Amino Acid Sequence of Bovine Carboxypeptidase A. Tryptic and Chymotryptic Peptides of the Cyanogen Bromide Fragment F_I*

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ABSTRACT: In continuation of the structural analysis of bovine carboxypeptidase A, the largest fragment of the protein, F_I, obtained by cleavage with cyanogen bromide, was analyzed.

This fragment, containing 198 amino acid residues, was subjected to hydrolysis by trypsin and chymotrypsin. The acid-soluble peptides from each enzymatic digest were fractionated on ion-exchange columns and characterized by a combination of Edman degradations, digestion with leucine aminopeptidase and carboxypeptidases A and B, and high-voltage electrophoresis at pH 6.5. The tryptic peptides accounted for 90 of 198 residues (45%) and chymotryptic peptides for 143 (72%). A 38-residue segment, surrounding the two half-cystinyl residues and containing phenylalanine 151, was structured from these data. The identity of this latter residue, which has been questioned on the basis of X-ray diffraction data, has been confirmed by sequence analysis of chromatographically purified carboxypeptidase A.

Following the isolation of the four fragments of bovine carboxypeptidase A produced by cleavage with cyanogen bromide (Bargetzi *et al.*, 1964; Nomoto *et al.*, 1969), experiments were initiated to determine the amino acid sequence of each fragment in order to solve the complete primary structure of this enzyme. So far, data have been reported for the amino- (F_N) and carboxyl- (F_C) terminal fragments (Sampath Kumar *et al.*, 1964; Bargetzi *et al.*, 1964) and for one of the internal fragments, F_{III} (Bradshaw *et al.*, 1969a; Bradshaw, 1969). The present series of reports (Bradshaw *et al.*, 1971a,b) documents the sequence analysis of the largest fragment, F_I. In all, five different enzymatic digests were employed to determine the final structure of this fragment, which contains 198 amino acid residues. The isolation and characterization of the soluble peptides resulting from digestion with trypsin and chymotrypsin, respectively, are described in this communication.

Experimental Procedure

The materials and methods used in these experiments are the same as those described previously (Bradshaw *et al.*, 1969a). The F_I fragment, in which the two half-cystinyl residues were converted into the *S*-carboxymethyl derivative, was prepared as described by Nomoto *et al.* (1969). Carboxypeptidase A_γ^{Val} was prepared from carboxypeptidase A (Anson), obtained from Worthington Biochemicals, by the method of Pétra and Neurath (1969).

Results

Tryptic and Chymotryptic Digests. The digestion of fragment F_I was carried out by the addition of an aliquot of enzyme to a 2% suspension of the fragment at 37° so that final weight concentration of trypsin or chymotrypsin was 2–3% that of substrate. Constant pH was maintained with a pH-Stat (pH 8.8 for trypsin and 8.5 for chymotrypsin) until no further significant increase in alkali uptake was noted. The soluble peptides were recovered after centrifugation of the reaction mixture which had been adjusted to pH 2.0 with 6 N HCl.

Isolation of the Soluble Tryptic Peptides. Fragment F_I of carboxypeptidase A (Anson) was subjected to tryptic degradation as previously described (Bradshaw *et al.*, 1969a). The soluble peptides obtained after acidification of the reaction mixture were chromatographed on a 2.0 × 25 cm column of Dowex 50-X8 (Spinco amino acid analyzer resin, AA-15). The column was developed with a double linear gradient of pyridine–acetic acid buffers (Bradshaw *et al.*, 1969b) over the pH range indicated. The elution profile is shown in Figure 1. The fractions were pooled according to the solid bars and peptides were recovered after rotary evaporation. Each peptide was subsequently subjected to further purification or, in the case of those fractions found to contain only a single peptide, committed directly to sequence analysis. The amino acid composition of each of the pure tryptic peptides is summarized in Table I. In addition, the number of residues and the per cent yield are also given in the table. Since the sum of the sequence of the soluble tryptic peptides did not account for the entire structure of the F_I fragment, the peptides have not been designated in the order that they were ultimately found to occur in the sequence. Rather, the position that each occupies (given in inclusive residue numbers based on the α form of the enzyme which contains 307 amino acids) is given in the bottom line of Table I to facilitate its location in the complete structure (Bradshaw *et al.*, 1971b). The treatment of each pool is described in detail below.

