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Immunoglobulin λ-Chains

THE COMPLETE AMINO ACID SEQUENCE OF A BENCE-JONES PROTEIN

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The total amino acid sequence of a λ Bence-Jones protein has been established. The protein contains 211 residues, which include two methionine residues. Splitting with cyanogen bromide gave three fragments, the largest of which included the *C*-terminal half, which is common to other Bence-Jones proteins of the same type. The peptides obtained by tryptic, chymotryptic and peptic digestion were isolated and purified by paper-electrophoretic and chromatographic techniques. Reduction followed by carboxymethylation of the cysteine residues with radioactive iodoacetate was found to be a powerful tool in the isolation of some insoluble peptides. Unusual features of the molecule are the fact that it contains six cysteine residues and not five as observed in both κ and λ Bence-Jones proteins studied previously, and its size, which seems two residues smaller than the smallest Bence-Jones protein studied hitherto. The similarities and differences between this and other Bence-Jones proteins are discussed.

Immunoglobulins are made up of light and heavy peptide chains. There are several types of both light and heavy chains, differing in their antigenic and chemical characteristics, but each immunoglobulin molecule has two or more light chains of a single type and two or more heavy chains of a single type (see review by Cohen & Milstein, 1967). The different classes of immunoglobulins are defined by their different heavy chains, but the nature of the light chain subdivides each class. Thus an immunoglobulin G can be either type K or type L (containing γ heavy chains and κ or λ light chains respectively), and an immunoglobulin M can also be type K or type L depending on the light chain (κ or λ respectively) attached to the μ heavy chains.

In humans two types of light chains, κ and λ, have been described. Chemically, however, light chains isolated from normal humans are a very heterogeneous population of κ and λ molecules. But in some pathological conditions, where monoclonal proteins are produced (myeloma proteins), pure preparations of a single species can be easily obtained, especially from patients who release the light chains in their urine as Bence-Jones proteins.

In this paper we report the detailed study of the sequence of a type L Bence-Jones protein (λ-chain). Progress reports of this study have been published previously (Milstein, 1965, 1966*c*,*d*; Milstein, Clegg & Jarvis, 1967*a*; Milstein, Frangione & Pink, 1967*b*; Cohen & Milstein, 1967). Sequences of other Bence-Jones proteins of the same type have been reported by Wikler, Titani, Shinoda & Putnam (1967).

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MATERIALS AND METHODS

Bence-Jones protein X (also referred to as Pet) was kindly provided by Dr A. Feinstein as a freeze-dried powder prepared from urine by $(NH_4)_2SO_4$ precipitation. The fully reduced and carboxymethylated protein gave a single band on gel electrophoresis (Feinstein, 1966), but was slightly coloured. It could be fractionated into dimers (protein XA) and monomers (protein XB) by Sephadex filtrations (Milstein, 1965) to yield a white powder after freeze-drying. Experiments were done on protein XA or on unfractionated protein, the results being identical.

Cyanogen bromide fragmentation (*Gross & Witkop*, 1962). Protein XA was dissolved in 80% (v/v) trifluoroacetic acid [or 80% (v/v) formic acid] to a concentration of 20mg./ml., and treated with three to four times its weight of CNBr at room temperature for 30hr., after which time solvent and excess of CNBr were removed by freeze-drying. With neither solvent was the reaction more than 90% complete as indicated by the methionine content after total hydrolysis and amino acid analysis. The yields of the various fragments on subsequent purification were not very reproducible, and they were lower when the formic acid solvent was used. However, this was preferred to trifluoroacetic acid because it was suspected to give fewer side reactions. The freeze-dried material was dissolved in 5% (v/v) formic acid and fractionated on columns of Sephadex G-100. Each component was further purified by further chromatography on Sephadex G-100 (or G-75 for the smallest component) after performic acid oxidation.

For the estimation of homoserine present in the CNBr fragments the technique described by Ambler & Brown (1967) was adopted.

Cleavage of disulphide bridges. Several methods were used. Performic acid oxidation was carried out as described by Hirs (1956). Total reduction followed by carboxymethylation with iodo[^{14}C]acetate was performed as described by Milstein (1966*b*).

S-β-Aminoethyl derivatives of totally reduced protein X were made by the procedure described by Clegg, Naughton & Weatherall (1966).

Enzymic digestion of protein X and its fragments. Unless otherwise stated, tryptic digests were done in 1% (w/v) NH_4HCO_3, pH 8·0 (enzyme/substrate ratio 1:50, w/w), for 5hr. at 37°. Chymotryptic digestion in the presence of soya-bean trypsin inhibitor was done in 1% (w/v) NH_4HCO_3 (enzyme/substrate ratio 1:75, w/w) for 6hr. at 37°. Peptic digestion was done in 0·02M-HCl (enzyme/substrate ratio 1:50, w/w) for 5hr. at 37°. In each case, digestion was stopped by freeze-drying. The digests obtained with NH_4HCO_3 as solvent were freeze-dried again from water once or twice to ensure complete removal of the volatile salts.

Digestions of peptides with trypsin, chymotrypsin, pepsin, subtilisin, Pronase and carboxypeptidases A and B were carried out as described by Ambler (1963).

Fractionation of enzymic digests. The initial fractionation of the digests was done by high-voltage paper electrophoresis at pH 6·5. In most cases this was followed by descending chromatography in BAWP* and finally, by electrophoresis at pH 3·5. In some cases an electrophoresis run at pH 2 was also necessary, and some peptides were further purified by additional procedures as described in the text. Details of the electrophoretic techniques and buffers were given by Milstein (1966*a*). For large-scale preparations a preliminary fractionation on Sephadex G-50 columns with 0·1M-NH_3 as eluent was preferred. The peptides eluted from the Sephadex columns were identified by paper electrophoresis and then subjected to the ordinary purification procedures.

N-*Terminal analyses and* N-*terminal sequence determinations of peptides.* These were performed by the 'dansyl' and 'dansyl'–Edman methods (Gray, 1967). In the later stages 'dansyl' derivatives were identified as described by Frangione & Milstein (1968).

Hydrazinolysis. This was carried out under the conditions used by Offord (1967). About 50 nmoles of peptide were dried in a Durham tube and dissolved in 40μl. of anyhdrous hydrazine. The reaction was carried out at 70° for 15–18hr. in an evacuated desiccator previously flushed with N_2. The excess of hydrazine was removed *in vacuo* and the dried reaction mixture subjected to paper electrophoresis at 60v/cm. at pH 6·5 for 1hr. This separated the hydrazides, which are basic at pH 6·5, from any neutral or acidic amino acids released. The neutral band was cut out, sewn on to another paper and subjected to electrophoresis at pH 2 for 15–18min. at 6kv. The amino acids were detected by staining the paper with 0·25% (w/v) ninhydrin in acetone containing 3% (v/v) of collidine and heating it at 100° for 2–3min. No attempts were made to identify any basic amino acid released.

Mobilities of peptides in paper electrophoresis. These were expressed relative to various markers. The following markers were used in all runs: Cyanol FF (blue), red Pentel-pen ink, ε-DNP-lysine, taurine, and an amino acid mixture including all the acidic and basic amino acids plus glycine, valine and glycylalanine. A mobility of +1 at pH 6·5 was defined as the distance between ε-DNP-lysine and aspartic acid, −1 being the same distance in the opposite direction. When aspartic acid had been run off the paper the mobility of Cyanol FF (0·4) was used as reference. At pH 3·5, mobilities were expressed relative to the mobility of glycylalanine (taken as +1 and measured from the ε-DNP-lysine position). At pH 2, mobilities were expressed relative to valine taken as +1 and measured from the position of a taurine marker.

Other methods. Tryptophan was determined quantitatively only in the intact protein by the method of Spies & Chambers (1948), as described by Ambler & Brown (1967).

The techniques used for amino acid analysis and other analytical procedures were the same as those used and described by Milstein (1966*a*).

RESULTS

Cyanogen bromide fragmentation

Treatment of the isolated dimer (protein XA) of Bence-Jones protein X with cyanogen bromide gave three fragments, which were separated by chromatography on Sephadex G-100 (Fig. 1); fraction X-I contained unfragmented chains. After oxidation with performic acid fraction X-III gave rise to a large fragment IIIa and a much smaller fragment IIIb, which were well separated from each other by chromatography on Sephadex G-75. Fragment

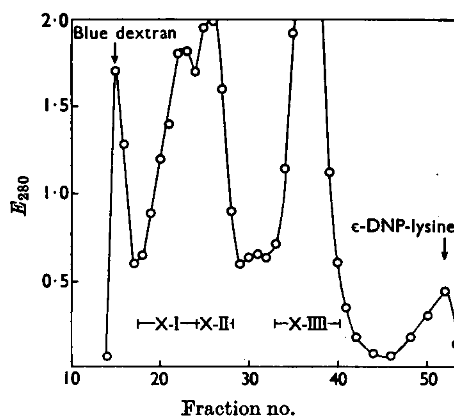

Fig. 1. Separation of cyanogen bromide fragments of Bence-Jones protein X on a Sephadex G-100 column in 5% formic acid.

* Abbreviations: BAWP, butan-ol–acetic acid–water–pyridine (15:3:12:10, by vol.); AE, aminoethyl; CMCys, carboxymethylcysteine; Cya, cysteic acid; Hsr, homoserine; Hsl, homoserine lactone; Glx, glutamic acid or glutamine; Asx, aspartic acid or asparagine; $MetSO_2$, methionine sulphone; $CMCysSO_2$, carboxymethylcysteine sulphone.

Table 1. *Amino acid analyses of protein X and its cyanogen bromide fragments*

N.D., Not determined.

	Amino acid composition (residues/mol.)						
						Whole protein	
Fragment ...	II	IIIa	IIIb	III	II+III (to nearest integer)	Found‡	Computed from sequence (Fig. 9)
Lys	7·7	1·7		2·4	10	9·9	10
His	1·8				2	2·0	2
Arg	1·9	2·6		2·8	5	5·1	5
Cya	2·9	1·5	1·1	2·7	6	5·9	6
Asp	5·4	8·0	2·9	9·7	15–16	16·3	16
$MetSO_2$	—	—	—	—		2·0	2
Thr	12·2	6·0		5·6	18	18·7	20
Ser	16·4	10·7	0·9	11·5	28	29·0	31
Glu	11·7	9·0	2·1	11·2	23	23·0	23
Pro	9·6	6·5		5·7	15–16	15·3	15
Gly	5·6	6·8		7·9	13–14	14·1	14
Ala	10·6	3·7	2·0	5·7	16	16·1	16
Val	10·5	4·3†		4·5	15–17	17·1	17
Ile	1·1	3·4†		3·4	4–5	5·1	5
Leu	7·7	3·5		4·2	12	11·9	12
Tyr	3·5	2·8	1·9	2·4	6	7·9	9
Phe	3·1	1·0		1·3	4	4·2	4
Trp	N.D.	N.D.	N.D.	N.D.		3·7	4
Hsr*	—	0·8	0·7	§	2		
N-Terminus	Ser	Asp	Tyr			Tyr	

* Calculated by using the serine constant.

