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The Disulphide Bridges of Immunoglobulin κ-Chains

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The arrangement of the disulphide bridges of the major component of the light chains of immunoglobulins (κ-chains) has been studied in the Bence-Jones proteins. Three disulphide bridges have been found. An interchain bridge at the *C*-terminus has been shown to occur in the dimers of all the proteins studied and was characterized by symmetrical peptides. In the monomer form, the *C*-terminal half-cystine of the corresponding peptides was linked to a lone half-cystine residue. A second common disulphide-bridge peptide in which a single amino acid difference could be related to the Inv factors of the individual proteins was found in Bence-Jones proteins and in the κ-chains of normal and abnormal immunoglobulins. Peptides characteristic of a third disulphide bridge studied in three specimens were found to have differences in some residues, but also striking similarities. A methionine peptide has also been characterized in two specimens as a by-product of the technique employed. It is suggested that a general manner of folding may be a common feature of the heterogeneous population of κ-chains: one bridge which folds an invariable stretch of the chain, another bridge which folds a stretch that varies from protein to protein, and a bridge at the *C*-terminus which is the interchain link.

The antigenic determinants characteristic of the two types of light chains (κ and λ) have been associated with all the immunoglobulin fractions: the macroglobulins (IgM), the β_2a-globulins (IgA) and the γ-globulins (IgG) (Fahey, 1962; Fudenberg & Franklin, 1963). In the IgG it has been shown that the light chains are linked to the heavy chains through one disulphide bridge (Fleischman, Porter & Press, 1963). In spite of this homogeneous structural pattern the light chain isolated from normal individuals is a highly heterogeneous population of molecules (Cohen & Porter, 1964; Poulik, 1964). This heterogeneity has been a major problem in sequence studies. However, the immunoproteins isolated from patients with multiple myeloma do not share this heterogeneity (Cohen & Porter, 1964; Edelman, 1962). Further, it has been shown that the proteins excreted through the urine of some of these patients (Bence-Jones proteins) were very similar to (Putnam, 1962) and possibly identical with (Schwartz & Edelman, 1963) the light chains of the same patient. It is possible that the myeloma proteins are normal components produced by an increased population of cells derived from a single clone. The arrangements of disulphide bridges in normal light chains and in Bence-Jones protein are in agreement with such a scheme.

In the Bence-Jones proteins, which have been more fully characterized, five cysteine residues have been identified (Milstein, 1966*a*,*b*). Detailed knowledge of the complete sequence of Bence-Jones proteins is not yet available. However, a block sequence of one type K specimen (Hilschmann & Craig, 1965) suggests the presence of five cysteine residues in positions 20–30 (CyS 1), 80–90 (CyS 2), 132 (CyS 3) and 189 (CyS 4); CyS 5 has been shown to occur at the *C*-terminus of most or all type K chains (Milstein, 1964). In the present paper detailed evidence is presented that supports the idea (Milstein, 1964) that a general manner of folding of the molecule may be a common feature of the heterogeneous population of κ-chains: CyS 3 and CyS 4 being linked by a disulphide bridge to fold an invariable stretch of the chain, the *C*-terminal cysteine providing for the interchain link, and the other two cysteine residues, located in an environment that is different in individual proteins, giving a 'variable' disulphide-bridge peptide.

MATERIALS AND METHODS

Abnormal proteins. These were kindly supplied by Dr S. Cohen (BJ and F) and by Dr A. Feinstein (Rad and Ker). Carboxymethylated chains were prepared by the procedure of Fleischman, Pain & Porter (1962), by using iodoacetate as the blocking agent and fractionating on a Sephadex G-100 column. In some experiments the car-

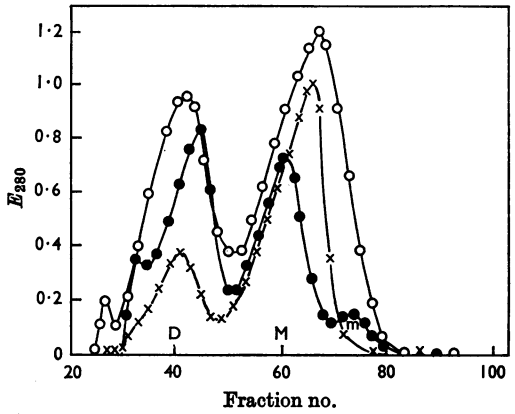

Fig. 1. Fractionation of Bence-Jones proteins in Sephadex G-100: ○, protein BJ (200mg.); ×, protein Rad (100mg.); ●, protein Ker (150mg.). Protein Ker was the only specimen which gave the minor component Km. The ε-DNP-lysine peak emerged in fraction 105.

boxymethylation step was omitted, to obtain non-carboxymethylated chains (LSH). The separation of LSH chains on a Sephadex G-100 column (in M-propionic acid) is less satisfactory, possibly because of a higher degree of aggregation, the yields being therefore lower.

Sephadex fractionation. A column (3·5cm. diam. × 70cm. long) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) was run in N-propionic acid. ε-DNP-lysine was added to the protein dissolved in N-propionic acid and used as marker to standardize the position of the peaks. The pattern of the Bence-Jones protein separations was dependent on protein concentration and on the individual specimen. This is probably due to the formation of aggregates, the stability of which depended on the individual sample. Fig. 1 shows the fractionation profile of the three proteins studied in this paper.

'Map' of disulphide bridges. The method is based on the use of performic acid oxidation of paper strips in the vapour phase (Brown & Hartley, 1963).

The peptic digest (protein concn. 10mg./ml.; digestion with 0·25mg. of pepsin/ml. in 5% formic acid for 14–16hr. at 37°) was applied to a large sheet of Whatman paper (both no. 1 and 3MM paper were used). The loading was not higher than 0·8mg./cm. of no. 1 paper and 1·2mg./cm. of 3MM paper. Enough Xylene cyanol FF was added to the digest to give a pale-blue band after application. This dye has been previously used as an external marker (Milstein & Sanger, 1961) and has a very convenient mobility at pH6·5 (mobility 0·38 of that of aspartic acid). A spot made with a red Pentel pen was also used as external coloured marker. It gives a spot with a mobility very similar to that of glutamic acid at pH6·5 and is therefore very useful to indicate the length of the runs.

The pepsin digest was submitted to electrophoresis at pH6·5 for 60min. After the run a 3cm.-wide strip was cut from the length of the paper and placed in a desiccator containing 40ml. of formic acid and 2ml. of 30% (w/v) H_2O_2. Oxidation was carried out for 2½hr. at room temperature, after which time the paper was left in an air draught in the fume cupboard for about an hour and then sewn on to a new sheet of the same paper. Electrophoresis in the second dimension was then carried out in a direction at right angles to the original, under the same conditions as for the first run. The peptides were detected with 1% ninhydrin or with cadmium–ninhydrin (100ml. of 1% ninhydrin in acetone plus 15ml. of a solution of 6g. of cadmium acetate in 30% acetic acid). Peptides containing cystine gave rise to new peptides that departed from the diagonal, as a result of the increased negative charge of the cysteic acid formed on performic acid oxidation. The position of the disulphide-bridged peptides in the first dimension could be located from these 'fingerprints' and used for preparative purposes.

Purification and analysis of peptides. The remainder of the paper containing the unoxidized disulphide-bridged peptide was cut out and oxidized in a desiccator as described above. Special care is necessary in this step to avoid any traces of chloride present in the desiccator that will give rise to chlorotyrosine and dichlorotyrosine. In some experiments considerable conversion was observed because of traces of HCl present in the desiccator. The bands were eluted and subjected to electrophoresis at pH6·5. The resulting cysteic acid peptides were further purified by electrophoresis at pH3·5, unless otherwise indicated. For details of all electrophoretic conditions see Milstein & Sanger (1961). The electrophoretic mobility at pH6·5 (*m*) was calculated by taking the mobility of aspartic acid as unity (Ambler, 1963).

Peptides were named as follows. Letters were used to identify the protein (BJ, R, K, F etc.) and the Sephadex fraction (D, M and m). The first number identified the region containing the cystine peptide in the first dimension of electrophoresis at pH6·5. The letter following identified the position of the cysteic acid peptide obtained in the electrophoretic fractionation of the performic acid-oxidized peptide at pH6·5. The next number, when present, indicated that two or more cysteic acid peptides having the same mobility at pH6·5 were fractionated at pH3·5. For example, BJM3c2 indicates a peptide isolated from protein BJ, Sephadex fraction M, region 3, position c of the peptide 'map', finally fractionated into peptides 1 and 2 at pH3·5.

