Immunoglobulin κ -Chains

COMPARATIVE SEQUENCES IN SELECTED STRETCHES OF BENCE-JONES PROTEINS

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Three type K Bence-Jones proteins have been fully reduced and carboxymethylated with high-specific-activity iodo[^{14}C]acetate. A tryptic digest and a chymotryptic digest of each protein were fractionated on a Sephadex column and the radioactive peptides were purified by paper electrophoresis. All the proteins studied had five unique carboxymethylated cysteine sequences. Three of these were identical with the exception of a single substitution, and two had some variations. The common peptides could be placed in the *C*-terminal half of the molecule. The variations around the other two half-cysteine residues provided information about the nature of the variability of the primary sequence of immunoglobulin κ -chains. The results are consistent with the hypothesis that the chains derive from a common ancestor by somatic mutation of a small number of genes or by gene doubling and selection in the course of evolution. The isolation of the *N*-terminal peptide in methionine-containing Bence-Jones proteins is also described.

The light chains of IgG immunoglobulins have been shown to contain a *C*-terminal sequence that is a common feature (Milstein, 1964, 1965) in the heterogeneous population of light chains of the same type (Cohen & Porter, 1964). Comparative sequence studies of Bence-Jones proteins of the same type K performed in different Laboratories lead to the conclusion that the *C*-terminal half of the κ -chains may be invariant (except for variations under genetic control like the valine/leucine variation related to the Inv types (Milstein, 1966*a,b*; Baglioni, Zonta, Cioli & Carbonara, 1966), and variations in the other half account for the heterogeneity of the κ -chains (Hilschmann & Craig, 1965; Titani, Whitley, Avogardo & Putnam, 1965; Milstein, 1966*a*). Comparative sequences in the variable stretch are of particular interest because they throw light on the nature of this variability. Sequence studies around the disulphide bridges have been used to compare equivalent stretches of individual chains and have given evidence indicating that the variability is in itself of a very limited nature, similar to the variability observed in an individual protein in the course of evolution (Milstein, 1966*a*). The present paper gives the detailed experimental evidence on which those sequences were established, and also extends some of them, including studies on the *N*-terminal part of the chains.

MATERIALS AND METHODS

The Bence-Jones proteins BJ, Rad and Ker were the same as those used previously (Milstein, 1966*b*).

Total reduction and alkylation with iodo[^{14}C]acetate. For the isolation and purification of the cysteine-containing peptides, a labelling technique was used. Full reduction and alkylation was done in 5–6*M*-guanidine-HCl and about 0.5*M*-mercaptoethanol. The reaction with iodo[^{14}C]acetate of high specific activity was performed under N_2 in the presence of about 2*mm*-mercaptoethanol.

A.R.-grade guanidine carbonate was dissolved with conc. HCl until the apparent pH was about 3–4 and then filtered (guanidine hydrochloride). Tris-guanidine was made by adding about 1.3*g.* of tris and 2*ml.* of 0.1*M*-EDTA to 100*ml.* of guanidine hydrochloride (apparent pH taken to 8.1). Acid-guanidine was made by adding to 100*ml.* of guanidine hydrochloride 2*ml.* of acetic acid, 2*ml.* of 0.1*M*-EDTA and mercaptoethanol to a final concentration of 2*mm*.

The reduction and carboxymethylation procedure in a typical preparation was as follows: 100*mg.* of protein was dissolved in 10*ml.* of tris-guanidine and N_2 was bubbled through for a few minutes. All subsequent operations were done under N_2 . After the addition of 300 $\mu\text{l.}$ of mercaptoethanol, the solution was incubated at room temperature for 2–3 hr. after which time 1*ml.* of acetic acid was added. The solution was dialysed against sufficient changes of acid guanidine so that the concentration of mercaptoethanol was lowered to 2*mm*. The solution was transferred to a screw-cap tube. A second solution was prepared with

an equal volume of the diffusate to which was added 400 μ moles of iodoacetate, 200 μ C of iodo[14 C]acetate (specific activity 6.5 mc/m-mole; The Radiochemical Centre, Amersham, Bucks.) and enough solid tris to bring the final pH (after mixing with the protein solution) to 8.0-8.2 as determined by titration of the diffusate (about 1.70 g. of tris to 10 ml. of diffusate). The two solutions were mixed after bubbling N_2 for 5 min., precautions being taken to exclude air. The reaction was allowed to proceed for 3 hr. at room temperature, after which time 0.1 ml. of mercaptoethanol was added. The solution was dialysed against large volumes of 0.1% pyridine containing acetic acid (0.003%, v/v) and freeze-dried. Amino acid analysis of a sample was carried out to check that half-cystine was undetectable, ensuring that carboxymethylation was at least 95% complete.

Purification of the radioactive peptides. A solution (10 mg./ml.) of radioactive protein in 1% (w/v) NH_4HCO_3 was digested at 37° with trypsin (6 hr.) or chymotrypsin (15 hr.) by using a 1:100 enzyme/substrate ratio (except in the chymotryptic digest of protein Ker; see below). The

digest was freeze-dried, dissolved with 0.1 N- NH_3 to one-third of its original volume and applied to a Sephadex G-50 column (2 cm. $\times$ 100 cm.) equilibrated with 0.1 N- NH_3 . The eluate of the column was collected in 5 ml. fractions and samples were taken for determination of radioactivity in a Nuclear-Chicago scintillation counter. The bulk of the peaks (a, b, c etc.) were pooled separately from the intermediate fractions (ab, bc etc.). After freeze-drying they were applied side by side on Whatman 3MM paper and subjected to electrophoresis (60 v/cm. at pH 6.5 for 1 hr.). In this way peptides of different size but the same mobility were readily separated. An example of this fractionation procedure is illustrated in Fig. 1. The radioactive bands were cut out, sewn on to another paper and run by electrophoresis at pH 3.5 for about 2 hr. Unless otherwise stated the purified bands were then cut out and subjected to performic acid oxidation in the vapour phase (Brown & Hartley, 1963). This procedure was considered convenient to produce quantitative transformation of the carboxymethylcysteine peptides to their sulphone derivatives (Harris, 1966). The peptides were finally purified by electrophoresis at pH 3.5, taking advantage of the change in the pK introduced during the oxidation procedure. CMCySO $_2^*$ is very labile to alkali and is partly destroyed during acid hydrolysis in 5.7 N-HCl in evacuated tubes. In general about 50-60% was recovered after 20 hr. at 105°, but sometimes the destruction was almost complete. In such cases the radioactivity of the peptide was used to measure the CMCySO $_2$ content.