† Values after 72hr. hydrolysis.

‡ Values extrapolated from four hydrolyses at 25, 49, 72 and 96hr. of performic acid-oxidized and one 25hr. hydrolysis of untreated protein.

§ Not sufficiently resolved from glutamic acid on analysis.

X-II was purified by further fractionation on Sephadex G-100 after performic acid oxidation. Amino acid analysis of the three oxidized fragments indicated that they constituted the whole of the molecule (Table 1). Fragment II contained no homoserine and also contained the tryptic peptide *C*-terminal in the whole molecule, and was therefore the *C*-terminal fragment. *N*-Terminal analysis of fragment IIIa, and the presence of the same tryptic peptide beginning with tyrosine both in the whole molecule and in fragment IIIa, showed that this was the *N*-terminal fragment. The order of the fragments can therefore be written as IIIa–IIIb–II.

Tryptic peptides of fragment II

Table 2 shows the amino acid composition of the tryptic peptides present in fragment II. The sequences were established as described below and are shown in Fig. 2.

*Peptide T*1. The sequence was established by the 'dansyl'–Edman method and confirmed by the sequence of the *C*-terminal peptic peptides (Milstein, 1965).

*Peptide T*2. Tables 3 and 4 show the subtilisin and pepsin peptides isolated to establish the sequence of peptide T2 shown in Fig. 2. The pepsin digest established the order of peptides T2S3d and T2S2b. The *N*-terminal sequence of the whole T2 peptide by the 'dansyl'–Edman method was shown to be Ser-Tyr-Ser-Cya-Glx, which gave the order of peptides T2S3b1 and T2S1 as shown. The peptide Cya-Glx (peptide T2S1A2) was slower than cysteic acid. The mobility of Cya-Glu run as marker was 1·2 (slightly faster than cysteic acid), and therefore the glutamic acid residue of peptide T2S1 was in the amide form.

Peptides T2S2b and T2P2 were acidic and the only possible acidic residue was a glutamic acid (and not the amide) in the sequence -His-Glx-. Finally, the *C*-terminal sequence of the peptide must be -Glu-Lys, since peptides T2S3e and T2S3g2 were neutral.

*Peptide T*3. The sequence was established by the 'dansyl'–Edman method (Fig. 2).

*Peptide T*4. This peptide was not isolated in digests of performic acid-oxidized protein. It was

Table 2. *Amino acid analyses of tryptic peptides of fragment II*

Mobilities (*m*) at pH 6·5 are expressed relative to aspartic acid.

	Amino acid composition (residues/mol.)												
Peptide	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11a	Total of peptides	Fragment II
Lys		0·9		1·0	0·9	1·0	0·9	1·0	0·9	0·9		7·5	7·7
His		0·9	1·0									1·9	1·8
Arg			1·0								1·0	2·0	1·9
Cya	0·9	0·9						0·8*				2·6	2·9
Asp					1·9		1·1	1·1	1·1			5·2	5·4
Thr	1·7	2·1		1·0		2·8		1·9	1·0	1·0	0·9	12·4	12·2
Ser	1·1	3·1	0·9	2·9	1·1	1·0	2·1	1·1	3·2	1·1	1·0	18·6	16·4
Glu	1·0	3·1		2·0	1·1	1·0			3·0	1·1		12·3	11·7
Pro	1·1			0·9		1·1	1·1	1·1	2·8	1·0		9·1	9·6
Gly		1·0				0·9		1·1			3·0	6·0	5·6
Ala	0·9			2·0		1·0	1·0	2·8	3·0			10·7	10·6
Val	0·8	2·1				0·9	1·0	2·8	1·1	0·9	1·9†	11·5	10·5
Ile								1·0				1·0	1·1
Leu				2·0				2·0	1·9	1·7		7·6	7·7
Tyr		0·9		1·9				1·0				3·8	3·5
Phe								1·0	1·0		0·9	2·9	3·1
Trp				+				+					
N-Terminus	Thr	Ser	Ser	Tyr	Glx	Ala	Ala	Ala	Ala	Leu	Ser		Ser
m (pH 6·5)	0·60	0·25	−0·6	0	−0·33	0	0	Insol.	0·15	−0·28	−0·28		

* CMCys
† Value after 90 hr. hydrolysis.

T1 Thr-Val-Ala-Pro-Thr-Glu-Cys-Ser
→ → → → → → → →

T2 Ser-Tyr-Ser-Cys-Gln-Val-Thr-His-Glu-Gly-Ser-Thr-Val-Glu-Lys (Tables 3 and 4)
→ → → → →

T2S2a, T2S3d, T2S2b, T2S3e

T2S3b1, T2S1, T2S3b2, T2S3g2

T2P4, T2P2, T2P1(?)

T2P3

T3 Ser-His-Arg
→ → →

T4 Tyr-Ala-Ala-Ser-Ser-Tyr-Leu-Ser-Leu-Thr-Pro-Glu-Gln-Trp-Lys (Tables 5 and 6)
→

T4C3, T4C5, T4PN2

T4C4, T4PN2Pr1, T4PN2PrN, T4PN2Pr3

T4PN2Pr2

Fig. 2 (*continued on p.* 635).

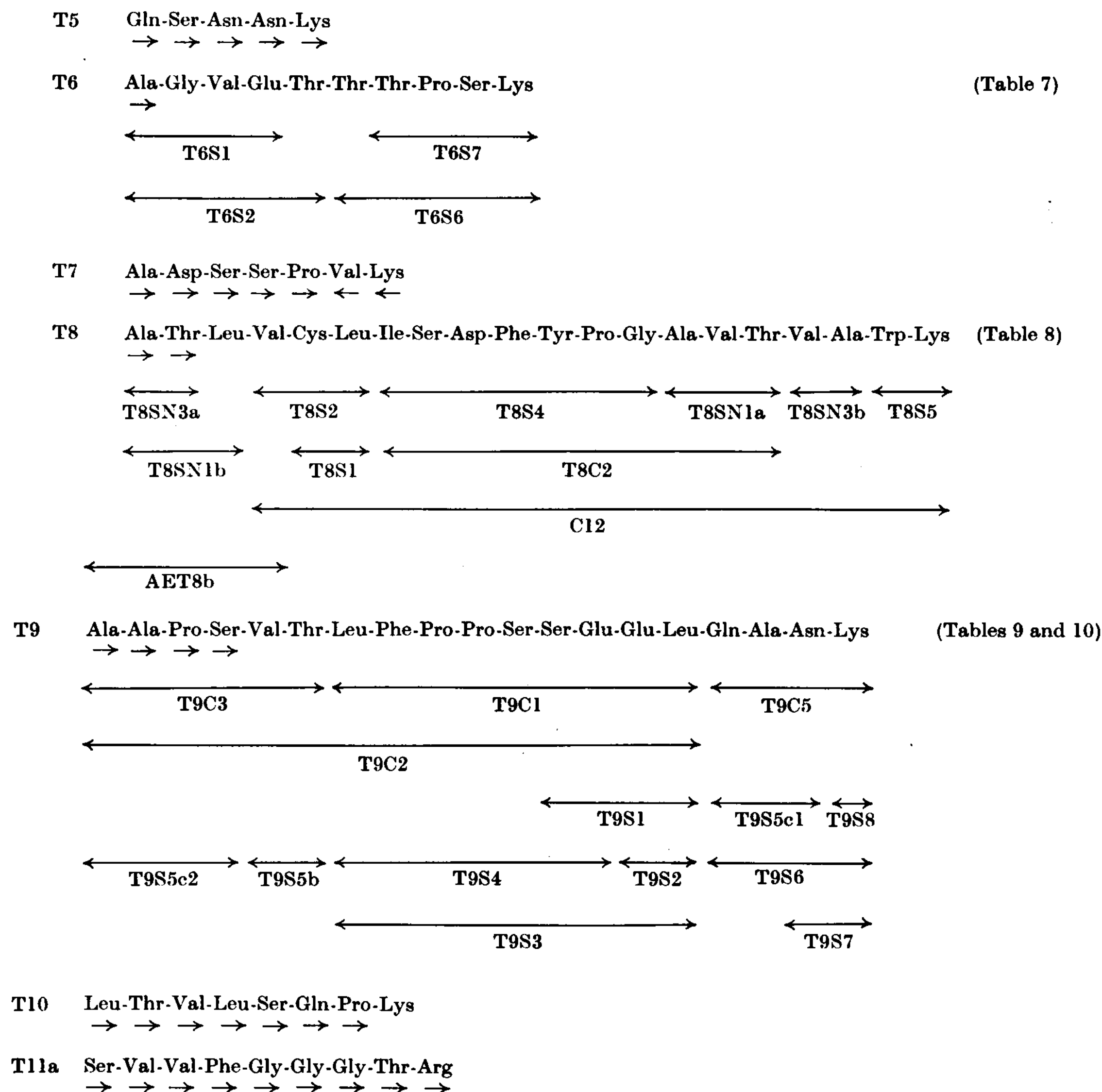

Fig. 2. Sequences of the tryptic peptides of cyanogen bromide fragment II. The arrows show the results of the 'dansyl'–Edman procedure (→) and of carboxypeptidase treatment (←) on the original peptides.

isolated in good yields from tryptic digests both of whole protein and of cyanogen bromide fragments reduced and blocked with either iodoacetate or ethyleneimine. The simplest purification procedure was electrophoresis at pH 6·5 (it is a neutral peptide) followed by chromatography in BAWP ($R_{Val.}$ 1·1) and electrophoresis at pH 3·5 (mobility 0·2). The sequence of this peptide was established from a chymotryptic and a peptic digest (Table 5). The mobility of peptides T4PN2Pr1, T4PN2Pr2 and T4PN2PrN obtained from a Pronase digest of peptide T4PN2 (Table 6) established clearly the sequence -Glu-Gln-Trp-.

Peptide T5. The peptide was basic, indicating no acidic residues. The sequence was established by the 'dansyl'–Edman method (Fig. 2).

Peptide T6. The sequence (Fig. 2) was established from the two major products, peptides T6S2 and T6S6, of subtilisin digestion, as shown in Table 7.

