50 positions from the rabbit IgG sequence in spite of an incompletely known section in rabbit of about 25 residues. Evidence for the recent origin of the human γ-chain subclasses is also provided by the almost identical sequences around the disulphide bridges of the four subclasses (Milstein *et al.* 1967; Frangione *et al.* 1969*b*). The sequences of the *C*-terminal octadecapeptides of the γ-chain subclasses of several species provide additional support for this view. For example the two bovine subclasses of γ-chains (Milstein & Feinstein, 1968) contain three residues (threonine, lysine and alanine) at positions 2, 4 and 6 from the *C*-terminus common to both but absent from any of the other species studied, whereas a valine residue is common only to the two equine γ-chain subclasses (Weir, Porter & Givol, 1966). This indicates that the two bovine and equine γ-chain subclasses arose by gene duplication after the separation of the evolutionary lines leading to cattle and horses.

It might be argued that very strong selective pressures forced a convergence of amino acid sequences of different subclasses in various species. We consider this unlikely because of the accumulating evidence that most interspecies amino acid-sequence differences are not the consequences of strong selective pressures (Kimura, 1968; King & Jukes, 1969). The selective pressure on the sequence of immunoglobulin chains is unlikely to be very high, because of the high frequency of allotypic sequence alternatives noted in several species (Kelus & Gell, 1967; Potter & Lieberman, 1967; Cohen & Milstein, 1967). Recent gene doublings appear to be sufficiently common in mammalian systems (Hilse & Popp, 1968; Schroeder *et al.* 1968) to enable subclasses to evolve independently in several lines.

When did the human subclasses evolve? From the differences between mouse, human and rabbit sequences of light- or heavy-chain *C*-regions it can be calculated that on average one difference per 100 residues became incorporated about every 4×10^6–5×10^6 years. This evolutionary rate (which appears to be roughly constant for a given protein type) is more rapid than that of cytochrome *c* (Margoliash & Smith, 1965) or haemoglobin (Zuckerkandl & Pauling, 1965) (see Table 12). (In these calculations we have deliberately excluded the section of the molecule where the interchain bridges occur. This is a puzzling section where the variation is extremely large, and includes deletions and additions. If those differences are also included this would make the evolutionary rate even faster than calculated, but not affect significantly the following arguments.) Thus the 14 differences per 220 residues between the Fc sections of the human γ1 and γ4 chains may have arisen in the last 2×10^7–3×10^7 years, putting the time of divergence of human subclasses just before the divergence of *Homo sapiens* from the present-day Old and New

World monkeys. van Loghem, Shuster & Fudenberg (1968) detected allotypic markers typical of $\gamma 1$ chains only in great apes. Markers (b^+ or Gm 5) typical of human $\gamma 3$ chains were found in Old World monkeys but not in New World monkeys.

It thus appears that particular IgG subclasses are shared by only a small group of related mammalian species (e.g. Old World monkeys, great apes and men). On the other hand, the immunoglobulin classes IgM, IgA and IgG appear to have arisen early in the evolution of the immune system (Marchalonis & Edelman, 1965, 1966; Good, Gabrielsen, Pollara, Gewurz & Finstad, 1968). The recentness of subclass evolution thus provides a rationale for the distinction between class and subclass, at present based empirically on degrees of immunological cross-reactivity between immunoglobulins of different species. It seems likely that the immunoglobulins are only one example of protein complexes consisting of many closely related and recently-evolved gene products. The multiple haemoglobins of several species appear to have a similar origin.

It is most likely that separate genetic systems code for the *C*- and *N*-regions of immunoglobulin chains (Milstein, 1967; Hood & Ein, 1968; Milstein, Milstein & Feinstein, 1969; Prahl, Mandy, David, Steward & Todd, 1969). So far we have discussed only the *C*-regions of immunoglobulin chains, but the arguments have implications for the variable sections of these chains as well (Milstein & Pink, 1969).

If the appearance of new *C*-regions is a relatively common process it seems very likely that all the subclasses can share variable regions derived from a common pool of variable-region genes; this is in accord with the results of Prahl *et al.* (1969) and Wilkinson (1969), suggesting that rabbit γ-, μ- and α-chains can share variable regions. The findings of identical 'switch peptide' sequences for $\gamma 4$ and $\gamma 1$ chains gives support to this assumption. Such a sequence could reflect a recognition site for the integration of *N*- and *C*-region genes or gene products.

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