Characterization of the Soluble Tryptic Peptides. FRACTION I. Although this pool contained a large breakthrough fraction, chromatography on Dowex 1-X2 failed to reveal the presence

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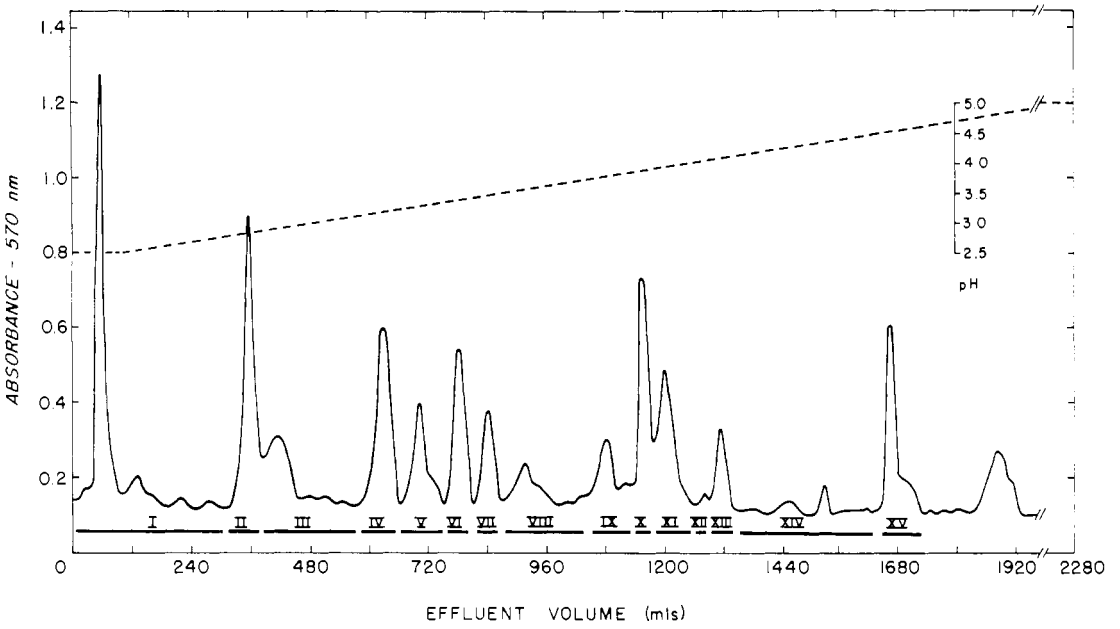

FIGURE 1: Elution profile of the soluble tryptic peptides from fragment F_I of bovine carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. The column was developed at 80 ml/hr with a double linear gradient of pyridine–acetic acid, as described in the text. Fractions of 6.0 ml were collected and monitored by ninhydrin analysis at 570 nm after alkaline hydrolysis. Fractions were pooled according to the solid bars. The pH gradient is indicated by the dashed line.

of any peptide from fragment F_I in sufficient yield to warrant further characterization. However, a low yield (~5%) of peptide T-6a[1] from fragment F_{III} (Bradshaw *et al.*, 1969a) was observed. In view of the recovery of other F_{III} peptides as well (*vide infra*), it is clear that the large-scale preparations of F_I used for sequence analysis were not totally devoid of F_{III}. Whether this contaminant arises from occlusion in the polymeric forms of F_I (Nomoto *et al.*, 1969) obtained by chromatography on Sephadex G-75 in 0.1 M propionic acid or from incomplete cleavage by cyanogen bromide was not conclusively established. Some evidence for incomplete fragmentation was obtained from the thermolytic peptides of this fragment in which a peptide which bridged the F_I and F_C fragments was isolated. In this case, the methionyl residue was present as homoserine (Bradshaw *et al.*, 1971a).

FRACTION II. Further purification of this fraction by chromatography on Dowex 1-X2 indicated the presence of only a single peptide, T-II-1. Sequence analysis of this peptide is given in Table II. [Primary data for Edman degradations are not included. Details of the criteria employed have been reported previously (Bradshaw *et al.*, 1969a).]

FRACTION III. Chromatography of this pool on Dowex 1-X2 did not yield any peptides in sufficient quantity to allow further purification.

FRACTION IV. Chromatography of this fraction on Dowex 1-X2 produced one peptide in high yield, T-IV-1, as well as low-yield peptides identified as being derived from F_{III}. The characterization of T-IV-1 is described in Table II. By a combination of Edman degradations and carboxypeptidase B hydrolysis, the complete sequence of this octapeptide was established. The electrophoretic mobility at pH 6.5 indicated that there were one acid and two amide residues.