Peptide T7. The sequence was established by the

Table 3. *Peptides isolated from peptide T2 after digestion with subtilisin*

The products were fractionated by paper electrophoresis at pH 6·5 (mobilities, *m*, relative to aspartic acid, are given) and further purified at the pH indicated.

Peptide	Purification pH	*m* (pH 6·5)	Relative yield	Amino acid composition (residules/mol.)	'Dansyl'–Edman results
T2S1		0·75	+++	Cya (1·0), Ser (1·0), Glu (1·1)	
T2S1A2*		0·87		Cya (1·0), Glu (1·0)	Cya-
T2S2a		0·38	+	Cya(+), Ser(++), Glu(+), Tyr(+)	
T2S2b	2·1	0·38	+	His (0·5), Ser (1·0), Glu (1·1), Gly (1·0)	His-Glx-Gly-Ser
T2S3b1	3·5, 2·1	Neutral	++	Ser (1·0), Tyr (1·0)	Ser-
T2S3b2	3·5, 2·1	Neutral	+	Ser (1·0), Gly (1·0)	Gly-
T2S3d		Neutral	+	Val(++), Thr(++)	Val-
T2S3e		Neutral	+	Lys (0·8), Thr (1·0), Glu (1·2), Val (1·2)	Thr-Val-Glx-Lys
T2S3g2		Neutral	+	Lys(+), Glu(+)	

* Residual peptide after one step of Edman degradation of peptide T2S1.

Table 4. *Peptides isolated from peptide T2 after digestion with pepsin*

The products were fractionated by paper electrophoresis at pH 6·5; mobilities (*m*), relative to aspartic acid, are given. N.D., Not determined.

Peptide	*m* (pH 6·5)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T2P1	0	++	N.D.	
T2P2	0·15	+	His (0·9), Thr (1·1), Ser (1·1), Glu (0·8), Gly (0·9), Val (0.8)	Val-
T2P3	0·30	Trace	His(+), Gly(+), Val(+), Ser(++), Thr(++) Glu(+), Tyr(+), Cya(+)	
T2P4	0·42	+	Ser (2·0), Glu (1·0), Cya (1·0), Tyr (0·8)	

Table 5. *Peptides isolated from peptide T4 after digestion with chymotrypsin or pepsin*

Mobilities (*m*) at pH 3·5 and pH 6·5, relative to aspartic acid, are given.

Peptide	*m* (pH 6·5)	*m* (pH 3·5)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T4C3	0	0·4	++	Ser (2·1), Ala (1·9), Tyr (1·7)	Tyr-Ala-Ala-
T4C4	0	0·5	+	Ser (2·1), Ala (2·0), Tyr (0·9)	Ala-Ala-Ser-Ser-Tyr
T4C5	0	1·0	++	Thr (1·0), Ser (1·1), Glu (2·1), Pro(+), Leu (1·6), Trp(+), Lys (0·9)	Leu-
T4PN2	0	0·90	++	Thr (1·0), Ser (1·1), Glu (2·0), Pro (1·1), Leu (1·7), Trp(+), Lys(+)	Leu-Ser-Leu-Thr-Pro-Glx-

'dansyl'–Edman method. Carboxypeptidase (a mixture of A and B enzymes) released lysine and valine, which were identified by paper electrophoresis; the yields of lysine and valine were not quantitatively determined.

Peptide T8. This peptide remained at the origin under all conditions of paper electrophoresis. It could, however, be purified by chromatography. The peptide was isolated from protein X that had been reduced and blocked with iodo[^{14}C]acetate, as the [^{14}C]carboxymethyl derivative. The piece of paper from the original pH 6·5 electrophoretic run containing the peptide was sewn on to a new sheet of paper and subjected to descending chromatography in BAWP. It was the fastest peptide in this solvent system, moving almost with the solvent front with an R_F similar to that of the ϵ-DNP-lysine marker. Amino acid analysis after elution from the paper gave erratic results. Erratic amino acid analyses have been reported previously on peptides eluted after chromatography (Ambler, 1963). To overcome this difficulty, the area containing the peptide was first washed with acetone, dried and then eluted with water followed by

Table 6. *Peptides isolated from peptide T4PN2 after digestion with Pronase*

The products were fractionated at pH 6·5; mobilities (*m*) at this pH and at pH 3·5, relative to aspartic acid, are given.

Peptide	*m* (pH 6·5)	*m* (pH 3·5)	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T4PN2Pr1	0·53		Thr (0·9), Glu (1·1), Pro (0·9)	(Yellow–pink with cadmium–ninhydrin)
T4PN2Pr2	0·45		Thr (0·9), Ser (1·1), Glu (1·0), Pro (1·1), Leu (0·9)	
T4PN2PrN	0	0·4	Glu(+), Trp(+)	Glx-
T4PN2Pr3	As lysine		Free lysine	

Table 7. *Peptides isolated from peptide T6 after digestion with subtilisin*

The products were fractionated by electrophoresis at pH 6·5 (mobilities, *m*, relative to aspartic acid, are given) and further purified by electrophoresis at pH 3·5.

Peptide	*m* (pH 6·5)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T6S1	0·50	+	Glu (1·0), Gly (1·1), Ala (1·0), Val (1·0)	
T6S2	0·43	+++	Thr (1·0), Glu (1·0), Gly (1·1), Ala (1·1), Val (1·0)	Ala-Gly-Val-Glu-Thr
T6S4	0	+	Free threonine	
T6S6	−0·38	++	Lys (1·0), Thr (2·0), Ser (1·3), Pro (0·9)	Thr-Thr-Pro-Ser-Lys
T6S7	−0·45	+	Lys (1·0), Thr (1·0), Ser (1·5), Pro (1·0)	

Table 8. *Peptides isolated from peptide T8 after digestion with subtilisin*

The products were fractionated by electrophoresis at pH 6·5; mobilities (*m*), relative to aspartic acid, are given. The peptides were further purified as indicated by electrophoresis at pH 2 and chromatography on BAWP.

Peptide	Purification	*m* (pH 6·5)	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T8S1	pH 2	0·62	Leu, CMCys	
T8S2	pH 2	0·50	Val (1·0), Leu (1·0), CMCys	Val-Cys-Leu
T8S4		0·31	Asp (0·8), Ser (1·0), Pro (1·0), Gly (1·1), Ile (1·0), Tyr (0·9), Phe (1·1)	Ile-Ser-Asx-Phe-Tyr-Pro-Gly
T8SN1a	pH 2, BAWP, pH 2	0	Thr (0·9), Ala (1·0), Val (1·0)	Ala-Val-Thr
T8SN1b	pH 2, BAWP, pH 2	0	Thr (1·0), Ala (1·0), Leu (1·0)	Ala-Thr-Leu
T8SN3a	pH 2, BAWP, pH 2	0	Ala(+), Thr(+)	Ala-Thr
T8SN3b	pH 2, BAWP, pH 2	0	Val (1·0), Ala (1·0)	Val-Ala
T8S5		−0·44	Lys, Trp	

pyridine–acetate buffer, pH 6·5 (as used in paper electrophoresis) and several fractions were collected and counted. The bulk of the material was eluted in the first fractions of the pH 6·5 elution. The peptide was digested with subtilisin (Table 8). The overlap of the subtilisin peptides T8S2 and T8SN1b was established from a tryptic peptide (peptide AET8b, mobility −0·3 at pH 6·5), isolated from the aminoethylated protein, which had the following composition (residues/mol. of peptide): AECys (0·8), Thr (1·0), Ala (0·9), Val (1·0), Leu (1·1). The 'dansyl'–Edman method established the sequence Ala-Thr-Leu-Val-AECys. Peptide T8 was also
⟶ ⟶ ⟶ ⟶
digested with chymotrypsin and an acidic (mobility 0·2) non-radioactive tyrosine-positive peptide was isolated by paper electrophoresis at pH 6·5. Its composition (peptide T8C2) was (residues/mol.): Asp (1·0), Thr (1·0), Ser (1·0), Pro (1·0), Gly (1·0), Ala (1·0), Val (1·0), Ile (1·0), Tyr (0·9), Phe (0·9); this is the combined composition of peptides T8S4 and T8SN1a. Its *N*-terminal residue was isoleucine, giving the order shown in Fig. 2. Further evidence for the overlaps in Fig. 2 is provided by the composition of the chymotryptic peptide C12 (Table 14) and of the peptic peptide PN4a (see Fig. 9). Peptide PN4a does not contain threonine in its composition

Table 9. *Peptides isolated from peptide T*9 *after digestion with chymotrypsin*

The products were fractionated by paper electrophoresis and further purified at the indicated pH. Mobilities (*m*) at pH 6·5, relative to aspartic acid, are given.

Peptide	Purification pH	*m* (pH 6·5)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T9C1	6·5	0·50	0·6	Ser (2·0), Glu (2·2), Pro (1·8), Leu (1·9), Phe (0·9)	Leu-Phe-Pro-Pro-Ser-Ser-Glx-Glx-Leu
T9C2	6·5	0·35	0·3	Thr (1·0), Ser (2·9), Glu (2·0), Pro (2·4), Ala (2·0), Val (1·1), Leu (2·0), Phe (1·0)	Ala-
T9C3	6·5, 3·5, 2	0	0·2	Thr (0·9), Ser (1·1), Pro (1·4), Ala (1·8), Val (1·0)	Ala-Ala-Pro-Ser-Val-Thr
T9C5	6·5	−0·35	0·6	Lys (1·0), Asp (1·1), Glu (0·9), Ala (1·1)	Glx-Ala-Asx-Lys

Table 10. *Peptides isolated from peptide T*9 *after digestion with subtilisin*

The products were fractionated by electrophoresis at pH 6·5 (mobilities, *m*, relative to aspartic acid, are given) and further purified as indicated.

Peptide	Purification	*m* (pH 6·5)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T9S1		0·84	0·3	Ser (1·0), Glu (2·1), Leu (1·0)	Ser-
T9S2	pH 3·5	0·62	0·06	Leu (1·0), Glu (1·0)	Glu-
T9S3	pH 3·5	0·48	0·5	Ser (1·9), Glu (2·1), Pro (2·1), Leu (1·9), Phe (0·75)	Leu-
T9S4	pH 3·5	0·28	0·06	Ser (2·0), Glu (1·0), Pro (2·2), Leu (0·9), Phe (0·9)	
T9S5b	pH 2	0	Trace	Thr(+), Val(+)	Val-
T9S5c1	BAWP, pH 2	0	Trace	Asp (0·9), Glu (1·0), Ala (1·0)	
T9S5c2	BAWP, pH 2	0	Trace	Ser (1·0), Pro (0·9), Ala (1·9)	
T9S6		−0·40	0·17	Lys (0·9), Asp (1·0), Glu (1·0), Ala (0·9)	
T9S7		−0·48	0·10	Lys (0·9), Asp (1·0)	
T9S8		−0·76	Trace	Lys	

(Table 20) and must include part of peptide T8, because PN4a has valine in the *N*-terminal position and contains tryptophan; therefore peptide T8SN3b (Val-Ala) must be next to Trp-Lys.