Amino acid analysis, electrophoretic techniques, terminal analysis, Edman degradations etc. were carried out as described previously (Milstein, 1966b).

Cyanogen bromide cleavage and separation of the N-terminal peptide. The protein was dissolved in 70% (v/v) formic acid and treated overnight with 1.5 times its weight of CNBr. It was freeze-dried and fractionated in a Sephadex G-50 column (1.3 cm. $\times$ 45 cm.) equilibrated with 5% (v/v) formic acid. The breakthrough of the column was separated from the remaining small ninhydrin-positive material that had no absorption at 280 m μ . This second fraction was pooled and fractionated by electrophoresis at pH 6.5 for 1 hr. and the neutral band, containing the N-terminal peptide, was further purified by electrophoresis at pH 3.5 for 2 hr.

RESULTS

Protein BJ

Qualitative N-terminal analysis by the 'dansyl' method (Gray & Hartley, 1963) gave aspartic acid as sole N-terminus of the protein. The N-terminal peptide was prepared by cyanogen bromide cleavage. Its amino acid analysis was: Asp, 0.95; homoserine, 0.50; Glu, 1.10; Val, 0.99. The N-terminus of the high-molecular-weight pooled fraction from the breakthrough of the Sephadex G-50 column was threonine, plus a trace of aspartic

* Abbreviations: CMCySO $_2$, carboxymethylcysteine sulphone; in amino acid sequences, Asn and Gln refer to asparagine and to glutamine respectively, and Asx and Glx to aspartic acid or asparagine and to glutamic acid or glutamine respectively.

Fig. 1. Fractionation of a chymotryptic digest of [14 C]-carboxymethylated Bence-Jones protein BJ. (a) Initial fractionation on a Sephadex G-50 column. (b) Paper-electrophoresis (pH 6.5) fractionation of the Sephadex fractions.

acid. The results suggest the following *N*-terminal sequence:

since protein BJ contains only one methionine residue.

A trypsin digest (BJT, 90mg.) and a chymotrypsin digest (BJC; 40mg.) were fractionated on Sephadex followed by paper electrophoresis as described in the Materials and Methods section.

Table 1. *Carboxymethylcysteine sulphone peptides from tryptic digestion of protein BJ, fractionated on Sephadex followed by paper electrophoresis*

Peptide BJT3a contains a Val-Val sequence, which is not completely hydrolysed in 24hr. The high leucine value is due to a contaminating peptide, differing by the presence of an extra leucine in the *C*-terminus (see Fig. 14).

Peptide.....	BJT1	BJT2	BJT3a	BJT3b	BJT4	BJT5
Amount analysed (μ mole).....	0.020	0.015	0.015	0.025	0.022	0.016
Lys					0.96	1.08
His				1.03		1.15
CMC ₃ SO ₂	0.40	0.50	0.45	0.53	0.50	0.48
Asp	0.96	1.00			1.91	
Thr			1.02	1.02	1.88	1.94
Ser			1.85		1.15	2.07
Glu	3.11	3.11		1.90	2.05	1.98
Pro	0.93	1.26				0.73
Gly			1.09	1.13		1.06
Ala			1.03	1.05	1.04	1.04
Val			1.33	0.96	1.00	1.88
Ile					1.82	
Leu	0.95	0.99	1.37	1.85		1.67
Tyr	2.08	3.07		1.02		0.97
Yield (μ mole)	0.25	0.50	0.15	0.25	0.24	0.31
Mobility (pH 6.5)	0.50	0.43	0.30	0.30	0.24	0.08

Table 2. *Carboxymethylcysteine sulphone peptides from chymotryptic digestion of protein BJ, fractionated on Sephadex followed by paper electrophoresis*

Peptide BJC4a includes the sequences Ile-Phe and Val-Val.

Peptide.....	BJC2	BJC3b	BJC4a	BJC5	BJC6	BJC7
Amount analysed (μ mole).....	0.011	0.03	0.02	0.02	0.02	0.009
Lys			0.93			0.9
His				0.98		
Arg		0.97				1.0
CMC ₃ SO ₂	0.96*	0.58	0.53	0.51	0.51	0.55
Asp	1.11	0.88	1.04			3.1
Thr			1.01	1.06		2.0
Ser			2.91			3.1
Glu	3.14	1.06	2.03	2.08	2.03	2.3
Pro	0.9		2.03			
Gly		1.05	1.03	1.03		1.2
Ala			1.02	1.00		2.2
Val			1.17	1.02		2.0
Ile			0.64			2.0
Leu	1.03		2.13	1.04		
Tyr	2.00				2.0	1.1
Phe			0.70			
Yield (μ mole)	0.025	0.20	0.20	0.40	0.22	0.020
Mobility (pH 6.5)	0.50	0.45	0.35	0.38	0.35	0.16

* Determined from the radioactivity.

Table 3. *Peptides from peptic digestion of peptide BJT2, fractionated by electrophoresis at pH 2 (45 min. at 4 kv)*

Peptide	Relative yield	Composition	N-Terminus (A1)	N-Terminus after Edman degradation	
				A2	A3
BJT2P1*	0.11	CMCySO ₂ , 0.5; Glu, 2.1; Tyr, 1.15			
BJT2P2	0.17	Asp, 1.0; Glu, 1.0; Pro, 0.9; Leu, 1.0; Tyr, 2.0			
BJT2P2A2†	—	Asp, 1.0; Glu, 1.0; Pro, 0.8; Leu, 1.0; Tyr, 1.2	Glu	Asp	Leu

* Radioactive peptide.
† Peptide BJT2P2 after one step of Edman degradation.

The amino acid analyses of the different peptides are given in Tables 1 and 2.

The peptides that can be identified as belonging to the C-terminal stretch common to different Bence-Jones proteins are discussed below.

Peptide BJC6. The composition (Table 2) was (CMCySO₂,Glu₂,Tyr₂). Carboxypeptidase released 0.8 mol. of tyrosine/mol. and 0.3 mol. of glutamine/mol.