The enzymic digestion and location of peptides were carried out as described by Ambler (1963) unless otherwise stated.

Amino acid analysis. This was carried out in a Spinco amino acid analyser fitted with a high-sensitivity unit purchased from Evans Electroselenium Ltd., Halstead, Essex. This system gives a sensitivity 8·2 times as high as the ordinary sensitivity of the Spinco analyser. Quantitative determinations on samples containing 0·01μmole of peptide are considered to be within about 10% error. Glutamic acid (0·005μmole) gives a peak height of about 0·075 *E* unit. The simpler peptides were run through a column 25cm. long, which gave a reasonable analysis. Paper-electrophoretic analysis at pH2 was also performed in some cases (Milstein & Sanger, 1961).

Proline stain. After the separation of amino acids at pH2 the paper was developed in 0·25% ninhydrin in acetone containing 2–3% of collidine. After the results had been recorded the paper was dipped in a 0·2% (w/v) solution of isatin in 4% (v/v) acetic acid in acetone, and placed in a 100° oven for 5min. Proline becomes a bright-red spot (but sometimes blue, depending on the batch of

collidine), which is much more sensitive than the normal yellow obtained after ninhydrin–collidine.

N-Terminal analysis. This was performed by the 'dansyl' method of Gray & Hartley (1963*a*). Electrophoresis at pH 4·4 does not always resolve some DNS* residues: Ala, Pro and Ser, and Val, Ile and Phe. These derivatives were separated after electrophoresis at pH 4·4 as follows. The area containing them was sewn on to Whatman 3MM paper, which was subjected to ascending chromatography in PeAW (light petroleum–acetic acid–water; 10:9:1, by vol.) (Boulton & Bush, 1964) for 24 hr.

Other difficulties encountered were: (1) slight changes of pH caused the glycine derivative and the dimethylaminonaphthalenesulphonic acid to run together on electrophoresis; (2) after chromatography in PeAW the serine derivative remained at the origin together with the dimethylaminonaphthalenesulphonic acid; (3) if ammonia had not been carefully eliminated before the 'dansylation', the arginine derivative was hidden under the dimethylaminonaphthalenesulphonamide spot after electrophoresis at pH 4·4. All these difficulties could be resolved by subjecting the suspected spots to electrophoresis at pH 2 for 20 min. at 110 v/cm.

Peptides containing *N*-terminal tyrosine with few exceptions gave no visible amounts of α-DNS-tyrosine. The bis-DNS-tyrosine is insoluble and not easily detected.

Edman degradation. The procedure of Doolittle (1965) was modified and followed by the determination of the new *N*-terminal amino acid (Gray & Hartley, 1963*b*) and in some cases by amino acid analysis of the peptide left after degradation. The *N*-terminal residue of a peptide is named as A1, the *N*-terminal residue of the peptide released after the first Edman degradation procedure as A2 and so on. Details of the procedure used are as follows. The peptide was placed in a 3 ml. screw-cap tube and dissolved in 300 μl. of 30% (v/v) pyridine. Then 50 μl. of 0·2 M-*N*-ethylmorpholine (redistilled)–acetate buffer, pH 9·0, was added and the tubes were flushed for a few minutes with N_2. Then 0·3 ml. of 5% (v/v) phenyl isothiocyanate in redistilled pyridine was added and the mixture incubated for 3 hr. at 37°. Next 200 μl. of water was added and the solution extracted four times with 2 ml. portions of benzene. Finally the aqueous phase containing the coupled peptide was freeze-dried. Cyclization was performed by dissolving the dry residue in 100 μl. of trifluoroacetic acid and incubating for 1½ hr. at room temperature. The solution was dried over NaOH and the remaining peptide dissolved in 30% pyridine. A sample containing approx. 5 mμmoles of peptide was taken for *N*-terminal analysis and the rest was kept for further degradation.

RESULTS

Fig. 2 shows a photograph of the disulphide-bridge peptides of the peptic digest of some fractions obtained by Sephadex-gel filtration. Representative examples are presented to illustrate the general pattern, but the individual proteins are discussed below.

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; in amino acid sequences, $CySO_3H$, $MetSO_2$, Asn and Gln refer to cysteic acid, methionine sulphone, asparagine and glutamine residues respectively.

For convenience five regions can be considered in the patterns by dividing them into vertical bands. Each region included several cysteic acid-containing peptides lying off the diagonal, which indicated the position of the unoxidized cystine peptides in the first dimension. Equivalent Sephadex fractions (Fig. 1) of different Bence-Jones proteins gave an identical pattern in region 5, but this varied when the Sephadex fractions of any one particular protein were compared. This region was shown to contain cysteic acid peptides, which differed in the dimer and the monomer forms (and were missing altogether in the smaller fraction Km of Fig. 1). These peptides were used to characterize the amino acid sequence around the interchain disulphide bridge. Some peptides derived from this part of the molecule were also present in the region 3 (neutral in the first dimension, i.e. before oxidation). Region 1 contained peptides of the common intrachain disulphide bridge, and was present in two patterns: the one shown in the photograph of KD had a slower acidic component than the one of RD (Fig. 2). The first was present in all the Inv(1, 2) and the second in all the Inv(3) type proteins. Finally, the regions 2, 3 and 4 varied from specimen to specimen, indicating differences in the sequences around the relevant disulphide bridges. In some cases the pattern was reproducible, but in other cases the intensity of some spots varied considerably from one digest to the next.

The interchain bridge

Fig. 3 shows a diagram of the disulphide-bridge 'maps' of the two Sephadex fractions of protein BJ. Both gave very similar patterns except for two sets of peptides. These peptides were basic or neutral in the first dimension (before the performic acid oxidation) and gave rise to two very fast-moving acidic spots (3a and 5a) on the M fraction, which were either absent from or very faint in the D fraction. Both spots were identified as free cysteic acid by their amino acid analysis and by their mobility at pH 3·5 and 6·5. Since peptides in region 5 were basic in the first dimension, peptide 5a must have been linked to one of the other two peptides (5b and 5c) moving out of the diagonal. This was confirmed by running a sample of unoxidized band 5 at pH 3·5. The paper was then subjected to performic acid vapours and a new diagonal was performed at pH 3·5. A spot with the mobility of cysteic acid departed from the diagonal in line with another cysteic acid peptide. Traces of cysteic acid were sometimes observed in fraction D, probably due to contamination with fraction M. The two basic peptides, 5b and 5c, from fraction M were also obtained from fraction