FRACTION V. This pool contained only one major peptide after purification on Dowex 1-X2. The composition and characterization of this peptide are given in Tables I and II, respectively. The peptide was completely structured by a combination of Edman degradations and hydrolyses by carboxypeptidases A and B. The presence of at least two acidic residues was indicated by high-voltage electrophoresis at pH 6.5.

FRACTION VI. This pool was subjected to purification on Dowex 1-X2 and yielded two peptides, as indicated in the elution profile in Figure 2. The compositions of these peptides, T-VI-1 and T-VI-2, are given in Table I and the sequence data are found in Table III. Peptide T-VI-1, which was completely structured, is identical with one of the allotypic peptides (peptide $CPA_\gamma{}^{Leu}F_ITp$-VII-1) isolated from the chromatographically purified enzyme (Pétra *et al.*, 1969). Peptide T-VI-2 was subjected to eight rounds of the Edman degradation and was shown to terminate in lysine. Chymotryptic digestion of this peptide, followed by isolation of the resulting peptides on a column of Dowex 1-X2, allowed the elucidation of the sequence of the three carboxyl-terminal residues.

FRACTION VII. This pool contained a single peptide, T-VII. As shown by the data in Table IV, this peptide appears to correspond to the same region as T-VI-1. Indeed, comparison of these data with those of peptides obtained from chromatographically purified carboxypeptidase A_γ (Pétra *et al.*, 1969) indicates that this peptide is identical with peptide $CPA_\gamma{}^{Val}F_ITp$-VII-1 isolated in that study.

FRACTION VIII. This fraction was found to contain only one major peptide, T-VIII-1, after purification on Dowex 1-X2. Although electrophoretically pure, the fractional ratios of valine and isoleucine suggested that this fraction was a mixture of peptides. Partial resolution was accomplished by paper chromatography and it was ultimately established (Bradshaw *et al.*, 1971b) that those peptides contained the site of the second allotypic interchange present in the F_I fragment (Pétra *et al.*, 1969).

FRACTION IX. This pool contained only a single peptide, T-IX-1, after chromatography on Dowex 1-X2. The complete structure of this tetrapeptide is documented in Table IV. The

[1] Abbreviations used are: T-, tryptic peptide; C-, chymotryptic peptide.

TABLE 1: Amino Acid Composition of the Soluble Tryptic Peptides of Fragment F_1.[a]

Amino Acid	T-II-1	T-IV-1	T-V-1	T-VI-1	T-VI-2	T-VII	T-VIII-1	T-IX-1	T-X-1	T-XI-3	T-XII	T-XIII-2	T-XV	Total[c]	F_1[d]
Lysine		1.07 (1)	0.82 (1)	1.00 (1)	0.98 (1)	1.00 (1)	0.98 (1)		0.68 (1)	0.67 (1)	0.83 (1)	0.74 (1)		10	11
Histidine					0.96 (1)							0.83 (1)		2	4
Arginine	0.89 (1)							0.99 (1)			1.03 (1)		1.23 (1)	3	6
S-Carboxymethyl-cysteine	0.78 (1)				0.76 (1)									2	2
Aspartic acid	2.24 (2)	1.08 (1)	1.00 (1)				1.03 (1)	0.96 (1)	1.86 (2)			1.96 (2)		10	17
Threonine	0.97 (1)	0.96 (1)			0.90 (1)			0.86 (1)		0.97 (1)	0.87 (1)		1.00 (1)	6	16
Serine	3.65 (4)		0.84 (1)	0.84 (1)	3.41 (4)	0.95 (1)	0.92 (1)			1.82 (2)				13	24
Glutamic acid		2.04 (2)	1.98 (2)	1.01 (1)	0.97 (1)									5.5	15.5
Proline					1.02 (1)									1	6
Glycine	1.16 (1)				2.02 (2)			1.00 (1)	2.15 (2)	1.30 (1)		1.09 (1)		8	16
Alanine	0.98 (1)	1.13 (1)	1.02 (1)	1.98 (2)	1.98 (2)	2.93 (3)			1.02 (1)					8.5	13.5
Valine	3.18 (3)	0.96 (1)	1.98 (2)	1.26 (1)		1.06 (1)	2.19 (2.5)[e]							8.5	11.5
Isoleucine							0.57 (0.5)[e]							0.5	12.5
Leucine	1.12 (1)	0.98 (1)		0.97 (1)		1.00 (1)				1.10 (1)				4	15
Tyrosine			0.53 (1)		0.60 (1)					1.68 (2)				4	13
Phenylalanine							0.97 (1)		0.97 (1)			1.00 (1)		3	10
Tryptophan									0.75 (1)					1	4
Homoserine and lactone														0	1
Total	15	8	9	7	15	7	7	4	8	8	3	6	2	90	198
% yield	57	26	45	38	47	72	28	46	48	21	24	19	57		
Purification process[b]	D X1	D X1	D X1	D X1	D X1		D X1	D X1	D X1	D X1		D X1			
Residue No.	131–145	217–224	169–177	225–231	154–168	225–231	178–184	273–276	146–153	232–239	128–130	185–190	129–130		