The sequence is: (Tyr,CMCySO₂,Glx)-Gln-Tyr. *Peptide BJC3a.* Paper analysis gave: CMCySO₂, +; Glu, ++; Tyr, +.

Peptide BJT2. The composition (Table 1) was (CMCySO₂,Asp,Glu₃,Pro,Leu,Tyr₃). The C-terminus (by hydrazinolysis) was tyrosine.

A pepsin digest was fractionated and analysed (Table 3). Digestion of peptide BJT2P2 with leucine aminopeptidase released: Tyr, ++; Glu, ++; Asn, +; Leu, trace. Its N-terminus was tyrosine (Table 3).

The above peptides (see also Tables 1 and 2), combined with previous studies of peptide BJ2a (Milstein, 1966b), indicate the sequence given in Fig. 2.

Peptide BJT4. The composition (Table 1) was (Lys,CMCySO₂,Asp₂,Thr₂,Ser,Glu₂,Ala,Val,Ile₂) and the N-terminus was valine.

The peptide was digested with pepsin and the digest fractionated by paper electrophoresis at pH 6.5 (Table 4). Peptide BJT4P3 is neutral, indicating that one of the two aspartic acid residues is in the amide form.

The results, combined with the sequence results obtained previously (peptide BJ2c; Milstein, 1966b), indicate the sequence given in Fig. 3.

Peptide BJC7. The yield of this peptide was very small and no further work was possible. Its amino acid analysis (Table 2) and the fact that it was obtained by chymotrypsin digestion, suggest that it contains an identical sequence in the N-terminus to the homologous peptide from peptide Ker (see below).

Fig. 2. Proposed sequence of peptide BJT2 (see the text) and peptide BJ2a (Milstein, 1966b).

The following sequence seems plausible on these grounds: Ser,Ala,Ser,Val,Gly,Asp,Arg-BJT4-Tyr.

Protein Ker

The N-terminal residue was aspartic acid. The N-terminal peptide prepared by cyanogen bromide cleavage gave the following amino acid analysis: Asp, 1.0; homoserine, 0.75; Glu, 1.1; Ile, 0.85.

A trypsin digest (KT; 75 mg.) and a chymotrypsin digest (KC; 35 mg.) were fractionated on Sephadex followed by paper electrophoresis. Owing to a mistake the chymotrypsin digest was made with 10 times the normal amount of enzyme (a 1:10 ratio instead of 1:100 was used and the digestion was carried out for 15 hr. at 37°).

Peptide KT1. The composition (Table 5) was (Lys,CMCySO₂,Asp₂,Thr₂,Glu₂,Pro₃,Gly₂,Leu,Tyr₂,Phe₁).

The peptide was digested with pepsin, with a high enzyme/substrate ratio. The results are summarized in Table 6 and Fig. 4. Peptide KT1P4 after one step of Edman degradation gave an amino acid analysis indicating N-terminal tyrosine (Asp, 2.0; Thr, 1.9; Pro, 2.9; Gly, 2.0; Leu, 1.0;

* Radioactive peptide.

Fig. 3. Proposed sequence of peptide BJT4 (see the text) and peptide BJ2c (Milstein, 1966b).

* Values obtained after 86 hr. hydrolysis; threonine corrected for destruction during hydrolysis.

Peptide KC5. The composition (Table 8) was

Table 6. *Peptides from peptic digestion of peptide KT1, fractionated by paper electrophoresis at pH 6.5 and 3.5*

A 0.2 μ mole sample of peptide in 400 μ l. of 0.02N-HCl was digested for 6 hr. at 37° with 50 μ g. of pepsin.

Peptide	Mobility (pH 6.5)	Relative yield	Composition	N-Ter- minus (A1)	N-Terminus after Edman degradation		
					A2	A3	A4
KT1P1*	0.42	0.6	CMCySO ₂ , 0.45; Glu, 2.0; Tyr, 1.0				
KT1P2*	0.30	0.15	CMCySO ₂ , 0.48; Glu, 2.0; Tyr, 2.0				
KT1P3	0.22	0.15	Lys, 0.9; Asp, 1.9; Thr, 2.1; Pro, 3.0; Gly, 2.1; Leu, 1.0; Phe, 1.0	Asp	Asp	Leu	Pro
KT1P4	0.18	0.6	Lys, 1.0; Asp, 2.0; Thr, 2.0; Pro, 2.8; Gly, 1.9; Leu, 1.0; Tyr, 1.1; Phe, 1.1	Tyr†			
KT1P5	−0.40	0.10	Lys, 0.9; Thr, 1.0; Pro, 1.2; Gly, 2.0	Gly			

* Radioactive peptide.
† Determined by subtractive Edman degradation.

Fig. 4. Proposed partial sequence of peptide KT1 (see Table 6).

Table 7. *Peptides from subtilisin digestion of peptide KT1P4, fractionated by paper electrophoresis at pH 6.5*

Peptide	Purifica- tion	Mobility (pH 6.5)	Relative yield	Composition	N-Ter- minus (A1)	N-Terminus after Edman degradation	
						A2	A3
KT1P4S1		0.60	0.20	Asp, 1.9; Thr, 1.0; Pro, 2.0; Leu, 1.0; Tyr, 1.1			
KT1P4S2		0.50	0.15	Asp, 2.1; Thr, 1.2; Pro, 2.6; Gly, 1.7; Leu, 1.3; Tyr, 1.1; Phe, 1.0			
KT1P4S3	3.5	0	0.25	Gly, 2.2; Pro, 1.1; Phe, 0.8	Phe	Gly	Pro
KT1P4S4		−0.50	0.35	Thr, 1.0; Lys, 1.0	Thr

Fig. 5. Proposed sequence of peptide KT1P4.

(CMCySO₂,Glu₂,Tyr₂). Carboxypeptidase released tyrosine and glutamine in a 1.0:1.3 ratio. Edman degradation was performed and the residual

peptide analysed. The following result was obtained: after the first step: CMCySO₂, 0.41; Glu, 2.0; Tyr, 1.3; after the second step: CMCySO₂, 0.2; Glu, 2.0; Tyr, 0.2.

The probable sequence of peptide KC5 is therefore: Tyr-CMCySO₂-Gln-(Gln,Tyr).

All these results and those obtained previously on the peptic peptide K3d1 (Milstein, 1966b) can be summarized as given in Fig. 6. Peptide KC3b contained only one tyrosine residue, which could be either N-terminal or C-terminal.