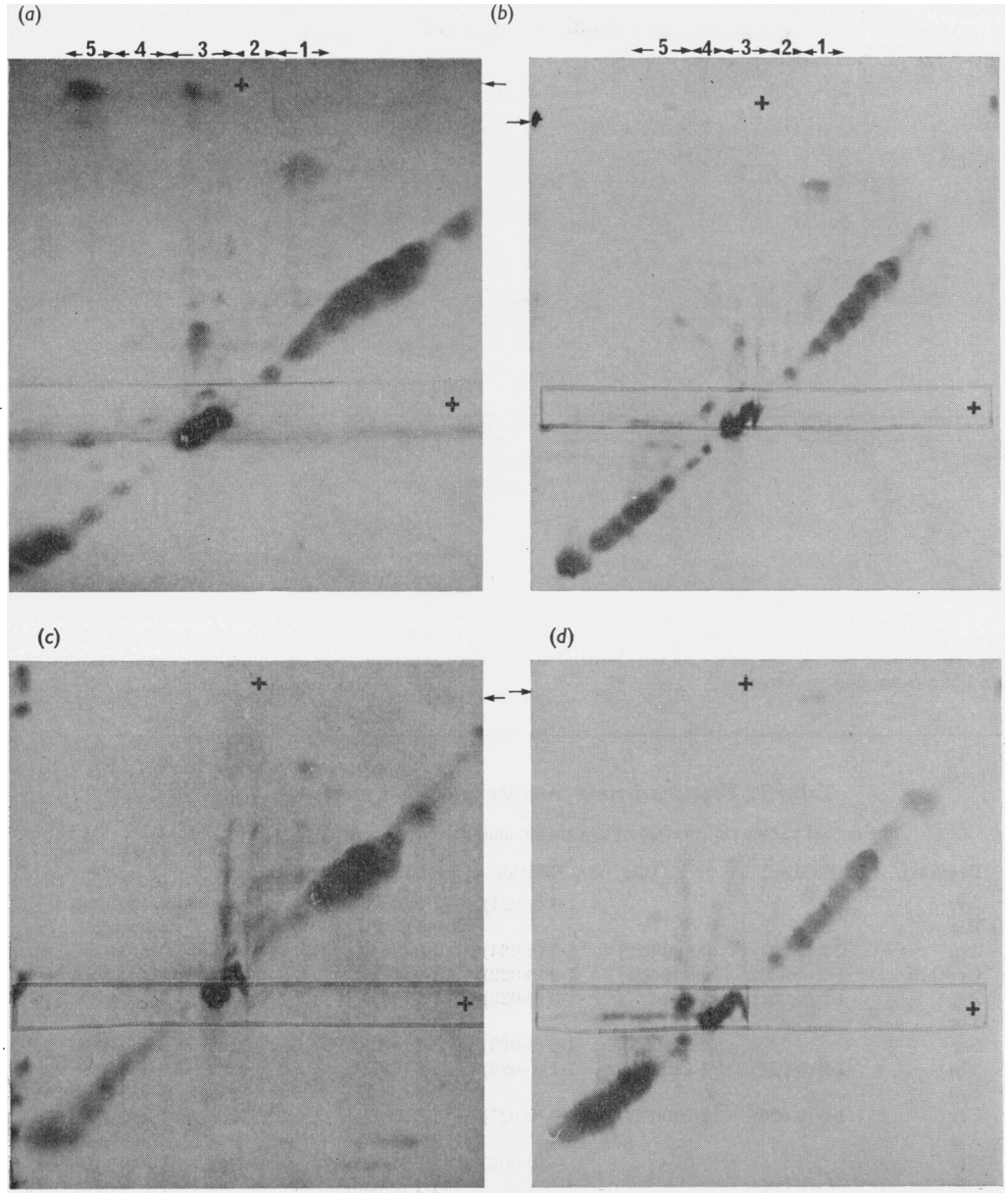

Fig. 2. 'Map' of the disulphide bridges of several fractions obtained as shown in Fig. 1: (*a*) KM; (*b*) KD; (*c*) Km; (*d*) RD. Each 'map' was divided into five regions, which included the peptides present in a vertical line. A peptic digest of each protein was fractionated by electrophoresis at pH 6.5. A marker strip cut out along the paper was oxidized with performic acid vapour. The strip was then sewn on to a new sheet of paper, as shown in the Figure, and run again at pH 6·5 at right angles to the original direction. A mixture of amino acids or a spot made with red Pentel pen or both were used as markers on each side of the paper. The position to which the red spot had moved at the end of the run is shown by an arrow.

D. The cystine peptides from which they were derived by oxidation had a different position in the first dimension, suggesting that these peptides had different mobilities from the ones in fraction M only when the disulphide bridge was intact.

Peptides BJ M3c2 and BJ D5a. The mobility

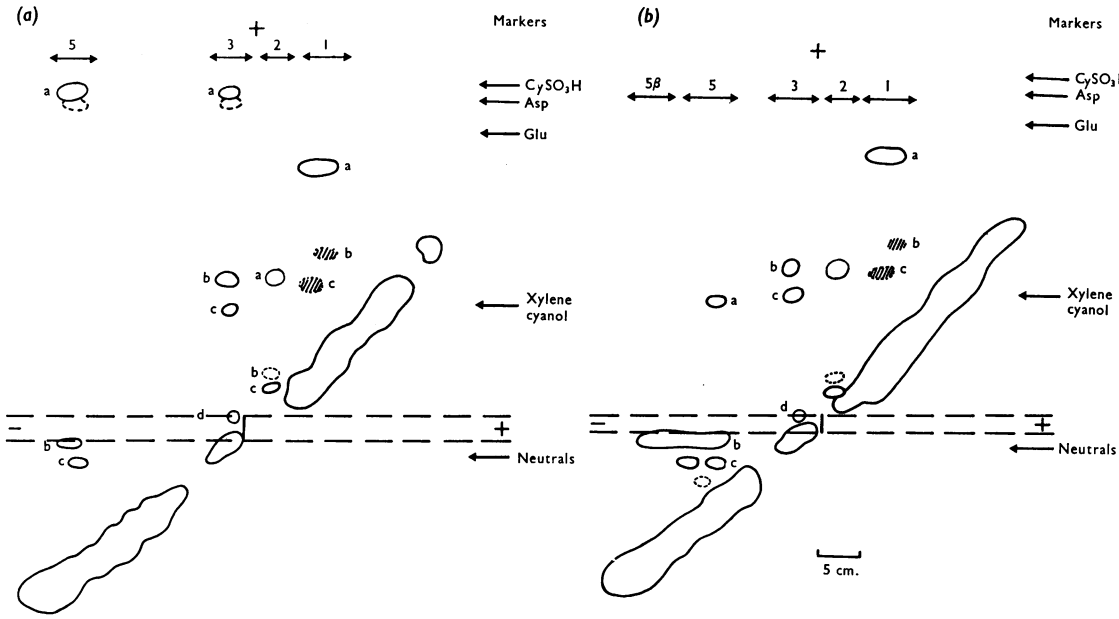

Fig. 3. Diagram of the 'map' of the disulphide bridges of the two fractions obtained by Sephadex fractionation (Fig. 1) of protein BJ: (*a*) BJM; (*b*) BJD. Hatched areas indicate peptides giving an almost negative ninhydrin stain but a clear chlorine reaction.

Table 1. *Peptides derived from the interchain disulphide bridge*

The actual values (in μmoles) of the amino acid analysis are indicated in parentheses.

Peptide.....	BJM3c2	BJD5a	BJM5b	BJM5c	BJM5cT2	K5c
Lys			1·0 (0·024)	1·0 (0·036)		0·8 (0·020)
His				0·9 (0·034)		1·0 (0·025)
Arg	0·9 (0·024)	0·9 (0·022)	1·0 (0·024)	1·0 (0·036)	0·8 (0·014)	0·8 (0·019)
$CySO_3H$	1·0 (0·026)	0·9 (0·023)	1·0 (0·022)	1·0 (0·037)	0·9 (0·016)	1·0 (0·024)
Asp	1·2 (0·031)	1·1 (0·027)	1·0 (0·023)	1·1 (0·041)	1·2 (0·021)	1·1 (0·026)
Thr				2·0 (0·074)		2·1 (0·050)
Ser		(<0·004)	1·0 (0·023)	3·1 (0·115)	1·1 (0·019)	3·1 (0·074)
Glu	1·2 (0·031)	1·1 (0·028)	1·1 (0·026)	1·9 (0·071)	1·2 (0·021)	2·0 (0·048)
Pro				0·8 (0·028)		1·2 (0·028)
Gly	1·0 (0·026)	1·1 (0·026)	0·9 (0·021)	2·0 (0·075)	1·1 (0·019)	1·9 (0·046)
Ala				(<0·005)	(<0·004)	(<0·004)
Val			(<0·002)	1·9 (0·071)	(<0·004)	1·9 (0·045)
Leu	(<0·005)			1·1 (0·038)		1·1 (0·026)
Phe	0·8 (0·021)	0·8 (0·020)	1·0 (0·022)	1·0 (0·037)	0·8 (0·014)	0·9 (0·023)

at pH 6·5 was 0·31. The composition (Table 1) was (Arg,$CySO_3H$,Asp,Glu,Gly,Phe) and the *N*-terminus was phenylalanine. The Edman degradation procedure gave identical results in both peptides; the *N*-terminal residues after each step were: A2, aspartic acid; A3, arginine; A4, glycine.

Peptide BJM3cA4 had mobility 1·1 at pH 6·5 and gave a grey colour after ninhydrin. From its mobility (slightly faster than free cysteic acid) it must have contained two acidic residues.

The cystine peptide from which peptide BJM3c2 was derived by oxidation was neutral, suggesting that it contained only one acidic residue.

The probable sequence of peptides BJM3c2 and BJD5a is: Phe-Asn-Arg-Gly-(Glu,$CySO_3H$).