*Peptide T*9. The peptide was digested with chymotrypsin (Table 9). A subtilisin digest (Table 10) was used as confirmation, especially of the amide groups. Peptides T9C5, T9S6 and T9S7 were basic. This places two amide groups in peptide T9C5; this was confirmed by peptide T9S5c1 being neutral. The whole of peptide T9 was acidic and therefore it must contain at least two acidic residues. They clearly occur in peptides T9S1, T9S2, T9S3, T9S4, T9C1 and T9C2. Further, peptide T9S1, though the larger peptide, was faster than peptide T9S2, indicating that peptide T9S1 contains two acidic residues and peptide T9S2 one.

*Peptide T*10. The sequence was established by the 'dansyl'–Edman method (Fig. 2).

*Peptide T*11*a*. This peptide was obtained from the cyanogen bromide fragment II but it was absent from tryptic digests of the whole protein, suggesting that it was the *N*-terminus of fragment II. It contains *N*-terminal serine as does the whole of fragment II, and confirmation that it is indeed the *N*-terminal peptide of fragment II is provided below (see Fig. 8 showing the overlap of fragments IIIb and II). The sequence of this peptide was established by the 'dansyl'–Edman method, and is shown in Fig. 2. During the standard purification (electrophoresis at pH 6·5 and 3·5, chromatography in BAWP) this peptide was contaminated with peptide T10. They were separated cleanly from each other by electrophoresis at pH 8·9.

In the tryptic digest of whole protein and of fragment II a basic peptide with the sequence Gly-Gly-Gly-Thr-Arg was observed in variable yields depending on the duration of the digestion. This peptide is the product of a very fast chymotryptic split to produce the *C*-terminal section of peptide T11a.

Tryptic peptides of fragment IIIa

The amino acid compositions of the tryptic peptides derived from this fragment are as shown in Table 1, and their sequences are shown in Fig. 3.

*Peptide T*16. This peptide was the *N*-terminal

Table 11. *Amino acid analyses of tryptic peptides of fragment IIIa*

Mobilities (*m*) relative to aspartic acid are given.

	Amino acid composition (residues/mol.)					
Peptide	T16	T15	T14	T12	T11c	TIbi†
Lys	0·9	1·0				2·2
Arg			1·0	1·0		2·1
Trp			+			+
Hsr+Hsl					0·7	
Cya	1·0		0·9			1·5*
Asp	2·1	1·1	1·0		2·0	5·6
Thr	2·9				3·9	2·8
Ser	4·8			1·8	4·0	6·0
Glu	2·1		2·2	1·0	1·0	6·7
Pro	2·8			1·0		4·8
Gly	2·1	1·0		1·0	3·2	4·2
Ala	1·1				2·1	1·2
Val	1·9		1·0			4·7
Ile	1·0			1·0	1·0	1·6
Leu	1·0	0·9			1·0	2·9
Tyr	0·9		1·0			2·5
Phe					0·8	
m (pH 6·5)	0·25	0	0·25	0·05	0	Insol.
N-Terminus	Tyr	Leu	Asp	Ser	Phe	

* As CMCys.

† Obtained after 2hr. digestion with trypsin (enzyme/substrate ratio 1:100 by wt.) from whole carboxymethylated protein.

Table 12. *Peptides isolated from peptide T16 after digestion with pepsin*

The products were fractionated by paper electrophoresis at pH 3·5 and further purified at the indicated pH. Mobilities (*m*) at pH 6·5, relative to aspartic acid, are given.

Peptide	Purification pH	*m* (pH 6·5)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
T16P1			0·2	Asp (1·3), Thr (0·9), Ser (1·7), Glu (1·1), Pro (2·0), Val (0·9), Leu (1·0)	Asx-
T16P23	2	0·3	0·4	Asp (1·0), Thr (0·8), Ser (1·7), Glu (1·1), Pro (2·1), Val (1·1), Leu (1·1), Tyr (1·0)	Thr-Asx-Leu-Thr-Glx-
T16P4	6·5	0·3	0·2	Asp (1·0), Thr (1·0), Ser (1·1), Glu (1·0), Pro (2·2), Val (0·9), Leu (1·0), Tyr (0·7)	Tyr-
T16P7a	6·5	0·5	0·2	Asp(+), Tyr(+), Leu(+)	Tyr-Asx-
T16P7b	6·5	0·35	0·7	Lys (1·1), Cya (0·9), Asp (1·0), Thr (0·9), Ser (1·3), Gly (1·0), Ile (0·9)	Ile-
T16P7c	6·5	0·3	1·1	Lys (1·0), Cya (1·0), Asp (1·1), Thr (1·0), Ser (2·2), Gly (1·0), Ala (1·0), Ile (0·9)	Ala-Ser-Ile-Thr-Cya-Ser-Gly-
T16P9			0·4	Thr (1·0), Ser (1·8), Glu (1·0), Pro (1·1), Gly (0·9), Val (1·0)	Ser-Val-Ser-Pro-Gly-Glx-Thr (Hydrazinolysis: Thr)

tryptic peptide of this fragment since it was the only one having tyrosine as the *N*-terminal residue. Pepsin digestion (Table 12) gave rise to several derivatives partially defining its sequence (Fig. 3). The evidence to complete the sequence was obtained from a subtilisin digest of the chymotryptic peptide

T16 Tyr-Asp-Leu-Thr-Gln-Pro-Pro-Ser-Val-Ser-Val-Ser-Pro-Gly-Gln-Thr-Ala-Ser-Ile-Thr-Cya-Ser-Gly-Asp-Lys (Tables 12 and 15 and Fig. 4)

T16P7a

T16P1,T16P23

T16P7b

T16P4

T16P9

T16P7c

T15 Leu-Gly-Asp-Lys

T14 Asp-Val-Cya-Trp-Tyr-Gln-Gln-Arg (Tables 13 and 15 and Fig. 4)

T14C1

T14C3

T12 Ser-Ser-Gly-Ile-Pro-Glu-Arg

T11c Phe-Ser-Gly-Ser-Asn-Ser-Gly-Asn-Thr-Ala-Thr-Leu-Thr-Ile-Ser-Gly-Thr-Gln-Ala-Hsr (Tables 14 and 17 and Figs. 6 and 7)

T11cC3

Fig. 7

T11cC3S1

T11cC3S3

T11cC3S2

Fig. 3. Sequences of tryptic peptides of fragment IIIa.

Table 13. *Peptides isolated from peptide T14 after digestion with chymotrypsin*

The products were fractionated by paper electrophoresis at pH 6·5; mobilities (*m*), relative to aspartic acid, are given.

Peptide	*m* (pH 6·5)	Relative yield	Composition	'Dansyl'–Edman results	Carboxypeptidase A results
T14C1	0·85	++	Cya (1·0), Asp (1·1), Val (0·9), Tyr (0·8), Trp(+)	Asx-Val-Cya-	Tyr
T14C3	−0·58	++	Arg (0·9), Glu (2·2)	Glx-Glx-Arg	

C1 (see Table 15 and Fig. 4). Assignment of amide groups is based on mobilities of both peptic [peptides T16P7a and T16P7b (Table 12)] and subtilisin [peptides C1S2b2, C1S4c1, C1S4c2 and C1S2b1 (Table 15 and Fig. 4)] derivatives; peptides containing aspartic acid are acidic and those containing glutamine but not aspartic acid are neutral.

*Peptide T*15. The sequence was established by the 'dansyl'–Edman method (Fig. 3).

*Peptide T*14. The yield of this peptide was very low and in some digests it was not found. It was obtained from the performic acid-oxidized protein. The peptide was digested with chymotrypsin (Table 13). Carboxypeptidase released tyrosine from peptide T14C1 and gave rise to a smaller tryptophan-containing peptide (mobility 0·9 at pH 6·5). The original peptide was acidic at pH 6·5 and peptide T14C3 was basic; therefore peptide T14C3 contained no acidic group and peptide T14C1 contained two acidic residues. The sequence is shown in Fig. 3. Further evidence was obtained by subtilisin digestion of a chymotryptic peptide (Table 15, peptides C1S1 and C1S2a; Fig. 4). Further, from the aminoethylated protein the two expected tryptic peptides were isolated (Asp,Val,AECys) (neutral) and (Trp,Tyr,Glu_2,Arg) (basic, mobility −0·3 at pH 6·5).

*Peptide T*12. The sequence was established by the 'dansyl'–Edman method (Fig. 3).

*Peptide T*11*c*. This peptide was purified from the peptides neutral at pH 6·5 by paper electrophoresis at pH 8·9. It contained homoserine and therefore is the *C*-terminal peptide of fragment III. Its sequence was deduced from the results of the 'dansyl'–Edman procedure (Fig. 3) and from the sequence of chymotryptic and peptic peptides (see Figs. 6 and 7).

*Peptide T*1*bi*. This was a large peptide containing several basic residues. It was obtained in good yields in short-time tryptic digests. It was purified on a Sephadex G-50 column in 0·1 M-ammonia (it was eluted near the breakthrough of the column) followed by electrophoresis at pH 8·9 and at pH 2. The peptide remained at the origin after pH 2 electrophoresis and was eluted with 0·01 M-ammonia. It was digested further with trypsin and shown to release peptides T16, T15 and T14; peptide T14 was released in trace quantities. However, there should have been a fourth peptide released since the combined composition of peptides T16, T15 and T14 does not make up the composition of peptide T1bi (Table 11). This fourth peptide was in fact never isolated and the sequence in that part of the molecule is discussed below (see also Fig. 10).

Chymotryptic digest of protein X

Performic acid-oxidized protein or fully reduced and carboxymethylated protein was digested with chymotrypsin. Not all the chymotryptic peptides were studied in detail, but only those providing information on overlaps of tryptic peptides or on unknown sequences. Amino acid compositions of the various peptides were obtained qualitatively by paper electrophoresis and from this information a number of peptides were selected for further study. The amino acid compositions of these peptides are shown in Table 14.

*Peptide C*1. The peptides produced by digestion of peptide C1 with subtilisin (Table 15) provided evidence for the overlaps of tryptic peptides T16, T15 and T14, thus establishing the *N*-terminal sequence of the protein as shown in Fig. 4.