Peptide KT2. The composition (Table 5) was (Lys,CMCySO₂,Asp,Thr₂Ser,Glu₂,Ala,Ile₃).

Table 8. *Carboxymethylcysteine sulphone peptides from chymotryptic digestion of protein Ker, fractionated on Sephadex followed by paper electrophoresis*

Peptide.....	K C1	K C2	K C3a	K C3b	K C4	K C5	K C6	K C7
Amount analysed (μ mole).....	0.01	0.009	0.022	0.008	0.015	0.011	0.011	0.016
Lys							0.76	0.78
His					0.94			
Arg			1.02					
CMCySO ₂	0.95*	0.46	0.51	0.93*	0.44	0.52	0.90*	0.55
Asp	1.97	2.05	0.82				1.90	
Thr	1.03	0.96			0.96			0.95
Ser							1.09	2.05
Glu	2.09	1.98	1.05	2.0	2.08	2.00	2.24	
Pro	2.14	1.80						
Gly			1.16		1.15			1.06
Ala					1.18		1.06	1.15
Val					1.04			1.25
Ile							0.83	
Leu	0.92	0.83			0.98			1.09
Tyr	1.04	1.60		0.85		1.92		
Phe	0.97	0.97					1.03	
Yield (μ mole)	0.08	0.07	—	0.08	0.46	0.13	0.15	0.35
Mobility (pH 6.5)	0.63	0.57	0.45	0.45	0.37	0.30	0.27	0

* Determined from the radioactivity of the peptide.

Fig. 6. Proposed sequence of peptide KT1 (see the text) and peptide K3d1 (Milstein, 1966b).

Table 9. *Peptides from peptic digestion of peptide KT2, fractionated by paper electrophoresis at pH 6.5*

Peptide	Purification	Mobility (pH 6.5)	Composition	N-Terminus (A1)	N-Terminus after Edman degradation		
					A2	A3	A4
KT2P2*		0.35	CMCySO ₂ ,† 0.8; Thr, 1.8; Glu, 1.1; Ile, 1.7	Ile	Thr		
KT2P3*		0.29	CMCySO ₂ , 0.5; Thr, 1.9; Ser, 1.1; Glu, 2.1; Ala, 1.0; Ile, 1.7				
KT2P4		0.20	Unchanged peptide KT2				
KT2P52	2.1	0	Lys, +; Ala, +; Ile, +; Ser, +; Glu, +; Asp, +	Ala	Ser	Glu	Asp
KT2P53	2.1	0	Lys, +; Asp, +; Ile, +	Asp	Ile		

* Radioactive peptide.

† Determined from the radioactivity of the peptide.

This peptide was digested with pepsin (Table 9). Assignment of the amide group of peptide KT2P52 (neutral) is made because peptide KT2P53 is also neutral.

The partial sequence of peptide KT2 is given in Fig. 7.

Fig. 7. Proposed partial sequence of peptide KT2.

Peptide KC6. The composition (Table 8) was (Lys,CMCySO₂,Asp₂,Ser,Glu₂,Ala,Ile,Phe). This peptide did not contain threonine and therefore should have CMCySO₂ as *N*-terminus (see peptide K3d2; Milstein, 1966b, and the sequence below). A split between threonine and CMCySO₂ is not common. However, this could be accounted for by the high enzyme/substrate ratio used for the chymotrypsin digest. The sequence Asn-Phe (see below) in the *C*-terminus is likely from the enzyme specificity. Asparagine rather than aspartic acid is indicated by the similarity in mobilities of peptides KC6 and KT2.

The results for peptides KT2 and KC6 and previous results obtained for the peptic peptides

Fig. 8. Proposed partial sequence relating peptides KT2 and KC6 (see the text), and peptides K3d2 and K3e (Milstein, 1966b).

Table 10. *Carboxymethylcysteine sulphone peptides from tryptic digestion of protein Rad, fractionated on Sephadex followed by paper electrophoresis*

Peptide RT0 was not performic acid-oxidized during its purification.

Peptide.....	RT0	RT1	RT2	RT3	RT4
Amount analysed (μmole).....		0.016		0.045	0.032
Lys				0.94	
His				1.00	
Arg			1.0		0.97
CMCyS	1.00				
CMCySO ₂		0.46	0.30	0.36	0.48
Asp		1.03	1.77		
Thr		2.88	1.20	2.03	1.05
Ser		1.14	1.93	2.07	1.08
Glu	1.03	5.28	0.4	2.07	
Pro		1.75	0.98	0.89	
Gly	0.97		1.20	1.02	
Ala		0.97	0.82	0.99	0.90
Val		0.94	1.15	2.85	
Leu		0.82	1.70	0.98	1.06
Tyr		3.06	0.72	0.93	
Phe		2.10	0.88		
Yield (μmole)	1.3	0.65*	0.05	1.2	1.4
Mobility (pH6.5)	0.98	0.67	0.15	0	0

* Partly insoluble at pH3.5, streaked at low-pH electrophoresis.

K3d2 and K3e (Milstein, 1966b) indicate the sequences given in Fig. 8.

Protein Rad

The *N*-terminal residue was glutamic acid. The protein does not contain methionine in any appreciable quantity. A tryptic digest (RT; 100mg.) and a chymotryptic digest (RC; 60mg.) was fractionated on Sephadex followed by paper electrophoresis, as indicated in the Materials and Methods section.

Peptide RT1. The composition (Table 10) was CMCySO₂, Asp, Thr₃, Ser, Glu₅, Pro₂, Ala, Val, Leu-, Tyr₃, Phe₂) and the *N*-terminus was leucine. After one step of Edman degradation the *N*-terminus was glutamic acid.

The peptide was digested with pepsin (Table 11 and Fig. 9). Peptide RT1P4a was digested with 100 μg. of leucine aminopeptidase for 5hr. Since neither serine nor threonine was present, glutamine

could be estimated. The analysis gave: CMCySO₂, 0.4; Gln, 1.6; Ala, 1.0; Val, 1.0; Tyr, 1.6. Both glutamic acid residues in the peptic peptide P4a are therefore glutamine.