Peptides BJM5b, BJD5b and BJD5β. These

Table 2. *Peptides derived from tryptic digestion of peptides BJM5b and BJD5β, fractionated by paper electrophoresis at* pH 6·5

Peptide	Mobility	Relative yield	Composition (paper analysis)
T1	+1·1	+++	Gly,Glu,$CySO_3H$
T2	+0·33	++	Arg,Gly,Ser,Glu,Phe, Asp, $CySO_3H$
T4	−0·43	+	Ser,Phe,Asp,Arg
T5	−0·67	++	Lys,Arg,Ser,Phe,Asp
T6	−0·86	+++	Lys

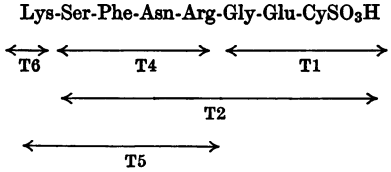

Fig. 4. Proposed sequence of peptides BJM5b, BJD5b and BJ D5β.

peptides were all neutral at pH 6·5. They all had the same electrophoretic mobility at pH 3·5 and the same amino acid composition (analysis by paper electrophoresis), namely (Lys,Arg,Gly,Ser,-Glu,Phe,Asp,$CySO_3H$). The quantitative analysis of peptide BJM5b is shown in Table 1. The *N*-terminus was lysine. Successive Edman degradations gave rise to the following *N*-terminal residues: A2, serine; A3, phenylalanine; A4, aspartic acid.

Digestion of peptides BJM5b and BJD5β with trypsin gave rise to two identical sets of peptides, as shown in Table 2. Edman degradation of peptide BJM5bT1 gave: A1, glycine; A2, glutamic acid; A3, cysteic acid.

Since peptide BJM5b was neutral at pH 6·5 only two acidic residues were present: cysteic acid and either aspartic acid or glutamic acid. Peptide T4 was basic and peptide T1 slightly faster than free cysteic acid, indicating the presence of asparagine in peptide T4 and glutamic acid in peptide T1. The proposed sequence of peptides BJM5b, BJD5b and BJD5β is given in Fig. 4.

Peptides BJM5c and BJD5c. The electrophoretic mobility at pH 6·5 was 0·12. These peptides were obtained in very small yields, and only the one from the M fraction was studied. The composition (Table 1) was (Lys,His,Arg,$CySO_3H$,Asp,-Thr_2,Ser_3,Glu_2,Pro,Gly_2,Val_2,Leu,Phe).

Trypsin digestion gave three major peptides, which were separated by electrophoresis at pH 3·5. The composition of peptide T1 (paper analysis) was (Gly,Glu,$CySO_3H$) and that of peptide T2 (Table 1) was (Arg,$CySO_3H$,Asp,Ser,Glu,Gly,Phe).

It therefore seemed to be a larger peptide including the peptides studied previously. The proposed partial sequence is: (His,Thr_2,Ser_2,Pro,Val_2,Leu)-Lys-Ser-Phe-Asn-Arg-Gly-Glu-$CySO_3H$.

The difference between the disulphide-bridge 'maps' of fraction M and fraction D could be explained in the following way. Fraction M (moving in the Sephadex column as the light chains) was the monomer, and fraction D the dimer. The structure of the various interchain disulphide-bridge peptides located by means of the 'maps' of Figs. 2 and 3 were as follows. In the monomer the interchain bridge occurred between the polypeptide chain and half-cystine residue. Oxidation of the M fraction gave rise to free cysteic acid (Milstein, 1965) and pepsin digestion to the following cystine peptides (see BJM; Fig. 3): CyS·S-BJM3c2, neutral in the first dimension, after oxidation giving peptide BJM3c2 (acidic) and free cysteic acid; CyS·S-BJM5b, basic in the first dimension, after oxidation giving peptide BJM5b (neutral) and free cysteic acid; CyS·S-BJM5c, basic in the first dimension, after oxidation giving peptide BJM5c (basic) and free cysteic acid. In the dimer the interchain bridge occurred through the same cysteine residue of two apparently identical polypeptide chains. The following peptic peptides characterized the interchain disulphide bridge (BJD; Fig. 3): BJD3c-S·S-BJD3c, a symmetrical peptide, neutral in the first dimension, which after oxidation should have given a single acidic peptide identical with peptide BJD5a; this was presumably peptide BJD3c, but the yield of this peptide was very low and it was not analysed. The following cystine peptides should have been present but not well resolved: BJD5a-S·S-BJD5b, basic in the first dimension, giving peptides BJD5a (acidic) and BJD5b (neutral) after oxidation; BJD5a-S·S-BJD5c, basic in the first dimension, giving peptides BJD5a (acidic) and BJD5c (basic) after oxidation; BJD5b-S·S-BJD5c, basic in the first dimension, giving peptides BJD5b (neutral) and BJD5c (basic) after oxidation; BJD5c-S·S-BJD5c, a symmetrical peptide, basic in the first dimension, giving peptide BJD5c after oxidation; BJD5b-S·S-BJD5b, a symmetrical peptide, basic in the first dimension, giving after oxidation peptide BJD5b, which was isolated as peptide BJD5β; this was the only disulphide peptide from fraction D that could be isolated free from the others at pH 6·5.

The separation of all these peptides was not satisfactory. However, it was clear that the dimer is formed through a disulphide bridge involving the same cysteine residue in each monomer. This was apparent not only because of the occurrence of peptide BJD5β on oxidation of a symmetrical disulphide-bridge peptide but, more especially, because of the presence of peptide BJ 5a (an acidic

peptide) in the basic region and by the shape and distribution of the different spots.

The characteristic pattern of peptic peptides in this region was observed in the 'maps' of all the type K Bence-Jones proteins studied (seven specimens). Table 1 gives the amino acid analysis of a peptic peptide of protein Ker, which was identical by its position in the 'map' (Figs. 2 and 5) with peptide BJ 5c. The carboxymethylated light chains obtained after partial reduction of pooled normal IgG gave a high yield of a carboxymethylated tryptic peptide that corresponded to the tripeptide T1 of the above-mentioned sequence. However, the yields were not quantitative and the significance of this has been discussed previously (Milstein, 1965). These results indicate that the interchain bridge is identical in most or all of the κ-chains.

There is one spot, present as a tail of the cysteic acid after performic acid oxidation, whose nature was obscure. The mobility of this spot was different from that of oxidized glutathione; at pH 6·5 and 2 it had the electrophoretic mobility of homocysteic acid. The yield was very low (about 5% of that of cysteic acid). It may have been an artifact originating during the oxidation.

The homologous disulphide bridge

Two of the specimens (K; Figs. 2 and 5; and BJ; Fig. 3) had a series of three cysteic acid peptides (numbered 1) that seemed identical in the peptide 'maps'. In particular, peptides 1b and 1c had a very faint ninhydrin colour yield, but gave a very strong chlorine stain. Specimen Rad (Figs. 2 and 6) and the light chain of the 7s myeloma protein F (not shown) gave a similar pattern in this region, except that the peptide 1a was considerably faster. These peptides were all present in normal human light chains (Fig. 7), but in this case both fast and slow peptides 1a were apparent. This difference was very consistent and seemed to be due to cleavage by pepsin at either side of a Tyr-Ala bond, depending on the specimen. The reason for this difference is discussed below.

Peptide BJ 1a. The mobility at pH 6·5 was 0·80.

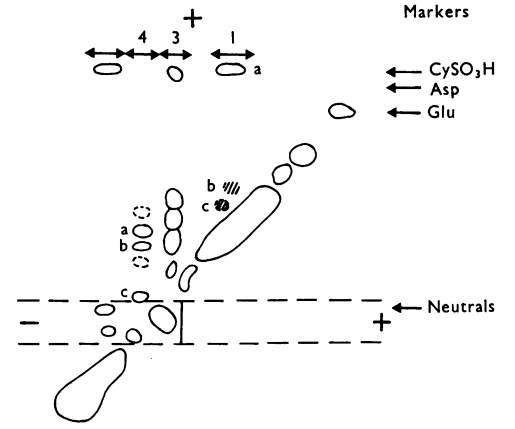

Fig. 6. Diagram of the 'map' of the disulphide bridges of the fraction M of protein Rad (RM).