*Peptide C*2. The *C*-terminal residue, found by hydrazinolysis, was phenylalanine. Digestion with trypsin (Table 16) gave two peptides. Peptide C2T1 included the *C*-terminal phenylalanine residue, and the composition and *N*-terminal residue indicated that it was the tryptic peptide T12 plus a phenylalanine residue at the *C*-terminus. To determine the distribution of amide groups, peptide C2T2 was subjected to Edman degradation and the electrophoretic mobilities of the 'dansylated' derivatives were determined at pH 6·5. Removal of the second residue produced a change of the mobility of the 'dansyl'-peptide from 0·3 to 0, this being the only significant change during the Edman degradation; 'dansylated' basic peptides or basic amino acids are neutral at pH 6·5. The results indicated that the sequence of this peptide was as shown in Fig. 5.

The only tryptic peptide containing *N*-terminal phenylalanine was peptide T11c and therefore peptide C2 established the overlap between peptides T12 and T11c and also the overlap between a

Table 14. *Amino acid analyses of selected chymotryptic peptides of protein X*

Mobilities (m) at pH 6·5, relative to aspartic acid, are given.

	Amino acid composition (residues/mol.)																	
Peptide ..	C1	C2	C3	C4a	C4b	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17
Lys	1·8										1·0			1·0			1·0	1·0
His																	1·1	1·0
Arg		2·1								0·9							1·0	
Trp	+					+	+	?					+			+		
Cya	2·0					†	0·7	†					†					†
Asp	4·1	1·9	2·0	1·9	2·1	1·1	1·0		1·0				0·9	2·0				
$MetSO_2$				0·9	0·8	1·0	0·7		0·8									
Thr	2·9		1·9	1·7	1·8					1·0	1·0		1·1			1·0		3·9
Ser	4·8	1·9	2·9	1·1	1·3	1·0	2·0		2·2		3·7	1·9	1·2	1·2	1·9	1·1	2·0	3·0
Glu	2·2	3·0		2·1	2·1	0·9	1·0	1·0			3·0	2·0	0·3			1·8		4·1
Pro	3·0	1·0									4·4	2·0	1·1			1·0		0·9
Gly	3·1	1·1	2·0	1·1	1·2					3·1			1·0					1·1
Ala	1·1		1·1	2·0	2·1	1·0	1·2	1·0			2·1		1·8		2·1			1·1
Val	3·0						1·3‡		1·3‡		1·0		2·7					3·0
Ile	0·9	0·9		0·9	0·9								0·9					
Leu	2·0		1·0							1·1	1·9	1·8	1·1			1·1		
Tyr	1·3			1·6	1·4								0·9	0·7	1·0		0·9	
Phe		0·9					0·7		0·9		1·0	0·9	1·0					
m (pH 6·5)	0·4	0	0	0·6	0·7	0·64	0·6	0·43	0·3	−0·40	0·15	0·6	Insol.*	−0·35	0	0·3	−0·54	0·53
C-Terminus	Tyr										Leu							
N-Terminus		Glu	Ser				Cya		Asp	Gly	Ser	Leu			Ala	Ser		

* Purified by paper electrophoresis at pH 8·9.

† Radioactive peptide purified after oxidation with performic acid $CMCysSO_2$ was not calculated.

‡ This value increased to approx. 2·0 after 90 hr. hydrolysis.

Table 15. *Peptides isolated from peptide C1 after digestion with subtilisin*

The products were fractionated by electrophoresis at pH 6·5 (mobilities, m, relative to aspartic acid, are given) and purified by electrophoresis at pH 3·5 and pH 2 or by chromatography in BAWP when indicated.

Peptide	Purification pH	m (pH 6·5)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
C1S1	2	0·6	—	Lys (1·0), Cya (0·9), Asp (2·0), Gly (1·0), Val (0·8)	
C1S2a	3·5, 2	0·5	0·2	Lys (0·9), Cya (0·9), Asp (2·0), Gly (1·0), Val (0·8), Tyr (0·7), Trp(+)	
C1S2b1	3·5, 2	0·5	0·7	Lys (1·0), Cya (0·9), Asp (1·1), Ser (1·0), Gly (1·1), Leu (1·0)	Cya-Ser-Gly-Asx-
C1S2b2	3·5, 2	0·5	0·4	Asp (1·1), Leu (0·9), Tyr (0·9)	Tyr-Asx-
C1S3a	3·5	0·3	0·8	Asp (1·0), Thr (0·9), Ser (1·9), Glu (1·0), Pro (2·1), Val (1·0), Leu (1·0), Tyr (1·0)	
C1S3b	3·5	0·3	0·2	Asp (1·0), Thr (0·9), Ser (0·6), Glu (1·1), Pro (1·7), Leu (0·9), Tyr (1·0)	
C1S4c1	3·5, 2	Neutral	0·4	Thr (0·9), Ser (1·9), Glu (1·1), Pro (2·1), Val (1·0)	
C1S4c2	3·5, 2	Neutral	0·3	Ser (1·1), Glu (0·9), Pro (0·8), Gly (1·0), Val (0·8)	Val-
C1S4d1	3·5, 2, BAWP	Neutral	0·4	Thr (0·9), Ser (1·0), Ile (1·0)	Ser-
C1S4d2	3·5, 2	Neutral	0·2	Thr (1·0), Glu (1·1)	Glx-
C1S4e1	3·5, 2	Neutral	0·8	Ser (1·0), Pro (1·0), Gly (1·0), Val (1·0)	Val-Ser-Pro-Gly
C1S4e2	3·5, 2	Neutral	0·4	Thr (1·0), Glu (0·9), Ala (1·1)	Glu-
C1S4f	3·5, 2	Neutral	0·1	Thr (1·0), Ala (1·0)	Thr-

fragment with *C*-terminal -Gln-Asp-Asn-Gln-Arg and peptide T12.

Peptide C3. The *N*-terminal sequence was established by the 'dansyl'–Edman method as shown in Fig. 6. The sequence was completed by isolating the three subtilisin peptides shown in Table 17. This peptide is obviously part of peptide T11c (Fig. 3). A peptide was also detected having

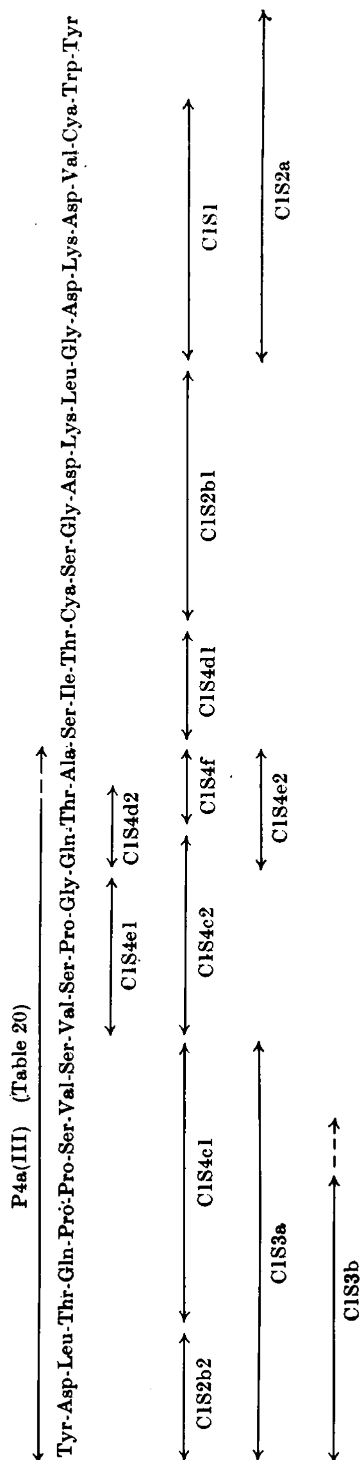

Fig. 4. Sequence of peptide C1. The products of subtilisin digestion are shown in Table 15.

an amino acid composition and partial sequence identical with those of peptide C3, but which was ninhydrin-negative. Its mobility was abnormal at all pH values, being always too acidic. At pH 2·0 it did not have a charge, and moved in electrophoresis with a taurine marker. The reason for this behaviour remains unexplained. It is possible that it is an artifact and that this peptide lacked a free α-amino group in that particular experiment, in which a preliminary fractionation on a Sephadex G-50 column in 0·1 M-ammonia was used. That both aspartic acid residues occur as the amides is in agreement with the mobility of the tryptic peptide T11c and with the peptic peptide PN3aTN3 (see Table 22).

Peptides C4a and C4b. These peptides had the same amino acid composition (Table 14). The low value for tyrosine was probably due to these peptides' being a mixture of two peptides containing either one or two *C*-terminal tyrosine residues as a result of an incomplete chymotryptic split. Confirmation of the presence of two tyrosine residues in this portion of the protein was obtained from the composition of fragment IIIb (Table 1), which includes the *C*-terminal portion of peptides C4a and C4b. Peptide C4b was very acidic at pH 3·5 (faster than glutamic acid) and peptide C4a, present in higher yields, was just faster than a valine marker. Peptide C4b was ninhydrin-negative, but peptide C4a contained threonine as *N*-terminal residue. It is possible that peptide C4b had no free *N*-terminus (see the comment on peptide C3 above). Their sequence, indicated by the results shown in Tables 18 and 19, is shown in Fig. 7. The peptide PN2 (see Table 20), obtained from fragment IIIa, is also shown in Fig. 7 with the results of the 'dansyl'–Edman procedure.

Peptides C5, C6, C7 and C8. The composition of these peptides is given in Table 14. The sequence of peptide C8 was established by the 'dansyl'–Edman procedure and the rest of the sequence was deduced from the sequence of the peptic peptide P4(W) (Table 20) to give the sequence shown in Fig. 8. Glutamine was indicated because the residual ninhydrin-positive peptide after two steps of Edman degradation of peptide P4 was neutral at pH 6·5. In addition the peptide AETN1c (Table 21 and Fig. 9) from aminoethylated fragment III was neutral at pH 6·5.

Peptide C9. The sequence of this peptide was established by the 'dansyl'–Edman method as: Gly-Gly-Gly-Thr-Arg-Leu. The peptide Gly-Gly-
$\longrightarrow \longrightarrow \longrightarrow \longrightarrow \longrightarrow$
Gly-Thr-Arg was released in tryptic digests of whole protein in very good yields with certain batches of trypsin. It appears that the split between phenylalanine and glycine in positions 96 and 97 is the fastest chymotryptic split in the protein.

Table 16. *Peptides isolated from peptide C2 after digestion with trypsin*

The products were fractionated by electrophoresis at pH 2 (mobilities, *m*, relative to valine, are given). Both derivatives were neutral at pH 6·5.