Peptide RT1P1a has a molecular weight of about 620 from its composition and a mobility of 0.90. Comparison with other peptides of similar mobility and molecular weight (Offord, 1966) indicate that it contains three charged acidic residues at pH 6.5. Therefore both glutamic acid and the aspartic acid residues of peptide P1 should be in the acid form. A partial acid hydrolysate of peptide P1 gave peptides (Table 12 and Fig. 10) indicating the sequence Pro-Glu after the sequence Leu-Glu (*N*-terminus of peptide RT1). The peptide Pro-Glu-Asp is indicated by the mobility of peptide RT1P1I, which is faster at pH 6.5 than Pro-Glu and has *N*-terminal proline, as indicated by its isatin reaction.

Peptide RC1. The composition (Table 13) was (CMCySO₂, Thr₃, Ser, Glu₃, Pro, Tyr, Phe). Carboxy-

Table 11. Peptides from peptic digestion of peptide RT1, fractionated by electrophoresis at pH 6.5

Peptide	Purification	Mobility (pH 6.5)	Relative yield	Composition	<i>N</i> -Terminus (A1)	<i>N</i> -Terminus after Edman degradation					
						A2	A3	A4	A5	A6	A7
RT1P1a	3.5	0.90	0.4	Asp, 1.2; Glu, 2.0; Pro, 1.1; Leu, 0.7							
RT1P1b	3.5	0.79	0.2	Asp, 1.0; Glu, 1.9; Pro, 1.0; Leu, 0.8; Phe, 0.8							
RT1P4a*	2.1	0.26	0.4	CMCySO ₂ , 0.57; Glu, 2.0; Ala, 0.9; Val, 0.9; Tyr, 2.1	Ala	Val					
RT1P4c	2.1	0.26	0.5	Thr, 2.8; Ser, 1.2; Glu, 1.0; Pro, 1.2; Tyr, 1.1; Phe, 1.0		Glu	Thr	Ser	Pro	Thr	Thr

* Radioactive peptide.

Leu-Glu-(Pro,Glu,Asp)-Phe-Ala-Val-(Tyr₂,CMCyS,Gln₂)-Tyr?-Glu-Thr-Ser-Pro-Thr-Thr-Phe?

Fig. 9. Proposed partial sequence of peptide RT1 (see Table 11).

Table 12. Peptides from partial hydrolysis (20 min., 6*N*-hydrochloric acid, 100°) of peptide RT1P1a, fractionated by electrophoresis at pH 6.5

Peptide RT1P1a is Leu-Glu-(Pro,Glu,Asp) (see Table 11 and Fig. 9).

Peptide	Purification	Mobility (pH 6.5)	Relative yield	Composition	Staining on paper
RT1P1I	3.5	0.96	Trace	—	Isatin (+)
RT1P1II	3.5	0.78	++	Leu, ++; Glu, +++; Pro, +	Ninhydrin (+); isatin (—)
RT1P1III	3.5	0.67	+	Glu, ++; Pro, +	Isatin (+)

peptidase (paper analysis) gave: Phe, +; Thr, +. The *N*-terminus was CMCySO₂. (After one step of Edman degradation the amino acid analysis of the remaining peptide was: CMCySO₂, 0.15; Thr, 3.1; Ser, 1.0; Glu, 3.1; Pro, 0.9; Tyr, 1.1; Phe, 1.1.) The peptide was very resistant to digestion, but

could be fractionated by using a high pepsin concentration (100 μg. of enzyme/0.1 μmole of peptide in 200 μl. of 0.02*N*-hydrochloric acid incubated for 15 hr. at 37°) (Table 14).

Peptide RC2. The composition (Table 13) was (CMCySO₂,Thr₃,Ser,Glu₃,Pro,Tyr₂,Phe). Carboxypeptidase released threonine and phenylalanine in the ratio 1.1:1.0.

Peptide RC6. The composition (Table 13) was (CMCySO₂,Glu₂,Tyr₂). Carboxypeptidase released glutamine and tyrosine in the ratio 1.3:1.0, indicating that both glutamic acid residues are present as amides: (Tyr,CMCySO₂)-Gln-(Gln,Tyr). The presence of glutamic acid in peptides RC1 and RC2 is indicated by the fact that the mobilities of these two peptides at pH 6.5 are greater than the mobility of peptide RC6.

Peptides RT1, RC1, RC2 and RC6 (see above)

Fig. 10. Proposed sequence of peptide RT1P1a (see Table 12).

Table 13. Carboxymethylcysteine sulphone peptides from chymotryptic digestion of protein Rad, fractionated on Sephadex followed by paper electrophoresis							
The composition of peptide RC7 was checked after 100 hr. hydrolysis to confirm the valine content.							
Peptide.....	RC1	RC2	RC3	RC4	RC5	RC6	RC7
Amount analysed (μmole).....	0.015	0.010	0.020	0.024	0.030	0.020	0.027
Lys				0.92			
His					0.86		
Arg			1.15				1.03
CMCySO ₂	0.48	0.55	0.55	0.44	0.44	0.60	0.49
Asp			0.85	0.98			0.91
Thr	3.07	3.10		0.95	0.94		
Ser	1.02	1.29		3.01			4.95
Glu	2.92	3.10	1.00	1.98	2.00	2.02	1.00
Pro	1.25	1.20		1.90			
Gly			0.98	1.03	1.05		
Ala				1.08	1.08		1.02
Val				1.14	0.98		0.94
Ile				0.73			
Leu				2.10	0.99		
Tyr	1.26	2.10				1.98	1.05
Phe	1.10	1.02		0.80			
Yield (μmole)	0.11	0.10	0.20	0.35	0.45	0.20	0.55
Mobility (pH 6.5)	0.42	0.38	0.35	0.30	0.33	0.36	0.05

Table 14. Peptides from peptic digestion of peptide RC1, fractionated by electrophoresis at pH 2.1
A high concentration of pepsin was used for the digestion (see the text).

Peptide	Relative yield	Composition	N-Terminus (A1)	N-Terminus after Edman degradation	
				A2	A3
RC1P1*	0.12	CMCySO ₂ , 0.5; Glu, 2.0; Tyr, 1.1			
RC1P2*	0.15	CMCySO ₂ , 0.5; Glu, 2.0			
RC1P3	0.37	Thr, 2.7; Ser, 1.3; Glu, 1.1; Pro, 0.9; Phe, 0.9	Glu	Thr	Ser
RC1P4	0.1	Thr, 1.9; Pro, 1.3; Phe, 1.0	Pro	Thr	Thr

* Radioactive peptide.