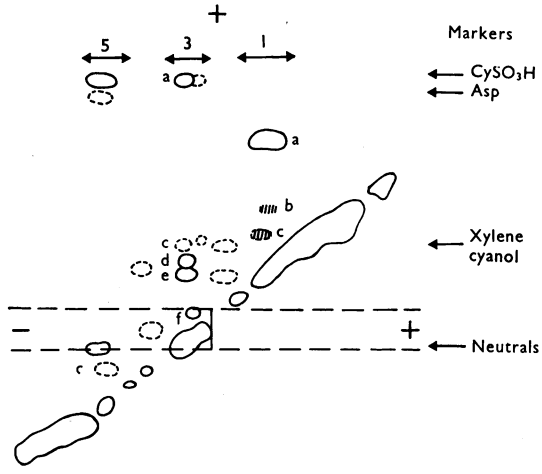

Fig. 5. Diagram of the 'map' of the disulphide bridges of the fraction M of protein Ker (KM).

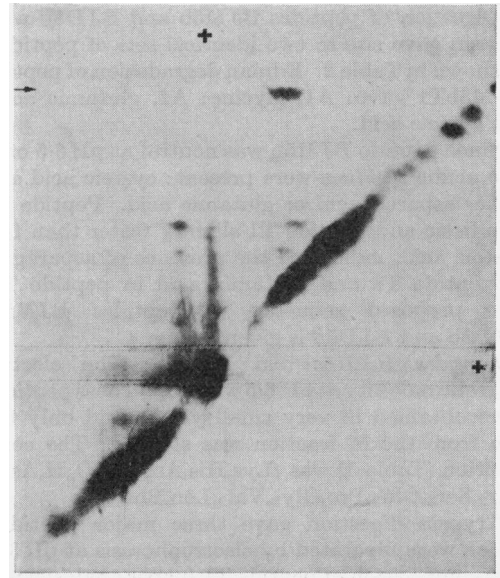

Fig. 7. 'Map' of disulphide bridges of light chains isolated from pooled IgG.

Table 3. *Cysteic acid peptides of the homologous intrachain bridge*

Details were the same as for Table 1.

Peptide.....	BJ 1a	BJ 1b†	BJ 1c*	K 1a	K 1b†	K 1c†	R 1a	R 1b†	R 1c†	L 1c	F 1a
$CySO_3H$	1·0 (0·047)	1·0 (0·031)	1·0 (0·11)	0·9 (0·086)	1·0 (0·020)	0·9 (0·016)	0·9 (0·015)	1·0 (0·006)	1·0 (0·013)	1·0 (0·008)	1·0 (0·069)
Asp	(0·009)			(0·002)	(0·003)		(<0·002)	(<0·003)	(0·002)		(<0·002)
Ser			(<0·02)			(0·003)					
Glu	1·1 (0·053)	(<0·002)		1·0 (0·103)			1·1 (0·017)			(<0·001)	1·1 (0·080)
Ala	0·9 (0·040)			1·0 (0·098)			1·0 (0·016)				0·9 (0·065)
Val		1·0 (0·030)	1·5 (0·16)		1·0 (0·020)	1·9 (0·032)		0·8 (0·005)	1·9 (0·025)	1·0 (0·008)	
Leu		1·0 (0·031)	1·0 (0·11)		1·0 (0·020)	1·1 (0·019)		1·0 (0·006)	1·0 (0·014)	1·0 (0·008)	
Tyr	0·9 (0·042)			1·0 (0·101)							

* 64 hr. hydrolysis. † 96 hr. hydrolysis.

The composition (Table 3) was ($CySO_3H$,Glu,Ala, Tyr).

For peptide BJ 1aA2 the composition (paper analysis) was (Ala,Glu,$CySO_3H$), the *N*-terminus was alanine, and the mobility at pH 6·5 was 1·1.

For peptide BJ 1aA3 the composition (paper analysis) was (Glu,$CySO_3H$), the *N*-terminus was cysteic acid, and the mobility at pH 6·5 was 1·2 (faster than cysteic acid).

For peptide BJ 1aA4 the mobility was identical with that of glutamic acid at pH 6·5.

The proposed sequence for peptide BJ 1a is: Tyr-Ala-$CySO_3H$-Glu.

Peptide BJ 1c. The mobility was 0·46. The composition (Table 3) was ($CySO_3H$,Val_2-Leu). This peptide gave a very faint colour with ninhydrin but a strong chlorine stain. The low yields of valine even after 64 hr. hydrolysis were explained by the presence of a Val-Val sequence. After 16 hr. acid hydrolysis a neutral spot was observed after electrophoresis at pH 2, possibly unhydrolysed Val-Val-$CySO_3H$ and Val-$CySO_3H$. Hydrolysis of the DNS-peptide gave rise to DNS-Val-Val and a trace of DNS-Val. DNS-Val-Val has the mobility of DNS-Ile on electrophoresis at pH 4·4. Chromatography in PeAW gave a very good separation of DNS-Val-Val and DNS-Val, the former being much slower. Carboxypeptidase released leucine.

Peptide BJ 1cA2 gave valine as *N*-terminus. A spot with the same mobility as DNS-carboxymethylcysteine (electrophoresis at pH 4·4) was also present after 20 hr. hydrolysis of the DNS-peptide, presumably DNS-Val-$CySO_3H$.

The proposed sequence for peptide BJ 1c is: Val-Val-$CySO_3H$-Leu.

Peptide BJ 1*b*. The mobility was 0·60. The composition (Table 3) was ($CySO_3H$,Val,Leu). The 16 hr. hydrolysate contained a peptide that was neutral after electrophoresis at pH 2 (probably Val-$CySO_3H$). It gave an almost negative ninhydrin reaction on paper. Staining with chlorine reagent gave a very strong spot. The *N*-terminal analysis was identical with that of peptide BJ 1cA2. Carboxypeptidase released leucine. Successive Edman degradations gave rise to the following *N*-terminal residues: A2, cysteic acid; A3, leucine.

The probable sequence for peptide BJ 1b is: Val-$CySO_3H$-Leu.

The following peptides of region 1 of other proteins were also analysed (Table 3).

Protein Ker. The three peptides from the region K 1 (Fig. 5) were identical in their mobilities and amino acid analyses with the three BJ 1 peptides. In addition, peptides K 1b and K 1c gave a very faint (almost negative) ninhydrin colour and a very strong chlorine reaction, as did the corresponding peptides of the BJ protein.

Protein Rad. Three cysteic acid peptides were isolated from band 1 (Fig. 6). Peptide R 1a had the amino acid composition ($CySO_3H$,Glu,Ala) (Table 3). It had a similar mobility to peptide BJ 1aA2. Peptide R 1c had the same mobility, staining characteristics and amino acid composition as peptide BJ 1c (Table 3). By the same criteria peptide R 1b was similar to peptide BJ 1b.

Normal light chains. Peptide L 1a had the same mobility at pH 6·5 and 3·5 as peptide BJ 1aA2.

Peptide L 1b had the composition (Table 3) ($CySO_3H$,Glu,Ala,Tyr) and the same mobility as peptide BJ 1a.

Peptide L 1c had the composition (Table 3) ($CySO_3H$,Val,Leu) and gave faint ninhydrin and

strong chlorine reactions; electrophoresis at pH 2 of a 16hr. acid hydrolysate showed a neutral spot [presumably (Val,$CySO_3H$)].

Peptides from light chains of a 7s myeloma protein (F) (diagram not shown). Peptide F1a had the composition (Table 3) ($CySO_3H$,Glu,Ala) and a mobility similar to that of peptide BJ 1aA2.

Peptide F 1b had the composition (paper analysis) (Val,Leu,$CySO_3H$) and mobility and staining similar to those of peptide BJ 1b.

The following cystine peptides were indicated by the above results:

Proteins BJ and Ker:

Tyr-Ala-CyS-Glu — Val-Val-CyS-Leu

Tyr-Ala-CyS-Glu — Val-CyS-Leu

Proteins Rad and light chains of F:

Ala-CyS-Glu — Val-Val-CyS-Leu

Ala-CyS-Glu — Val-CyS-Leu

The four peptides were probably present in light chains from pooled normal IgG. All were acidic in the first dimension. After oxidation they gave rise to acidic peptides of increased mobility.

The non-homologous disulphide bridge

A third disulphide bridge gave rise to a pattern of peptides fairly reproducible for each protein (even in the different Sephadex fractions) but which differed from sample to sample.