[a] Values are given in residues/mole. The assumed integral values are given in parentheses. [b] Abbreviations: D X1, Dowex 1-X2. [c] Peptides T-VI-1 and T-VII were averaged and treated as a single peptide; peptide T-XV was excluded from the total. [d] Taken from Nomoto *et al.* (1969). [e] Values obtained from 72-hr hydrolysates.

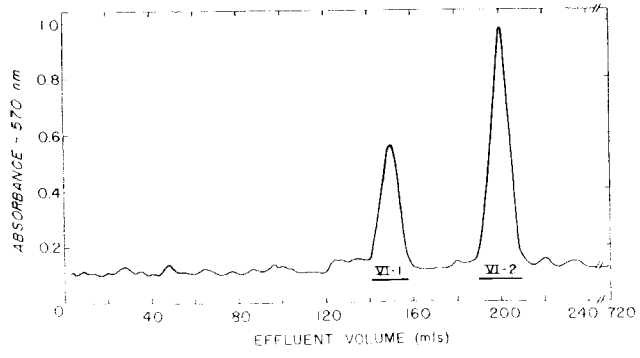

FIGURE 2: Elution profile of Fraction VI, separated on a 0.9 × 150 cm column of Dowex 1-X2 at 35°. The column was developed at 30 ml/hr with a gradient of pyridine–acetic acid, as described in the text. Fractions of 2.0 ml were collected and monitored by ninhydrin analysis at 570 nm after alkaline hydrolysis. Fractions were pooled as indicated by the solid bars.

TABLE II: Characterization of Peptides from Tryptic Fractions II, IV, and V.[a]

Fraction II:
Peptide T-II-1:
Sequence: Ser-Val-Thr-Ser-Ser-Ser-Leu-CMCys-Val-(Gly,Val,Asx,Ala,Asx)-Arg
Carboxypeptidase B: 3 hr; Arg, 0.63

Fraction IV:
Peptide T-IV-1:
Sequence: Thr-Glx-Leu-Asx-Glx-Val-Ala-Lys
Carboxypeptidase B: 3 hr; Lys, 0.76
HVE, pH 6.5: Neutral

Fraction V:
Peptide T-V-1:
Sequence: Tyr-Ala-Asx-Ser-Glx-Val-Glx-Val-Lys
Carboxypeptidase B: 3 hr; Lys, 0.93
Carboxypeptidase A: 3 hr; Val, 0.69
HVE, pH 6.5: Acidic

[a] Edman degradations are indicated by (→), whereas leucineaminopeptidase and carboxypeptidase A and B hydrolyses are indicated by (⇀) and (↼), respectively.

electrophoretic data indicate that the aspartyl residue is present in the acid form.

FRACTION X. Fraction X contained only a single peptide, T-X-1, after purification on Dowex 1-X2. The partial structure of this peptide is given in Table V. From the electrophoretic data, this peptide contains one acidic and one amide residue.

FRACTION XI. This pool was found to contain three peptides after purification on Dowex 1-X2. The elution profile is shown in Figure 3. Peptide T-XI-1 was found to have been derived from F_{III}, whereas peptide T-XI-2 was free lysine. The structure of peptide T-XI-3 is shown in Table V. All eight residues can be placed in sequence from the Edman degradation and carboxypeptidase data.

FRACTION XII. This pool contained a single peptide, T-XII, whose structure is shown in Table VI. This tripeptide was completely structured from a single Edman degradation and hydrolysis by carboxypeptidase B.