Peptide	*m* (pH 2)	Relative yield	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
C2T1	0·9	1	Arg (1·0), Ser (1·9), Glu (1·1), Pro (1·0), Gly (1·0), Ile (0·9), Phe (1·0)	Ser-
C2T2	1·05	1	Arg (0·9), Asp (2·0), Glu (2·1)	Glx-Asx-Asx-Glx-Arg

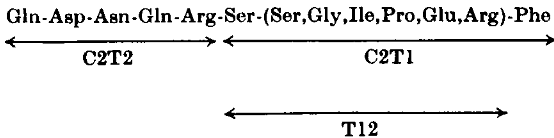

Fig. 5. Sequence of peptide C2 (Table 6).

Table 17. *Peptides isolated from peptide C3 after digestion with subtilisin*

The products were fractionated by electrophoresis at pH 6·5 (mobilities, *m*, relative to aspartic acid, are given) and purified by electrophoresis at pH 2.

Peptide	*m* (pH 6·5)	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
C3S1	0·1	Asp (1·0), Ser (1·0)	Asx-
C3S2	0·05	Thr (1·0), Leu (1·0)	Thr-
C3S3	0	Asp (0·9), Thr (0·9), Gly (1·1), Ala (1·1)	Gly-Asx-Thr-

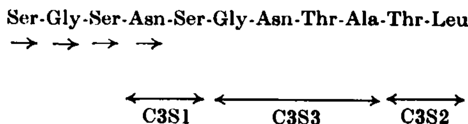

Fig. 6. Sequence of peptide C3 (see also Tables 14 and 17 and Fig. 3).

*Peptide C*10. Tryptic digestion of peptide C10 (Table 14) released a basic peptide with the sequence (Ser,Gln,Pro)-Lys, which established the overlap between tryptic peptides T10 and T9 (Fig 9.).

Pepsin digestion of fragment II

The analysis of the selected peptides is shown in Table 20. Peptide P2(II), which was identical with the chymotryptic peptide C9, established the overlap of peptides T11a and T10, since peptide T11a contained the characteristic sequence Gly-Gly-

Table 18. *Peptides isolated from peptide C4a after digestion with pepsin*

The products were fractionated by paper electrophoresis at pH 6·5 (mobilities, *m*, relative to aspartic acid, are given).

Peptide	*m* (pH 6·5)	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
C4aP1	1·0	Asp (1·0), Glu (1·0), Ala (0·9)	Glx-Ala-
C4aP2	0·8	Asp(+), Glu(+), Ala(+), Tyr(+)	
C4aP6	0·3	Asp (0·8), $MetSO_2$ (0·9), Thr (1·8), Ser (1·0), Glu (1·1), Gly (1·2), Ala (1·2), Ile (1·0)	Thr-Ile-Ser-

Gly-Thr-Arg and peptide T10 was the only tryptic peptide of fragment II with *N*-terminal leucine. Peptide P7a4 obtained from whole protein confirms this overlap (see Fig. 9). Peptide P1(II) as an overlap of peptides T10 and T9 (see also peptide C10, Table 14) is the only solution consistent with its composition. The same applies to peptide P6(II) as the overlap of peptides T9 and T8.

Peptide PN4a(II), after tryptic digestion, gave rise to the intact tryptic peptide T7, a basic peptide (composition by paper electrophoresis Ala,Val,Trp,Lys), and an acidic peptide (not analysed). The *N*-terminal residue of peptide PN4a(II) was valine. There are only two tryptophan-containing tryptic peptides (peptides T4 and T8) in fragment II. Peptide T4 did not contain valine in its sequence, and therefore the tryptophan residue of peptide PN4a(II) must belong to peptide T8. Peptide PN4a(II) seems therefore to be the overlapping peptide including the *C*-terminal sequence of peptide T8 (not including the threonine residue) with peptide T7 and with the *N*-terminal sequence of peptide T6 as shown in Fig. 9.

Peptide P7a1(II) includes the whole of peptide T5 and has *N*-terminal threonine. Because of its tyrosine content it must include part of peptide T4, leaving three threonine residues that must come from peptide T6 and therefore provides evidence for the overlap T6–T5–T4. Confirmation of the

Table 19. *Peptides isolated from peptide C4b after digestion with subtilisin*

The products were fractionated by electrophoresis at pH 6·5 (mobilities, *m*, relative to aspartic acid, are given).

Peptide	Purification pH	*m* (pH 6·5)	Relative yield	Composition	'Dansyl'–Edman
C4bS1		0·75	0·2	Asp (1·0), $MetSO_2$ (0·9), Glu (2·0), Ala (1·1)	Glx-Ala-$MetSO_2$-Asp-Glx
C4bS2		0·65	0·1	Asp (1·9), $MetSO_2$ (0·8), Glu (2·0), Ala (2·2), Tyr (0·9)	Glx-
C4bS4		0·4	0·06	Asp (0·9), Ala (1·1), Tyr (1·5)	Ala-
C4bSN2	2	0	0·4	Thr (1·0), Gly (1·0)	Gly-Thr

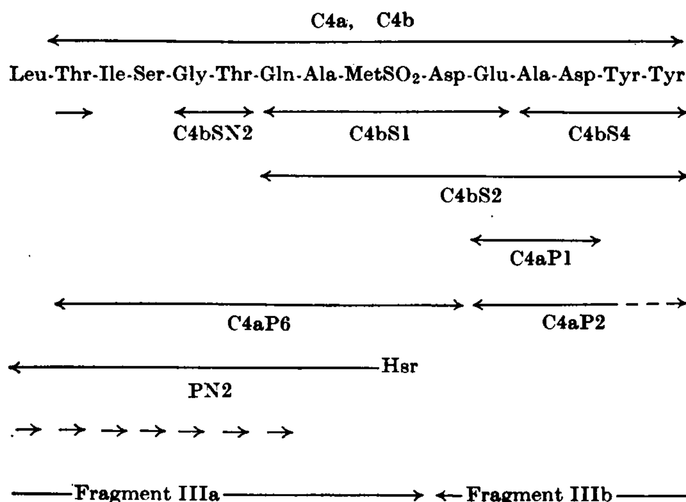

Fig. 7. Sequence of chymotryptic peptides C4a and C4b (Tables 18 and 19) and of peptic peptide PN2 (Table 20) showing the overlaps between fragments IIIa and IIIb.

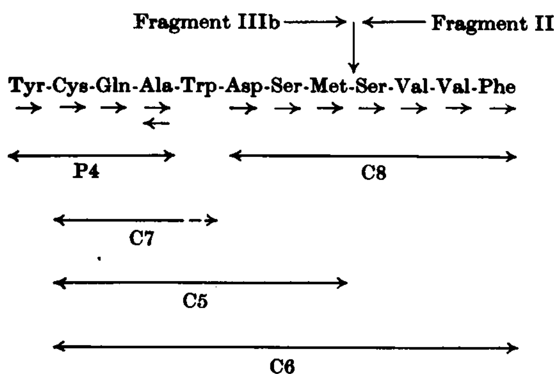

Fig. 8. Sequences of chymotryptic peptides C5, C6, C7, C8 and peptic peptide P4.

overlap of peptides T5 and T4 is provided by the composition of peptide C13 (Table 14). Peptide PB1(II), containing histidine, tryptophan and carboxymethylcysteine, is probably the overlap of the *C*-terminus of peptide T4 and peptides T3 and T2. The possibility that Trp-Lys is from peptide T8 is not consistent with the results obtained on peptide PN4a(II) discussed above. The overlaps of peptides T3, T2 and T1 have been published previously (Milstein, 1966*d*) and are confirmed by the compositions of peptides PN4b(II), C16 and C17 (Table 14).

Miscellaneous peptides

At this stage all the peptides described fell into a linear sequence, but there was an obvious gap between peptides T14 and T12. The end of the gap was obviously peptide C2T2 (Fig. 5), which must have been part of a sequence between two basic residues, but the linking tryptic peptide had not been found. In fact the hypothetical peptide was discussed above as the probable end of peptide TIbi (Table 11).

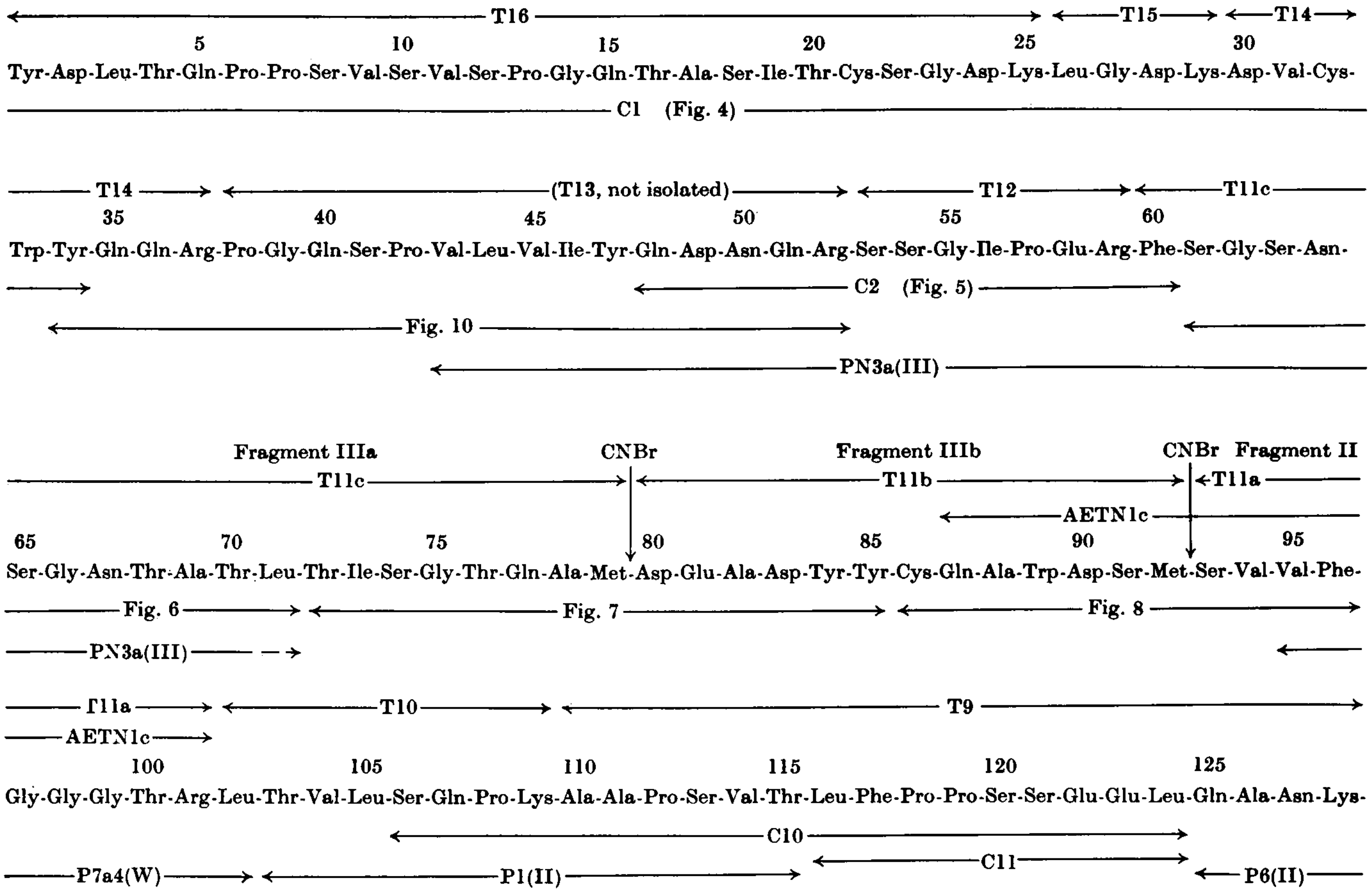

Fig. 9 (*continued on p.* 647).