Protein BJ. The cysteic acid peptides BJ 2a, BJ 2c and BJ 3b were isolated after oxidation.

Peptides BJ 2a and BJ 3b. The mobility was 0·43. The composition (Table 4) was ($CySO_3H$,Asp,Glu_3, Leu,Tyr_3). The low yield of tyrosine from peptide BJ 2a (Table 4) was due to the presence of chlorotyrosine, which originated in the performic acid oxidation step (see the Materials and Methods section). No *N*-terminal residue was detected by the 'dansyl' procedure. Successive Edman degradations gave the following *N*-terminal residues: A2, undetected; A3, cysteic acid; A4, glutamic acid; A5, glutamic acid. Carboxypeptidase released leucine, asparagine, glutamic acid and traces of tyrosine. The peptide was digested with pepsin with a high enzyme/substrate ratio (50 μg. of pepsin and 0·05 μmole of peptide in 100 μl. of 0·05N-hydrochloric acid incubated for 6hr. at 37°). The digest was fractionated by electrophoresis at pH 6·5 (Table 5). Amino acid analysis of peptide BJ 2aP3 (Table 5) after two steps of Edman degradation indicated that two tyrosine residues had been released. This agreed with *N*-terminal analysis since tyrosine was generally undetected by the 'dansyl' procedure. The mobility of peptide BJ 2aP3 (compare with peptide K 3d1 below) suggested that both glutamic acid residues were present as amides.

Table 4. *Cysteic acid peptides derived from the non-homologous disulphide bridge*

Peptides from this Table and from Table 6 are connected by disulphide bridges.

Peptide.....	BJ 3b	BJ 2a	K 3d1	R 4a	R 4b
$CySO_3H$	1·0 (0·028)	0·9 (0·021)	0·9 (0·010)	0·9 (0·008)	1·0 (0·004)
Asp	1·0 (0·027)	1·1 (0·026)		(<0·003)	
Ser	(<0·002)	(<0·003)	(<0·003)		(<0·001)
Glu	3·0 (0·081)	3·0 (0·071)	2·2 (0·025)	2·0 (0·017)	2·0 (0·008)
Ala					1·0 (0·004)
Val					1·0 (0·004)
Leu	1·0 (0·027)	1·0 (0·025)			
Tyr	2·6 (0·070)	1·9 (0·046)*	3·1 (0·035)	1·1 (0·009)	1·8 (0·007)

* Chlorotyrosine was detected on paper electrophoresis but not determined quantitatively in the analyser.

Table 5. *Peptides derived from peptic digestion of peptide BJ 2a, fractionated by electrophoresis at* pH 6·5

Peptide	Mobility (pH 6·5)	Composition	*N*-Terminus	*N*-Terminus after Edman degradation: A2	A3
BJ 2aP1	0·55	Asp, 1·0; Glu, 1·1; Leu, 0·8	Glu	Asp	Leu
BJ 2aP2	0·40	Asp, 1·0; Glu, 1·0; Leu, 0·8; Tyr*, 0·5	Not detected		
BJ 2aP3	0·30	$CySO_3H$, 1·0; Glu, 2·0; Tyr, 1·7	Not detected	Not detected	

* Chlorotyrosine not included.

The probable sequence of peptides BJ 2a and BJ 3b is given in Fig. 8. This sequence should be considered tentative from the data presented. However, further evidence has been obtained from a tryptic digest of the protein (Milstein, 1966*b*). In particular the assignment of tyrosine as *N*-terminal in peptide P2 has been confirmed by these studies.

Peptide BJ 2c. The mobility was 0·2. The composition (Table 6) was (Arg,$CySO_3H$,Asp,Thr_2,Ser,-Glu_2,Ala,Val,Ile), and the *N*-terminus was arginine. Carboxypeptidase (paper analysis) released aspartic acid and glutamine (serine and alanine in minor quantities). Digestion with subtilisin gave the results shown in Table 7. The mobility of peptide BJ 2cS1 at pH 6·5 was 0·73, considerably slower than $CySO_3H$-Glu (1·2) and Glu-$CySO_3H$ (1·3) markers. It was therefore $CySO_3H$-Gln. The

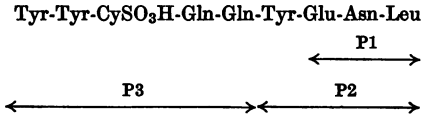

Fig. 8. Proposed sequence of peptides BJ 2a and BJ 3b.

mobility of peptide BJ 2cS3 (Table 7) at pH 6·5 after two steps of Edman degradation (A3) was 0·62, and after the third step (A4) it was identical with that of aspartic acid. Peptide BJ 2cS3 is therefore Ala-Ser-Gln-Asp.

The probable sequence of peptide BJ 2c is given in Fig. 9.

This disulphide bridge of protein BJ can be characterized by the two main peptides BJ 2a and BJ 2c. Peptide BJ 2a-S·S-BJ 2c was acidic (very low mobility) in the first dimension, and after oxidation gave peptides BJ 2a (acidic) and BJ 2c (acidic). Other peptides derived from or containing these sequences were probably also present in the digest. Some attempts to purify them were made with only partial success. It seemed that peptide BJ 2b did not contain the Ala-Ser-Gln-Asp sequence of peptide BJ 2a, but contained an aspartic acid residue in the *N*-terminal position. Neutral cystine peptides were also present, giving rise to cysteic acid peptides consistent with those mentioned above. These were likely to be produced by splitting the bond ...Gln-Ala... (see peptide BJ 2c) and the bond ...Tyr-Glu... (see peptide BJ 2a).

Protein Ker. The cysteic acid peptides K 3d1, K 3d2 and K 3e were isolated after oxidation.

Peptide K 3d1. The mobility was 0·32. The

Table 6. *Cysteic acid peptides derived from the non-homologous disulphide bridge*

Peptides from this Table and from Table 4 are connected by disulphide bridges.

Peptide.....	BJ 2c	K 3d2	K 3e	R 4c
Arg	0·8 (0·015)	0·9 (0·009)	0·8 (0·004)	0·8 (0·009)
$CySO_3H$	1·0 (0·018)	0·8 (0·008)	0·8 (0·004)	0·8 (0·007)
Asp	1·1 (0·020)	1·0 (0·011)	1·0 (0·005)	1·0 (0·011)
Thr	1·9 (0·034)	2·0 (0·021)	1·8 (0·009)	(0·005)
Ser	1·0 (0·019)	(<0·002)	2·0 (0·010)	5·0 (0·055)
Glu	2·1 (0·038)	1·1 (0·012)	1·0 (0·005)	1·2 (0·013)
Gly	(<0·002)		1·0 (0·005)	(0·005)
Ala	1·0 (0·019)		1·0 (0·005)	1·2 (0·013)
Val	1·0 (0·018)		1·0 (0·005)	1·1 (0·012)
Ile	0·9 (0·016)	2·0 (0·021)	1·8 (0·009)	
Tyr				1·0 (0·011)

Table 7. *Peptides derived from subtilisin digestion of peptide BJ 2c, fractionated by paper electrophoresis at* pH 3·5

Peptide	Relative yield	Composition	*N*-Terminus	*N*-Terminus after Edman degradation A2	A3	A4
BJ 2cS1	2	$CySO_3H$, 1·0; Glu, 0·9	$CySO_3H$			
BJ 2cS3	3	Asp, 1·1; Ser, 1·1; Glu, 1·0; Ala, 1·0	Ala	Ser	Glu	Asp
BJ 2cS4	1	Thr, 1·1; Ile, 0·9	Ile			
BJ 2cS5*	1	Arg, +; Val, +; Ile, +; Thr, ++	Arg	Val	(lost)	Ile
BJ 2cS6	1	Arg, 0·9; Thr, 1·0; Val, 1·0	Arg			

* Amino acid analysis by paper electrophoresis.

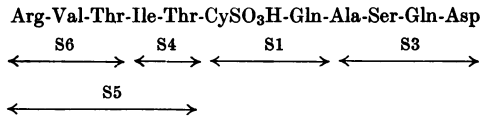

Fig. 9. Proposed sequence of peptide BJ 2c.