FRACTION XIII. After fractionation on Dowex 1-X2, this pool was found to contain two peptides, one of which was derived from F_{III}. The other peptide, T-XIII-2, contained six residues and was completely sequenced, as shown in Table VI.

FRACTION XIV. After fractionation on Dowex 1-X2, no peptides were recovered from this pool in sufficient yield to allow further characterization.

TABLE III: Characterization of Peptides from Tryptic Fraction VI.

Fraction VI:
Peptide T-VI-1:
Sequence: Ser-Ala-Val-Glu-Ala-Leu-Lys
Carboxypeptidase B: 3 hr; Lys, 0.96
Carboxypeptidase A: 3 hr; Leu, 0.87
HVE, pH 6.5: Neutral

Peptide T-VI-2:
Sequence: Ala-Gly-Ala-Ser-Ser-Ser-Pro-CMCys-(Ser,Glu,Thr,Tyr)-His-Gly-Lys
Carboxypeptidase B: 3 hr; Lys, 0.61
Chymotryptic Subdigest:
Peptide T-VI-2- C-1:
Sequence: His-(Gly,Lys)
Peptide T-VI-2 C-2:
Sequence: (Ala,Gly,Ala,Ser,Ser,Ser,Pro,CMCys,-Ser,Glx,Thr,Tyr)
HVE, pH 6.5: Neutral

TABLE IV: Characterization of Peptides from Tryptic Fractions VII, VIII, and IX.

Fraction VII:
Peptide T-VII:
Sequence: Ser-Ala-Val-Ala-Ala-Leu-Lys
Carboxypeptidase B: 3 hr; Lys, 1.00
Carboxypeptidase A: 1 hr; Leu, 0.54

Fraction VIII:
Peptide T-VIII-1:
Sequence: Ser-(Ile/Val,Val,Asx)(Phe,Val)-Lys
Carboxypeptidase B: 30 min; Lys, 0.69
Carboxypeptidase A: 30 min; Val, 0.95; Phe, 0.73
3 hr; Val, 0.96; Phe, 0.84

Fraction IX:
Peptide T-IX-1:
Sequence: Asp-Thr-Gly-Arg
Carboxypeptidase B: 3 hr; Arg, 0.77
HVE, pH 6.5: Neutral

TABLE V: Characterization of Peptides from Tryptic Fractions X and XI.

Fraction X:
Peptide T-X-1:
Sequence: Asx-Trp-Asx(Ala,Gly,Phe,Gly)-Lys
Carboxypeptidase B: 3 hr; Lys, 1.00
HVE, pH 6.5: Neutral

Fraction XI:
Peptide T-XI-2:
Sequence: Lys
Peptide T-XI-3:
Sequence: Ser-Leu-Tyr-Gly-Thr-Ser-Tyr-Lys
Carboxypeptidase B: 30 min; Lys, 0.70
Carboxypeptidase A: 30 min; Tyr, 0.96; Ser, 0.28; Thr, 0.18
3 hr; Tyr, 1.00; Ser, 0.85; Thr, 0.50

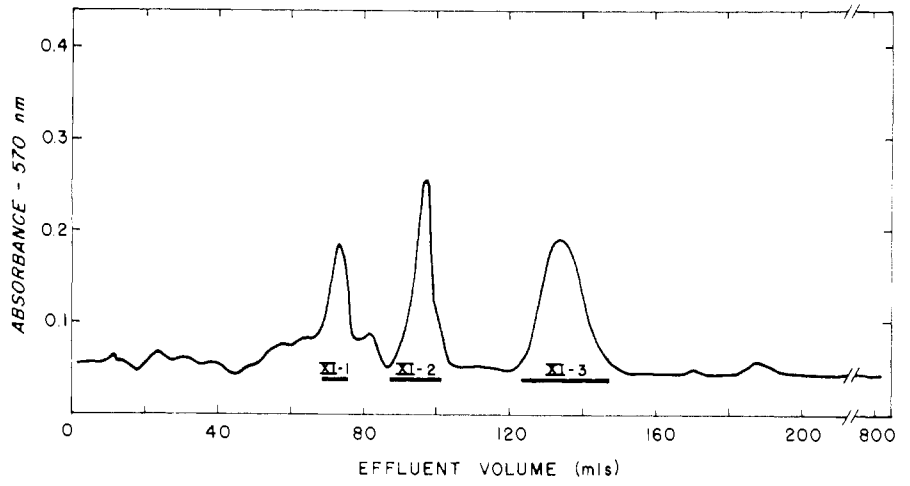

FIGURE 3: Elution profile of the separation of Fraction Th-XI on a 0.9 × 50 cm column of Dowex 1-X2. Details as in Figure 2.