Fig. 11. Proposed sequence relating peptides RT1, RC1, RC2 and RC6 (see the text) and peptides R4a and R4b (Milstein, 1966b).

Table 15. *Peptides from peptic digestion of peptide RC7, fractionated by paper electrophoresis at pH 3.5*

Peptide	Relative yield	Composition	N-Terminus (A1)	N-Terminus after Edman degradation				
				A2	A3	A4	A5	A6
RC7P2*	0-10	Arg, 1.0; CMCySO ₂ , +*; Ser, 2.0; Glu, 1.0; Ala, 1.0	Ser	(-)	Arg	Ala	Ser	Glu
RC7P3	0-17	Asp, 1.0; Ser, 2.8; Val, 1.0; Tyr, 1.0	Val	(see the text)				

* The peptide was radioactive but the CMCySO₂ peak was lost in the analysis.

and the peptic peptides R4a and R4b (Milstein, 1966b) are related as indicated in Fig. 11.

Peptide RT4. The composition (Table 10) was (Arg,CMCySO₂,Thr,Ser,Ala,Leu) and the *N*-terminus was alanine. Edman degradation gave: A2, threonine; A3, leucine; A4, serine; A5, nothing; A6, arginine. CMCySO₂ is destroyed on acid hydrolysis, especially if the hydrolysis is done in the presence of air (as is the case in the *N*-terminal analysis). CMCySO₂ is therefore suspected in peptide A5 by elimination.

Peptide RC7. The composition (Table 13) was (Arg,CMCySO₂,Asp,Ser₅,Glu,Ala,Val,Tyr). The aspartic acid and glutamic acid residues are in the amide form since the peptide was practically

neutral at pH 6.5. The slight acid mobility is probably due to the fact that serine was the *N*-terminal residue. The *N*-terminus was serine and the *C*-terminus (by hydrazinolysis) was tyrosine. Carboxypeptidase released: Val, +; Ser, ++; Asn, +; Gln, trace; Tyr, ++; Ala, trace.

The peptide was digested with pepsin (Table 15). Carboxypeptidase released from peptide RC7P2 (paper analysis): Ala, +; Ser, +; Gln, +. After five steps of Edman degradation a single peak was obtained in the amino acid analyser without prior hydrolysis of the peptide: it could have been either glutamine or serine by its position; it ran as glutamine on paper electrophoresis at pH 2. CMCySO₂ is assigned in the position of A2 because it is the

Table 16. *Peptides from subtilisin digestion of peptide RC7, fractionated by paper electrophoresis at pH 3.5*

No radioactive peptides were obtained. This is likely to be due to the instability of CMCySO₂ in the alkaline conditions used for subtilisin digestion.

Peptide	Purification	Composition	N-Terminus
RC7S3b	2.1	Ser, 1.0; Val, 2.0	Val
RC7S3c	2.1	Asp, 0.9; Ser, 1.0; Tyr, 1.0	Asp (trace)

only residue in the peptide that is undetectable by 'dansyl' N-terminal analysis. Direct evidence was obtained by preparing the cysteic acid-containing peptide R 4c (see Fig. 12). After one step of Edman degradation it gave cysteic acid as N-terminus. Peptide P3 gave a doubtful Edman degradation. The N-terminus was valine [identified both by electrophoresis at pH 4.4 of the 'dansyl' derivative and chromatography in PeAW (light petroleum-acetic acid-water; 10:9:1, by vol.)]. Further degradation gave: A2, Val, + + ; A3, Ser, + ; A4, Ser, +, and Asp, + ; A5, Asp, + + ; A6, Asp, +. In all cases a

Fig. 12. Proposed sequence relating peptides RC7 and RT4 (see the text) and peptide R 4c (Milstein, 1966b).

Fig. 13. Proposed sequence of the C-terminus containing CyS 4 and CyS 5. Peptides marked * are discussed by Milstein (1966b) and sequences in parentheses are taken from Hilschmann & Craig (1965) and Titani *et al.* (1965).

strong spot of chlorotyrosine was also observed. The same result was obtained in a second experiment with the only difference that in A2 traces of serine and aspartic acid were also detected. An analysis of peptide RC7P3 after three steps of Edman gave the following result: Asp, 1.0; Ser, 1.6; Tyr, 1.0. Both serine and aspartic acid were detected in the *N*-terminal analysis. The peptide seems to be at this stage a mixture of the following two: Ser-(Asn,Ser,Tyr) and Asn-(Ser,Tyr). The sequence Val-Val suggested by the 'dansyl'-Edman procedure is eliminated because after 100hr. hydrolysis of the peptide RC7 no increase in valine was observed. It seems therefore that the first Edman degradation step was incomplete either in the coupling step or the cyclization step, or in both. This is indicated by the fact that after three steps of Edman degradation, 1 valine and 1.4 serine residues had been subtracted from the original peptide, leaving the residual 60% of peptide with *N*-terminal serine and the A4 peptide containing aspartic acid as *N*-terminus.

The sequence of peptide RC7P3 suggested as

Val-Ser-Ser-Asn-Ser-Tyr was confirmed digesting peptide RC7 with subtilisin (Table 16).

This can be combined with the results for peptide RT4 (see above) and for peptide R4c (Milstein, 1966*b*) to yield the sequence given in Fig. 12.

The common peptides

Fig. 13 summarizes the results obtained on peptides derived from the 24 residues of the *C*-terminus, common to the three proteins, except for a valine/leucine substitution. The *N*-terminus of peptide BJT5 was leucine, that of peptide KT3 was also leucine and that of peptide RT3 was valine. This sequence includes one-half of the homologous intrachain bridge (CyS 4) and the interchain bridge (CyS 5) peptides (Roy numbering: CyS-192 and CyS-212 respectively; Hilschmann & Craig, 1965; Milstein, 1966*b*).

Fig. 14 and Tables 17, 18 and 19 show the results obtained on peptides containing the other half (CyS 3) of the homologous intrachain bridge (CyS-132, Roy numbering; Hilschmann & Craig, 1965;

Fig. 14. Proposed sequence around CyS 3. The sequence of the peptides marked * has been discussed previously (Milstein, 1966*b*). The order of the residues in parentheses is suggested on comparative grounds with similar proteins (Titani *et al.* 1965).