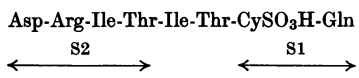

Fig. 10. Proposed sequence of peptide K 3d2.

composition (Table 4) was ($CySO_3H$,Glu_2,Tyr_3), and the *N*-terminal residue determined by the 'dansyl' method was negative. After one step of Edman degradation (A2) the result was again negative. Amino acid analysis of A2 gave: $CySO_3H$, 1·0; Glu, 2·2; Tyr, 2·0; and analysis of peptide A3 gave: $CySO_3H$, 1·0; Glu, 2·0; Tyr, 1·2; indicating the *N*-terminal sequence Tyr-Tyr. Peptide A3 gave cysteic acid as *N*-terminal residue, and A4 gave glutamic acid. Peptide A4 was neutral at pH 6·5, indicating that both glutamic acid residues were present as the amides before hydrolysis. It gave a positive reaction with α-nitroso-β-naphthol, indicating that tyrosine was present in the remaining peptide.

The probable sequence of peptide K 3d1 is: Tyr-Tyr-$CySO_3H$-Gln-(Gln,Tyr).

Peptide K 3d2. The mobility was 0·33. The composition (Table 6) was (Arg,$CySO_3H$,Asp,Thr_2, Glu,Ile_2), and the *N*-terminus was aspartic acid. The *N*-terminal residues after successive Edman degradations were: A2, arginine; A3, isoleucine; A4, threonine; A5, isoleucine; A6, threonine; A7, cysteic acid. After subtilisin digestion, two peptides were isolated. One was peptide K 3d2S1; its mobility was 0·74 and amino acid analysis gave: $CySO_3H$, 0·9; Glu 1·1 [glutamine was indicated by the mobility (compare with peptide BJ 2cS1 above)]. The other was peptide K 3d2S2, a neutral peptide at pH 6·5, that was purified by electrophoresis at pH 2; amino acid analysis gave: Arg, 1·0; Asp, 1·1; Thr, 1·0; Ile, 1·0.

The probable sequence of peptide K 3d2 is given in Fig. 10.

Peptide K 3e. This was purified by electrophoresis at pH 2 after the routine purification by electrophoresis at pH 6·5 and pH 3·5. The mobility was 0·24. The composition (Table 6) was (Arg,$CySO_3H$,-Asp,Thr_2,Ser_2,Glu,Gly,Ala,Val,Ile_2), and the *N*-terminus was serine. *N*-Terminal residues after successive Edman degradations were: A2, alanine; A3, serine; A4, valine; A5, glycine; A6, aspartic

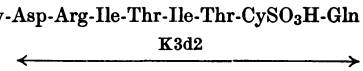

Fig. 11. Proposed sequence of peptide K 3e.

acid. Peptide K 3e seemed to be a larger peptide including peptide K 3d2 in it.

The proposed sequence for peptide K 3e is given in Fig. 11.

This disulphide bridge of protein Ker was characterized from the above peptides by two disulphide-bridged peptides that were not separated at pH 6·5, as follows: K 3d1-S·S-K 3d2, neutral in the first dimension, after oxidation giving rise to peptides K 3d1 and K 3d2, both of very similar mobility at pH 6·5, but well separated by electrophoresis at pH 3·5; K 3d1-S·S-K 3e, neutral in the first dimension, after oxidation giving rise to peptides K 3d1 and K 3e (both acidic).

Protein Rad. Several cystine peptides of similar mobility at pH 6·5 were present in the digest. They probably arose from the lack of specificity of pepsin. The cysteic acid peptides R 4a, R 4b and R 4c were isolated after oxidation.

Peptide R 4a. The mobility was 0·31. The probable composition (Table 4) was ($CySO_3H$,Glu_2,Tyr). This peptide may have contained two tyrosine residues (if significant conversion into chlorotyrosine, which was not estimated, occurred). After one step of Edman degradation the only significant change was that the tyrosine content was decreased to about 0·6 of a residue. From its mobility (compare with peptide K 3d1) and the mobility of the cystine peptide (basic) the two glutamic acid residues should have been present as amides.

Peptide R 4b. The mobility was 0·22. The composition (Table 4) was ($CySO_3H$,Glu_2,Ala,Val,-Tyr_2). Both glutamic acid residues should have been present as amides, for reasons similar to those given for peptide R 4a.

Peptide R 4c. This was neutral at pH 6·5 (in some runs it was just acidic with a mobility below 0·05). The peptide was not purified sufficiently but the following composition is probable (Table 6): (Arg,-$CySO_3H$,Asp,Ser_5,Glu,Ala,Val,Tyr). The glutamic acid should have been in the amide form since the peptide was neutral. It gave a positive stain with both the Sakaguchi and α-nitroso-β-naphthol reagents for arginine and tyrosine respectively.

The disulphide bridge was defined by the three peptides described above. It gave two cystine peptides that could not be separated by electrophoresis at pH 6·5, 3·5 or 2. The peptides were purified after running the band R 4 (Fig. 6) at pH 2. A strip of the pH 2 run was oxidized and run in a second dimension at the same pH. Two main spots

departing from the diagonal were in line. Both gave a positive stain for tyrosine. One of the spots was neutral at pH 2 and contained peptides R 3a and R 3b, which were separated by electrophoresis at pH 6·5. The other spot contained peptide R 3c. Further confirmation was obtained by submitting band R 4 (Fig. 6) to electrophoresis at pH 3·5 for 1 hr. The strip was then oxidized and run at pH 3·5 in the second dimension. Three main bands were observed, departing in line from the diagonal. The three were positive for tyrosine, and the less acidic was also Sakaguchi-positive. The sequences of these three peptides have been established from larger peptides isolated from *S*[^{14}C]-carboxymethylated protein (Milstein, 1966*b*). The results indicate the following cystine peptides: R 4a-S·S-R 4c, a slow basic peptide in the first dimension, giving after oxidation peptides R 3a (acidic) and R 3c (neutral); R 4b-S·S-R 4c, a slow basic peptide in the first dimension, giving after oxidation peptides R 3b (acidic) and R 3c (neutral). A cystine peptide such as R 4a-S·S-R 4b is precluded in this basic region since it would have been neutral in the first dimension.

The methionine peptide

The patterns of the disulphide-bridged peptides of BJ and Ker proteins showed a spot in the neutral region in the first dimension that after oxidation departed from the diagonal but was not quite in line with the cysteic acid peptides. These peptides were BJ 3d (Fig. 3) and K 3f (Fig. 5). It seems that methionine sulphone, when in an *N*-terminal position, has an amino group with a very low p*K*, giving a residual negative charge at pH 6·5.

Peptide BJ 3d. Amino acid analysis gave: $MetSO_2$, 0·9; Thr, 1·0; Ser, 3·3; Glu, 1·1; Pro, 0·9; Leu, 1·1. The composition was (Met,Thr,Ser_3,Glu,-Pro,Leu), and the *N*-terminus was methionine sulphone. The mobility was 0·1 (neutral before oxidation). Successive Edman degradations gave the following *N*-terminal residues: A2, threonine; A3, glutamic acid.

The probable sequence is: Met-Thr-Gln-(Ser,Ser, Ser,Pro-Leu).

Peptide K 3f. Amino acid analysis gave: $MetSO_2$, 0·8; Thr, 1·1; Ser, 3·4; Glu, 1·3; Pro, 0·9; Leu, 1·1. It was similar in mobility and composition to peptide BJ 3d.

The pepsin digest of protein Rad did not show such a peptide, indicating that the sequence in this area was different.

Other disulphide-bridge 'maps'

The Bence-Jones protein Ker gave a fraction Km that emerged from the Sephadex column (Fig. 1) after KM. A 'map' of the disulphide-bridge peptides of Km is shown in Fig. 2. Its main characteristic was the lack of the interchain disulphide-bridge peptides. Within the limitations of the 'fingerprinting' technique it seemed reasonable to say that the *C*-terminal sequence of the protein was missing. However, the homologous intrachain bridge and the non-homologous disulphide-bridge peptides seemed to be present. It was not possible to say whether this was due to splitting of the protein by proteolytic enzymes in the urine or kidney or whether it was due to incomplete chains being released. A more thorough analysis of this fraction was attempted, but it was unsuccessful because of the very small recoveries obtained.

Disulphide-bridge 'maps' of four other Bence-Jones proteins were made (not shown): Man, Day, Fr 3 and Fr 4. The interchain bridge pattern was present in all four protein preparations. No fractionation on Sephadex was performed on them, and therefore a mixture of the dimer and monomer was expected. From the patterns it was concluded that protein Man was predominantly monomer and protein Day predominantly dimer. The common intrachain bridge was present in the four proteins, although its intensity in protein Man was diminished. Proteins Man, Day and Fr 3 showed the fast component in region 1, as was found in protein Rad, whereas protein Fr 4 contained the slower one, as in proteins Ker and BJ.