T8 — T7 — T6

AET8b

130 135 140 145 150 155 160

Ala-Thr-Leu-Val-Cys-Leu-Ile-Ser-Asp-Phe-Tyr-Pro-Gly-Ala-Val-Thr-Val-Ala-Trp-Lys-Ala-Asp-Ser-Ser-Pro-Val-Lys-Ala-Gly-Val-Glu-Thr-

P6(II) — P4c(II) — PN4a(II)

T6 — T5 — T4 — T3 — T2

165 170 175 180 185 190

Thr-Thr-Pro-Ser-Lys-Gln-Ser-Asn-Asn-Lys-Tyr-Ala-Ala-Ser-Ser-Tyr-Leu-Ser-Leu-Thr-Pro-Glu-Gln-Trp-Lys-Ser-His-Arg-Ser-Tyr-Ser-Cys-

C13 — C14 — C15 — C16 — C17

P7a1(II) — PB1(II)

T2 — T1

195 200 205 210

Gln-Val-Thr-His-Glu-Gly-Ser-Thr-Val-Glu-Lys-Thr-Val-Ala-Pro-Thr-Glu-Cys-Ser

C17

PN4b(II)

Fig. 9. Amino acid sequence of protein X.

A series of peptides isolated from fragment IIIa was selected by staining for arginine and tyrosine, and those providing information on the unknown stretch of the chain are listed in Table 21 and in Fig. 10.

Peptide IIICN6 (*Table* 21 *and Fig.* 10). This peptide, obtained by chymotryptic digestion of fragment IIIa, stained very poorly with ninhydrin, but very well with the tyrosine stain. The 'dansyl'–Edman procedure established the sequence Val-Ile-Tyr.

Peptide IIIC3 (*Table* 21 *and Fig.* 10). The 'dansyl'–Edman method indicated the following N-terminal sequence after two steps of Edman degradation: (–)(–)Arg-Pro-Gly-Glx-Ser-Pro-Val-. Since the peptide was basic all the glutamic acid/glutamine residues should be glutamine residues.

Peptide IIIC4 (*Table* 21 *and Fig.* 10). This peptide appeared in higher yields when peptide IIIC3 was in lower yields after longer digestion with chymotrypsin. From its composition it is obvious that peptides IIIC4 and IIICN6 are the final products of the complete digestion of peptide IIIC3. Carboxypeptidase A released leucine quantitatively and no other residue in better yields than 0·2 mole/mole of peptide. Its N-terminus was glutamic acid/glutamine.

Peptide PN3a(III) (*Table* 20 *and Fig.* 9). The N-terminal sequence by the 'dansyl'–Edman method was Val-Ile. Tryptic digestion gave the results shown in Table 22. This peptide therefore contains peptide PN3aTN4, followed by tryptic peptide T12 (peptide PN3aTN5) followed by peptide PN3aTN3 (neutral), which corresponds to the N-terminal section of the tryptic peptide T11c of fragment IIIa with a split at either side of leucine (see Fig. 3). The N-terminal section of peptide PN3aTN4 (Table 22) includes peptides IIICN6 (Table 21) and C2T2 (Table 16; Fig. 10).

Peptide P67b(III) (*Table* 20). This was a basic peptide, and the 'dansyl'–Edman method gave a probable N-terminal sequence Tyr-Glx-Glx-Arg-Pro-Gly-Glx-Ser. However, the 'dansyl'–Edman method was not entirely satisfactory for the fourth residue (arginine); trace amounts of 'dansyl'–glutamic acid were also present throughout. However, this sequence is in perfect agreement with the sequence of peptide IIIC3 (see above and Fig. 10). The peptide was basic, indicating that the three glutamic acid/glutamine residues were all in the amide form.

Peptides AETN2, AETN1c and AET14a (*Table* 21). These peptides were present in the tryptic digest of aminoethylated fragment III. The yields were very low. All three gave a tryptophan-positive

Table 20. *Amino acid analysis of selected peptic peptides of protein X*

Peptides were isolated from whole protein X (W), from fragment II (II) and from fragment III (III). Mobilities (m) at pH 6·5, relative to aspartic acid, are given.

	Amino acid composition (residues/mol.)														
Peptide ...	Pc(W)	P4(W)	P7a4(W)	P1(II)	P2(II)	P4c(II)	PN4a(II)	PN4b(II)	P6(II)	P7a1(II)	PB1(II)	PN2(III)	PN3a(III)	P4a(III)	P67b(III)
Lys				1·0			2·0	1·0	1·0	1·9	1·2				
His											0·9				
Arg			1·0		1·0						1·0		1·7		+
Trp							+				+				
CMCys	0·8	0·6				0·5					0·5				
Asp							1·0		1·0	1·9			4·0	1·1	
Thr	0·8		1·0	1·7	0·9			0·8	0·9	2·6		1·6	2·0	1·8	
Ser	1·0			1·9			1·9			4·1	3·1	1·1	4·9	2·9	1·0
Glu	1·0	1·0		1·3			1·0	1·1	1·0	1·2	1·1	1·5†	3·2	2·1	2·8
Pro	0·9			2·0			0·8			0·8			1·0	3·1	1·5
Gly			3·3		2·9		1·2					1·1	3·3	1·2	1·3
Ala		1·0		2·0			3·1	1·0	2·0	2·0		1·1	1·0	0·6	
Val			1·1	1·9		0·8	2·8	1·8					0·6	1·7	1·0
Ile												0·8	1·6		
Leu			0·9	1·0	1·1	1·1			0·9			0·9	0·4	0·9	0·9
Tyr		0·8								1·8	0·8		0·8	0·5	0·5
Phe			0·9										1·0		
m (pH 6·5)	0·75	0·4	−0·3	0·50	−0·4	0·45			−0·25	−0·3	−0·25	−0·3*	0	0·2	−0·25
N-Terminus	Pro	Tyr	Val	Thr		Val	Val	Val		Thr		Leu	Val		

* A basic peptide was also isolated with the same composition as peptide PN2 and mobility −0·3; this probably represents the homoserine lactone form of peptide PN2.

† Includes homoserine.

Table 21. *Amino acid analyses of miscellaneous peptides isolated from fragment IIIa by chymotryptic digestion and by tryptic digestion of aminoethylated fragment IIIa*

Mobilities (m) at pH 6·5, relative to aspartic acid, are given.

	Amino acid composition (residues/mol.)					
Peptide ...	IIICN6	IIIC3	IIIC4	AET14a	AETN2*	AETN1c
Arg		1·0	1·2	1·0	1·7	1·0
Trp				+	+	+
Asp					2·0	1·0
Thr						1·0
Ser		1·1	1·2		1·5	1·9
Glu		2·7	2·8	2·0	4·6	1·0
Pro		1·7	1·9		2·3	
Gly		1·1	0·9		1·4	2·9
Ala						1·0
Val	0·6	1·7	0·9		1·9	1·3
Met						0·8
Ile	0·6	0·8			0·7	
Leu		1·0	0·9		1·1	
Tyr	1·0	0·8		0·9	1·7	
Phe						0·9
m (pH 6·5)	0	−0·15	−0·2	−0·2	Insol.	0

* This peptide was recovered from the origin after electrophoresis at pH 6·5, and subjected to chromatography in BAWP (R_F slightly lower than that of Cyanol FF) and to electrophoresis at pH 8·9 (eluted from the origin). It was hydrolysed for 40 hr.

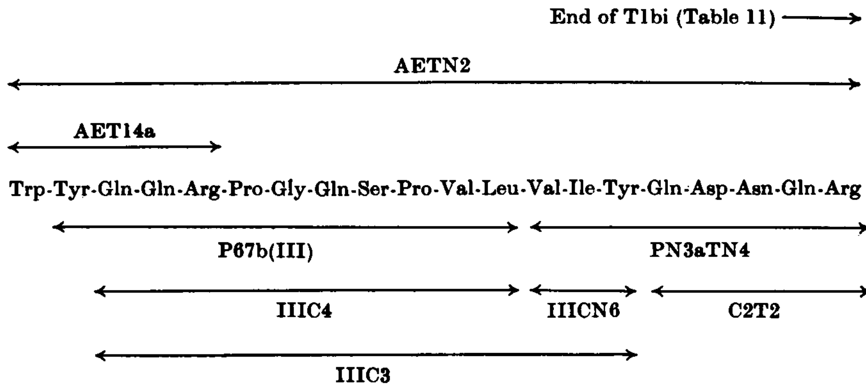

Fig. 10. Interrelation of some miscellaneous peptides.

stain. The composition of peptide AET14a was identical with the composition of the C-terminal section of peptide T14 (Fig. 3).

Peptide AETN2 contained two arginine residues in addition to tryptophan and therefore it must include the sequence of peptide AET14a. The rest of the composition of peptide AETN2 is in full agreement with the sequence shown in Fig. 10 and, therefore peptide AETN2 (as well as peptide TIbi) includes, between the two arginine residues, the hypothetical peptide T13 that could not be located in the tryptic digest.

Peptide AETN1c (Table 21) isolated from the same experiment provides additional confirmation for the sequence between residues 87 and 101 (see Fig. 9). It also shows that under the conditions used a considerable amount of Met-92 was not cleaved during the cyanogen bromide treatment.

DISCUSSION

All the results presented indicate that Bence-Jones protein X is a single chain of 211 residues (Fig. 9). The amino acid composition of the whole

Table 22. *Peptides isolated from peptide PN3a(III) after digestion with trypsin*

The products were fractionated by electrophoresis at pH 6·5 followed by electrophoresis at pH 3·5 of the neutral band. Mobilities, *m*, at pH 6·5, relative to aspartic acid, are given.