Milstein, 1966b). The tryptic peptide containing this half-cystine residue was insoluble and could not be obtained except for a trace amount in protein Rad (peptide RT2; Table 10). Because of the very small yields no further work was done on it, but it seems identical in amino acid composition with peptide T16 of protein Roy (Hilschmann & Craig,

1965) and with the equivalent peptide in other proteins. It should be noted that residue 120 is aspartic acid. Protein Ag has been reported to contain asparagine in that position (Titani *et al.* 1965). Protein Rad, on the other hand, gives aspartic acid in this position, which could be released with carboxypeptidase from peptide RC4P2 (Table 19). Further, peptide RC4P2 was an acidic peptide and aspartic acid is the only acidic residue in it. The mobility of RC4 was roughly equal to the mobility of peptide BJC4a at pH 6.5, suggesting that both peptides, which have identical amino acid analyses, have also the same charge distribution.

Table 17. *Peptides from peptic digestion (24 hr.) of peptide BJC4a, fractionated by paper electrophoresis at pH 6.5*

Peptide	Relative yield	Composition
BJC4aP1	1.0	Glu, 1.9; Leu, 1.0
BJC4aP2*	1.0	CMCySO ₂ , 0.6; Val, 0.9; Leu, 1.0
BJC4aP4	0.8	Lys, 0.8; Thr, 0.9; Ser, 2.0; Gly, 1.1; Ala, 1.1

* Radioactive peptide; the presence of two valine residues in a sequence Val-Val would give an analysis as shown after 20 hr. hydrolysis (Milstein, 1966b).

Table 18. *Peptides from subtilisin digestion of peptide KC7, fractionated by paper electrophoresis at pH 6.5*

Peptide	Mobility (pH 6.5)	Composition
KC7S1*	0.45	CMCySO ₂ , 0.5; Val, 1.1†; Leu, 1.0
KC7S2	-0.42	Lys, 0.9; Thr, 1.0; Ser, 2.0; Gly, 1.1; Ala, 1.2

* Radioactive peptide.

† 20 hr. hydrolysis; under these conditions the Val-Val sequence remains largely unhydrolysed giving a low valine recovery (Milstein, 1966b).

Table 19. *Peptides from peptic digestion of peptide RC4, fractionated by electrophoresis at pH 3.5*

Peptide	Composition	Released by carboxypeptidase
RC4P1*	CMCySO ₂ , +; Val, 1.7; Leu, 1.0	Leu, ++
RC4P2	Asp, 1.0; Ser, 1.0; Pro, 2.0; Ile, 0.7; Phe, 0.8	Asp, ++
RC4P3	Glu, 2.0; Leu, 1.0	Leu, ++; residual peptide had mobility 0.62: (Glu, Gln)-Leu
RC4P4	Lys, 0.9; Thr, 0.9; Ser, 2.0; Gly, 1.0; Ala, 1.0	

* Radioactive peptide; 80 hr. hydrolysis; the presence of CMCySO₂ is deduced from its radioactivity since after 80 hr. hydrolysis it is almost completely destroyed.

DISCUSSION

In the three proteins studied, five cysteine-containing sequences have been identified. By comparison with the block sequence of another protein (Roy numbering; Hilschmann & Craig, 1965) they can be preliminarily placed along the chain as follows: CyS 1, in position 23; CyS 2, in position 86; CyS 3, in position 132; CyS 4, in position 192. CyS 5 is in the C-terminus (Milstein, 1964). The disulphide bridges in the three proteins are established between CyS 1 and CyS 2 and between CyS 3 and CyS 4, the CyS 5 being involved in interchain binding (Milstein, 1964, 1966b). The sequences around the cysteine residues have now been extended, both in the variable and in the invariant stretch of the three proteins.

The 'invariant' stretch. Apart from the valine-leucine substitution in residue 189 (related to the Inv type; Milstein, 1966a,b; Baglioni *et al.* 1966), no well established difference within the C-terminal half of the different Bence-Jones proteins of type K studied has been found. A difference in residues 173-176 has been discussed by Titani *et al.* (1965) and confirmatory work is needed to show whether this is due to technical difficulties. In the present paper the evidence for an aspartic acid residue in position 120 (Roy numbering) is in contrast with an asparagine in the same position in protein Ag (Titani *et al.* 1965). However, this type of substitution should be taken with caution, not only because of the known difficulties in differentiating the acids from the amides, but also because often (as was the case in the present studies) acid conditions that might lead to deamidation are used in the purification of the proteins. However, the latter seems unlikely because the yield of the peptide (RC4 and BJC4a; see Fig. 14 and Tables 2 and 3) was not unduly low, indicating that if deamidation occurred it should have been very specific.

The 'variable' stretch. Fig. 15 presents the N-terminus and the sequence around the CyS 1 of

Protein Ker	Asx-(Ile, Glx)-(Met,Thr,Gln,Pro, Ser,Ser,Ser, Leu)-Ser- Ala- Ser-Val-Gly- Asp- Arg-Ile- Thr- Ile- Thr-Cys-Gln-Ala-Ser-Gln-Asp-Ile- Lys-(Asn,Phe)
Protein BJ	Asx-(Val,Glx)-Met- Thr-Gln-(Pro,Ser,Ser,Ser, Leu-(Ser,Ala, Ser, Val,Gly, Asp)-Arg-Val-Thr- Ile- Thr-Cys-Gln-Ala-Ser-Gln-Asp-Ile- Asn-Lys-(Tyr)
Protein Rad	Glx Ala-Thr-Leu- Ser- Cys-Arg-Ala-Ser-Gln-Val-Ser-Ser- Asn- Ser- Tyr Leu
Protein Ag	Asp- Ile- Gln- Met- Thr-Gln-Pro- Ser-Ser-Ser- Leu-Ser- Ala- Ser-Val-Gly- Asp- Arg
Protein Cu	Glx
Protein Roy	Asx-(Ile, Glx, Met, Thr,Glx,Pro, Ser,Ser,Ser)-Leu-Ser- (Ala,Ser,Val,Gly)-Asx- Arg- (Ile,Thr, Ile, Thr,Cys,Glx, Ala,Ser,Gln,Asp,Ile, Ser, Val, Phe)-Leu 10 20 30
Protein Day	Val-(Thr,Ile, Thr,Cys,Glx,Ala,Ser,Gln,Asp,Ile, Ser, Asx, Phe, Leu)
Protein 3	Asp- Ile- Val- Leu- Thr-Gln
Protein 12	Glu- Ile- Val- Val- Thr-Gln

Fig. 15. Proposed N-terminal sequence and sequence around Cys 1 in several type K Bence-Jones proteins. References: proteins Cu and Roy: Hilschmann & Craig (1965); protein Ag: Titani, Whitley & Putnam (1966); protein Day: Milstein (1966a); proteins 3 and 12: Hood, Gray & Dreyer (1966); proteins Ker BJ and Rad: this paper.