DISCUSSION

In all the specimens of Bence-Jones proteins of type K analysed so far, three disulphide bridges have been found (Fig. 12). The interchain bridge has been well characterized by pepsin digestion of both the monomer and the stable dimer forms of the protein. The monomer had an extra half-cystine residue, which was linked to the *C*-terminal cysteine of the chain (Milstein, 1964) and which gave free cysteic acid on oxidation. This served as a clear indication of the position of the interchain peptide in the 'map' of disulphide bridges. The lack of specificity of pepsin in this part of the molecule was a powerful tool for establishing the interchain bond of the symmetrical dimer. A symmetrical peptide is far less satisfactory than a series of non-symmetrical disulphide-bridged peptides, all of which can be explained on the basis of a common sequence, as was done with BJ protein. Thus a cysteic acid peptide that should have been neutral before oxidation, but that moved as a basic peptide, was a clear indication that it was linked to a basic peptide. However, the split with pepsin was very unspecific and peptides with similar mobilities did not allow a clear separation of all the cystine derivatives. A better example was found

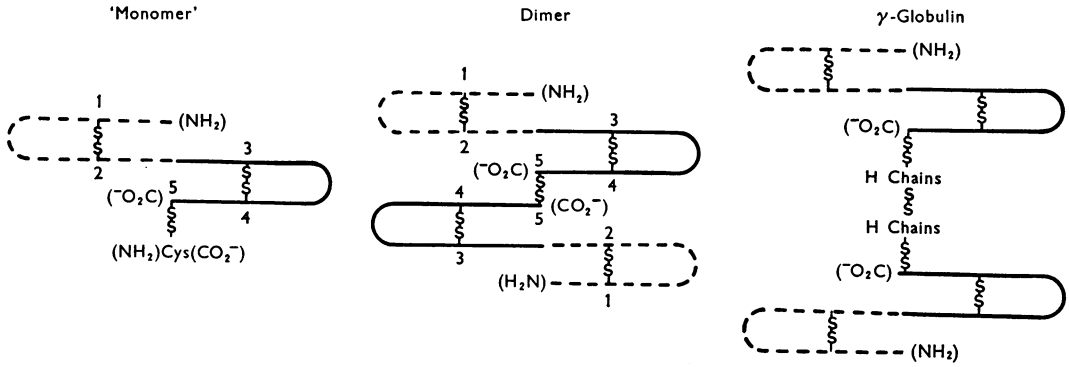

Fig. 12. Suggested arrangement of the disulphide bridges of type K Bence-Jones proteins (monomers and dimers) and of immunoglobulin κ-chains. The stretch where the amino acid sequence varies from protein to protein is indicated by a broken line. The half-cysteine residues are numbered 1–5 from the *N*- to the *C*-terminus of the chain. The amino acid soquences around the individual residues are discussed elsewhere (Milstein, 1966*a*,*b*).

in the study of the disulphide bridges of type L proteins, because the lack of specificity of pepsin involved two bonds, rather than three as in type K, thus providing a simpler picture (C. Milstein, unpublished work).

The common intrachain bridge seems to occur between the cysteine residues 21 and 81 positions removed from the *C*-terminal cystine residue. Evidence for the location of the former was provided by the peptic peptide 5c (in proteins BJ and Ker), which overlapped with a tryptic peptide containing Ala-CyS-Glu isolated by using radioactive techniques (Milstein, 1966*a*,*b*). This is in agreement with the sequence studies of proteins Roy and Cummings (Hilschmann & Craig, 1965) and of protein Ag (Titani, Whitley, Avogardo & Putnam, 1965). The position of the second cystine residue is inferred from the work on the above-mentioned proteins. The region 1 defining this disulphide bridge contains a fast-moving peptide (1a) that differs from sample to sample by the presence or absence of a tyrosine residue. This seems to be due to the presence of either valine or leucine before the tyrosine (Milstein, 1966*a*). Thus the pepsin split appears to be

...Leu-Tyr-Ala-CyS-Glu... or
↑
P
...Val-Tyr-Ala-CyS-Glu...
↑
P

Since both sequences are observed in individual Bence-Jones proteins (Hilschmann & Craig, 1965; Titani *et al.* 1965; Milstein, 1966*a*,*b*), this constitutes a simple way of distinguishing this particular variation in immunoproteins. It is noteworthy that the two patterns of disulphide-bridge peptides seem to agree with the Inv types of the proteins (Table 8). Further, the pooled population of normal light chains showed both peptides although the amount of the peptide from the *b* pattern was much larger. This suggests that the Inv(1, 2) and Inv(3) types are related to the presence of leucine or valine respectively in the position 24 removed from the *C*-terminus. Another protein, Roy, is Inv(2) and has leucine in that position (Hilschmann & Craig, 1965).

Table 8. *Inv factors and the pattern of the homologous disulphide bridge*

The Inv typing of proteins Ker, Rad, Man and Day was performed by Dr Steinberg. Proteins Fr3 and Fr4 were supplied by Dr Franklin, and protein BJ was typed by Dr S. Lawler. The patterns *a* and *b* of the disulphide bridges correspond to the patterns of proteins Rad and BJ respectively, which are discussed in the text.

Protein	Inv(+	Common intrachain disulphide bridge pattern	Residue at position 189 (Roy numbering) (Milstein, 1966*a*,*b*)
BJ	2	*a*	Leu
Ker	1*	*a*	Leu
Fr4	2	*a*	Leu
Rad	3	*b*	Val
Man	3	*b*	Val
Day	3	*b*	Val
Fr3	3	*b*	—
Normal pooled light chains	2, 3	*a*, *b*	—

* According to Terry, Fahey & Steinberg (1965), Inv(1) is a factor present whenever Inv(2) is present, but may also be found in the absence of Inv(2).

The third disulphide bridge showed at this stage considerable similarities in samples Ker and BJ. Sample Rad, on the other hand, seemed to be different. This technique indicated that a more extensive study around this area of the molecule could provide information about the nature of the variability of the light chains. A radioactive technique suitable for studies on a large number of samples is now being used for that purpose. The sequences of the peptides of the specimens discussed in this paper have been further studied by using it (Milstein, 1966*b*). The position of each half-cystine residue of the variable disulphide bridge is at the moment a matter of speculation. By comparison with the full block sequence of a Bence-Jones protein available (Hilschmann & Craig, 1965) they may be located in positions 23 and 86 (see Milstein, 1966*a*) of proteins Ker and BJ. This is based on the fact that (*a*) both the *N*-terminus and *C*-terminus of proteins Roy, Ker and BJ are the same, (*b*) the peptide containing the methionine residue located in position 4 in protein BJ (Milstein, 1966*b*) and protein Ag (Titani *et al.* 1965) (and presumably in proteins Ker and Roy, since they have the same amino acid composition) seems to be common, and (*c*) the sequences around the cysteic acid residues are almost identical. These four proteins seem to be very similar indeed. The positions of the two half-cystine residues of the non-homologous disulphide bridge in protein Rad, on the other hand, are difficult to state at the moment. One of them contains the sequence Tyr-CyS-Gln-Gln-Tyr (Milstein, 1966*b*), which has also been found in proteins BJ and Ker and may be present in protein Roy in position 86. The other half-cystine residue is in a different sequence when compared with any of the other two proteins studied (Milstein, 1966*b*). Whichever is its position, it seems that the bridges are established in such a way as to fold two independent stretches of the peptide chain: a 'variable' part in which the amino acid sequence is different from protein to protein, and an 'invariable' one in which the sequence is essentially identical in each protein. This also seemed to be the case when an individual light chain of a 7s myeloma protein (F) was studied. More noteworthy is the fact that normal pooled light chains also contained the homologous bridge. This gives support to the idea that the Bence-Jones proteins reflect the physiological variability of normal immunoproteins. The folding of the invariable region may provide the requirements for functions that are common to all light chains irrespective of the antibody-specificity of the individual immunoprotein. One of them seems to be the linkage to the heavy chains, which probably takes place through the *C*-terminal half-cystine residue. However, other common functions are likely.

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