Peptide	*m* (pH 6·5)	Amino acid composition (residues/mol.)	'Dansyl'–Edman results
PN3aTN3	0	Asp (2·0), Thr (1·8), Ser (3·1), Gly (2·1), Ala (1·1), Leu (0·4), Phe (0·8)	Phe-
PN3aTN4	0	Arg (0·8), Asp (2·0), Glu (2·0), Val (0·6), Ile (0·6), Tyr (0·8)	Val-
PN3aTN5	0	Arg(+), Ser (1·8), Glu (0·9), Pro (1), Gly (1·2), Ile (0·8)	Ser-

protein agrees quite well with the final composition deduced from the amino acid sequence (Table 1). We have not determined the tryptophan content in the isolated peptides, but from the total analysis of tryptophan it appears that no extra tryptophan residues (for instance in a sequence -Trp-Trp-) are present. The distinction between aspartic acid and glutamic acid residues and their amides was derived from mobility data, almost always from peptides containing either no acidic residue or a single acidic and no amide residues (Ambler, 1963). Once the sequence was established the mobilities of bigger peptides were checked against the mobilities of known peptides of similar mass and charge (Offord, 1966). In some cases, however [as in the sequence of peptide C2T2 (residues 48–51)], it was found necessary to determine the mobility of the residual peptide (Ambler & Brown, 1967) or of the 'dansylated' residual peptide (Gray, 1967) after each Edman degradation step.

No evidence of microheterogeneity of the type noted, for instance, in horse haemoglobin (Kilmartin & Clegg, 1967) was observed. Microheterogeneity in certain stretches of the molecule could have been overlooked owing to technical difficulties. Nevertheless, it can be safely concluded that the protein contains essentially a single sequence.

The protein gave rise to a completely soluble digest whichever method of digestion was used; even the cyanogen bromide fragments were fully soluble at neutral pH values. However, three 'core' regions, giving tryptic peptides that could not be fractionated on paper electrophoresis at pH 6·5 or below, were present even in the aminoethylated protein. These 'core' regions corresponded to residues 35–50, 60–86 and 134–147. These problems were mainly overcome by using paper electrophoresis at pH 8·9 or chromatography in BAWP and also by isolating the appropriate chymotryptic peptides.

By far the most difficult part of the sequence to determine was that between residues 37 and 52. Several attempts to find the peptides from this region were made, and it turned out that the time spent in determining this part and other minor details of the sequence was of the same order as the time needed to establish the rest of the sequence.

The split at Arg-37 by trypsin was very surprising and, to our knowledge, no split by trypsin at an arginine–proline bond has previously been reported. The split was never good since peptide T14 was not obtained in good yields. The tryptic peptide T13 (residues 38–52) having *N*-terminal proline was in fact never found. However, it should be noted that the split at Lys-29, also necessary to release peptide T14, was a very slow split and that the segment 38–52 contained the sites of two fast chymotryptic splits. It would therefore be expected that the yields of peptide T13 would be low. However, since a tryptic split at an arginine–proline bond is so unusual, other possibilities should be discussed. Contamination of the trypsin with some other proteolytic enzyme seems an unsatisfactory explanation because the split was found with two different batches of twice-recrystallized Worthington trypsin. The possibility of the presence of two alternative residues at position 38 is more difficult to exclude completely. The analysis of the chymotryptic peptides IIIC3 and IIIC4 (Table 21) gave a reasonable value for proline, but proline is particularly difficult to determine quantitatively and no other residue would appear to be as good a candidate for the alternative residue. It is not impossible that an alternative peptide (with the proline replaced by another residue) was lost during the purification procedure, but if the alternative peptide was present in about 30% of the amounts of peptide IIIC3, we would have expected to detect it. In summary, it seems reasonable to assume that trypsin did split the arginine–proline bond, though at a lower rate than the split at position 29 in the Asp-Lys-Asp sequence, but the evidence does not seem to be absolutely conclusive.

It has been shown by us (Milstein *et al.* 1967*a*) and by Wikler *et al.* (1967) that λ-chains, like κ-chains (Hilschmann & Craig, 1965; Titani, Whitley, Avogardo & Putnam, 1965; Milstein, 1966*b*), are made up of an 'invariable' *C*-terminal half (but see Milstein, 1967*a*; Ponstingl, Hess, Langer, Steinmetz-Kayne & Hilschmann, 1967) and an *N*-terminal half that differs from one Bence-Jones protein to another. Differences in selected stretches in the *N*-terminal half of Bence-Jones protein X and other λ-chains have been discussed

	90 95 100
κ-Chains	-Tyr-Tyr-Cys-Gln-Gln-var-var-var-var-Pro-var-Thr-Phe-Gly-var-Gly-Thr-Lys-
Protein X (λ-chain) (84–101)	-Tyr-Tyr-Cys-Gln-Ala-Trp-Asp-Ser-Met-Ser-Val-Val-Phe-Gly-Gly-Gly-Thr-Arg-
	-Lys-His-
Protein Sh (λ-chain) (84–103)	-Tyr-Tyr-Cys-Asn-Ser-Arg-Asp-Ser-Ser-Gly-∧ Val-Leu-Phe-Gly-Gly-Gly-Thr-Lys-

Fig. 11. Positions in protein X and κ-chains giving maximum similarity, with the necessary addition found in another λ-chain. The common residues of κ-chains are taken from Cohen & Milstein (1967), and the sequence of protein Sh is from Wikler *et al.* (1967). Positions in κ-chains containing several variants are shown by 'var'.

Table 23. *Positions that contain two pairs of common residues in four λ-chains (numbering of protein X)*

The data are from this paper and from Putnam *et al.* (1967).

Position no. ... ... Protein	11	20	26	Addition after 27	30	56	77	88
X	Val	Thr	Leu	No	Asp	Ile	Gln	Ala
Sh	Val	Thr	Leu	No	Asp	Ile	Gln	Ser
Bo	Gly	Ser	Ser	Yes	Tyr	Val	Arg	Ser
Ha	Gly	Ser	Ser	Yes	Tyr	Val	Arg	Ala

by Milstein *et al.* (1967*b*), including a difference in size. Additions or deletions (or both) in Bence-Jones proteins of the same type have been described in mouse and human κ-chains. They seem to occur always in the same stretch, around residue 30 (Gray, Dreyer & Hood, 1967; Hilschmann, 1967; Dreyer, Gray & Hood, 1967). In λ-chains a size difference has also been described. Two proteins (proteins Bo and Ha) had three extra residues after residue 27 in comparison with a third protein, Sh (Putnam, Shinoda, Titani & Wikler, 1967), and with protein X, which matches in this region with protein Sh. Another stretch where the number of residues does not match occurs after position 90 in protein X compared with other proteins (Milstein *et al.* 1967*b*). This would involve a probable addition or deletion of two residues. This difference in size is somewhat more surprising because protein X is so far the only λ-chain that contains the same number of residues as the κ-chains between the cysteine residue (residue 86 of protein X) and the beginning of the *C*-terminal half (see Fig. 11). Thus the *N*-terminal half of protein X gives maximum homology with other κ-chains if allowance is made for only one deletion (residue 10 of κ-chains) and one addition (residue 105 of protein X). However, until detailed evidence to support the preliminary report of the λ-chain sequences of proteins Sh, Bo and Ha is available, the comparison with the other λ-chains remains tentative.

Protein X contains six half-cystine residues. This is the first case in which such a large number has occurred in any light chain or Bence-Jones protein. Yet the ring structure that is characteristic of light chains (Milstein, 1966*a*,*c*) is preserved. CyS-210 is half of an interchain bridge (Milstein, 1965) and the two half-cystine residues in positions 133 and 192 are linked to produce the characteristic invariable loop (Milstein, 1966*c*). The second intrachain disulphide bridge involves CyS-86 (since fragment IIIa and IIIb are separated only after oxidation), but it is not clear if it is linked to CyS-21 as in other proteins. The remaining half-cystine residue (possibly CyS-32) occurs partly as a free thiol (Feinstein, 1966) and possibly in part blocked with a one half-cystine residue in a similar manner to the way the interchain CyS-210 is blocked in the monomer. This is suggested by the fact that more than 1 mol.mol. of free cysteic acid was recovered from the oxidized monomer (Milstein, 1965).

The presence of at least three fundamental sequences in κ-chains has been proposed, based on the occurrence of certain combinations of residues (linked group of residues) along the variable positions of the chain (Milstein, 1967*b*). The

available data for λ-chains do not offer as yet such clear-cut classification. There is some suggestion that well-defined fundamental sequences may occur in λ-chains also, but this is very tentative. Thus if one considers the four proteins Sh, Bo, Ha and X there are several positions where the residue shared by proteins X and Sh is different from that shared by proteins Bo and Ha. In Table 23 are tabulated positions that contain two varieties of residues, two proteins having residue A and the other two having residue B. There are seven such positions, and in six of them the same pairs (X–Sh and Bo–Ha) are involved, but in one (position 88) the pairs are different. The addition after residue 27 is also common to proteins Bo and Ha, though the residues added are different.

Cohn (1968) has in fact suggested three fundamental sequences for λ-chains. In our opinion the data are still insufficient to propose a meaningful and statistically significant group of linked residues in λ-chains, for any fundamental sequence. Until this is possible it cannot be established whether λ-chains are more or less variable than κ-chains.

Note added in proof. Since this paper was submitted the sequences of two λ Bence-Jones proteins have been published (Ponstingl, Hess & Hilschmann, 1968; Langer, Steinmetz-Kayne & Hilschmann, 1968). The size difference discussed in this paper is also observed in the two proteins studied by the above authors.

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APPENDIX

A Simple Procedure for the Fractionation of the Tryptic Peptides of the *C*-Terminal Half of Immunoglobulin λ-Chains

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(*Received* 27 *May* 1968)

Immunoglobulin light chains consist essentially of an ‘invariable’ and a ‘variable’ section, each being one-half of the chain. This conclusion has been substantiated in studies of both κ-chains (Hilschmann & Craig, 1965; Titani, Whitley, Avogardo & Putnam, 1965; Milstein, 1966*a*) and λ-chains (Milstein, Clegg & Jarvis, 1967; Wikler, Titani, Shinoda & Putnam, 1967). However, variants of the ‘invariable’ part of the molecule occur in both types of chain. Thus in κ-chains a substitution in position 191 (Hilschmann & Craig, 1965) was found to be correlated with the Inv antigenic marker (Milstein, 1966*a,b*; Baglioni, Alescio Zonta, Cioli & Carbonara, 1966). In λ-chains a substitution frequently occurs at the position equivalent to position 190 of κ-chains