Protein Ker	Tyr-Tyr- Cys-Gln-Gln- Tyr-Asp-Asp-Leu-Pro-Thr-Phe- Gly-Pro- Gly-Thr- Lys
Protein BJ	Tyr-Tyr- Cys-Gln-Gln- Tyr-Gln-Asn-Leu-Pro-Tyr
Protein Rad	Leu- Glu- Pro-Glu-Asp-Phe-Ala-Val- Tyr-Tyr- Cys-Gln-Gln- Tyr-Glu-Thr- Ser- Pro-Thr-Thr-Phe
Protein Ag	Leu- Gln- Pro-Glu-Asp-Ile- Ala-Thr-Thr- Tyr- Cys-Gln-Gln- Tyr-Glu-Thr- Leu-Pro-Arg-Thr-Phe- Gly-Gln- Gly-Thr- Lys
Protein Cu	Val- (Val,Gly,Glx,Asx, Met,Ala,Val,Tyr,Tyr, Cys,Glx,Glx)-Arg-Leu-(Glx,Ile, Pro,Tyr,Tyr,Phe, Gly,Glx, Gly,Thr)-Lys
Protein Roy	(Leu,Glx, Pro,Glx,Asx)-Ile- Ala-Thr-Thr-Tyr-(Tyr,Cys,Glx,Glx, Phe,Asx,Asx, Leu,Pro,Leu,Thr,Phe)-gly-(Gly,Gly,Thr)-Lys 80 100

Fig. 16. Proposed sequence around Cys 2 in several type K Bence-Jones proteins (for references see Fig. 15).

several proteins, and Fig. 16 the sequences around Cys 2. The most striking feature of this comparison is the similarity of the sequences of some of the proteins. The amino acid analysis of a peptide is often sufficient to make a reasonable guess at what the amino acid sequence is likely to be. This is well illustrated with protein Day (Fig. 15) and protein Roy (Figs. 15 and 16). However, there are also some proteins presenting a much larger variation in some stretches. This seems to be the case in protein Rad as shown in Fig. 15, and also with other proteins like Cu (Hilschmann & Craig, 1965), Day and Man (Milstein, 1966a). It is not yet clear what the significance of such a difference might be, but it must be carefully considered since it may invalidate some of the comparisons. With this word of caution, it is possible to summarize places where substitutions have been observed and to try to get some insight into the problems of the origin of the variability of the primary sequence of immunoglobulins. The sequences could be used to check some theories that have been proposed in the past (see, e.g., Milstein, 1966a). But in general the sequences resemble the type of variation that has been observed in the study of the species variations of proteins like haemoglobin, cytochrome *c* and others. Table 20 shows a summary of the places where substitutions have been observed. There are 33 such places in Table 20. However, this number is certainly larger, since several places where the sequence of the variant is not known have been omitted from the Table. For instance, position 78 in protein Cu cannot be glycine, since there is no glycine in the peptide, but the exact residue in its place is not known. Most of the variations could

arise by a one-step mutation but there are five exceptions (in boxes). However, the exceptions should be regarded with caution. Corrections to Table 20 are not excluded, especially for the proteins for which only preliminary results are available. But, more important, future sequences may change the significance of the variations. For example, an aspartic acid/threonine substitution requires a two-step mutation (see position 91; Fig. 16). But asparagine is present in a third protein at the same position. The substitutions asparagine/aspartic acid and asparagine/threonine are both possible by point mutations; a precursor protein containing asparagine could thus mutate to either threonine or aspartic acid in a single step.

The variable stretches are remarkably similar in the cases studied, as if derived from a common ancestor protein. Whether the common ancestor has given rise in the course of evolution to a large number of related proteins, or whether these arose from a single gene by somatic mutation, is an open question. If in both cases the same selective pressure is operative (requirement of a competent antibody), the resultant variety of sequences may be similar. However, the requirement of a two-gene-one-chain hypothesis seems a necessity if the multiple-genes hypothesis is accepted. An invariant half-chain will then have to join to one of a large set of variable halves (Hood *et al.* 1966; Cioli & Baglioni, 1966). On the other hand, if a mutation mechanism starting with a small number of genes is operative, the mechanism must include a way of selectively affecting only one stretch of the chain. Selective pressure is probably of primary importance (Milstein, 1966b) but may not be enough. The possibility of a special mechanism of

Table 20. *Substitutions observed in Bence-Jones proteins of type K*

Only substitutions up to residue 106 are included. Further details of each substitution are shown in Figs. 15 and 16 or in Titani *et al.* (1966).

Roy no.	Observed residues	Roy no.	Observed residues	Roy no.	Observed residues
1	Asp; Glu	31	Lys; Asn	83	Val; Thr
2	Val; Ile	32	Ser; Phe?; Tyr?	89	Tyr; Arg
3	Gln; Val	33	Tyr; Leu	90	Asp; Glu; Leu
4	Met; Leu; Val	46	Ile; Leu	91	Asp; Asn; Thr
19	Ile; Val; Ala	53	Asn; Lys	92	Leu; Ser
21	Ile; Leu	55	Glu; Ala	94	Pro; Thr; Arg; Tyr
22	Thr; Ser	72	Thr; Lys	98	Pro; Gln; Gly?
24	Gln; Arg	75	Gly; Arg; Ser?	102	Leu; Val
28	Asp; Val	76	Leu; Val	103	Glu; Asp
29	Ile; Ser	77	Glu; Gln	104	Ile; Phe
30	Lys; Asn; Ser	81	Phe; Ile	105	Lys; Arg

mutation based on specific recognition of a hypermutable fragment of DNA (Brenner & Milstein, 1966; Milstein, 1966c) could also explain this type of variability.

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