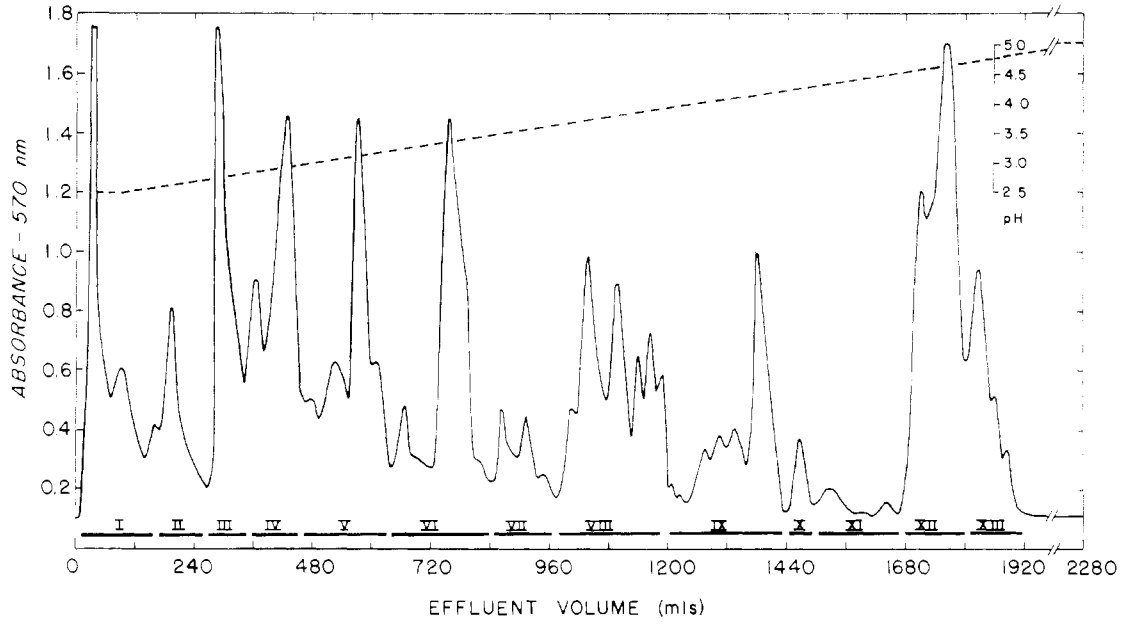

FIGURE 4: Elution profile of the soluble chymotryptic peptides from fragment F_1 of bovine carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. Details as in Figure 1.

TABLE VI: Characterization of Peptides from Tryptic Fractions XII, XIII, and XV.

Fraction XII:
Peptide T-XII:
Sequence: Lys-Thr-Arg
Carboxypeptidase B: 3 hr; Arg, 0.93

Fraction XIII:
Peptide T-XIII-2:
Sequence: Asx-His-Gly-Asn-Phe-Lys
Carboxypeptidase B: 30 min; Lys, 0.94
Carboxypeptidase A: 30 min; Phe, 0.67; Asn, 0.36

Fraction XV:
Peptide T-XV:
Sequence: Thr-Arg

FRACTION XV. This pool contained a single pure dipeptide, T-XV. Its structure is given in Table VI.

The last column of Table I summarizes the total composition of the soluble tryptic peptides. For this tabulation, the compositions of peptides T-VI-1 and T-VII were averaged and treated as a single peptide and peptide T-XV, which was assumed to be derived from T-XII, was omitted. From these data it was calculated that 90 of the 198 residues present (45%) were recovered in these fractions.

Isolation of the Soluble Chymotryptic Peptides. The soluble chymotryptic peptides of the *S*-carboxymethylated fragment F_1 were fractionated in the same manner as described for the tryptic peptides. The separation achieved on the Dowex 50-X8 column is shown in Figure 4. In each case, the indicated fractions (solid bars) were pooled and examined for purity by high-voltage electrophoresis. Subsequent treatment was dictated by these results. The composition of each of the pure peptides obtained is given in Table VII along with the total number of residues, the per cent yield, and the purification

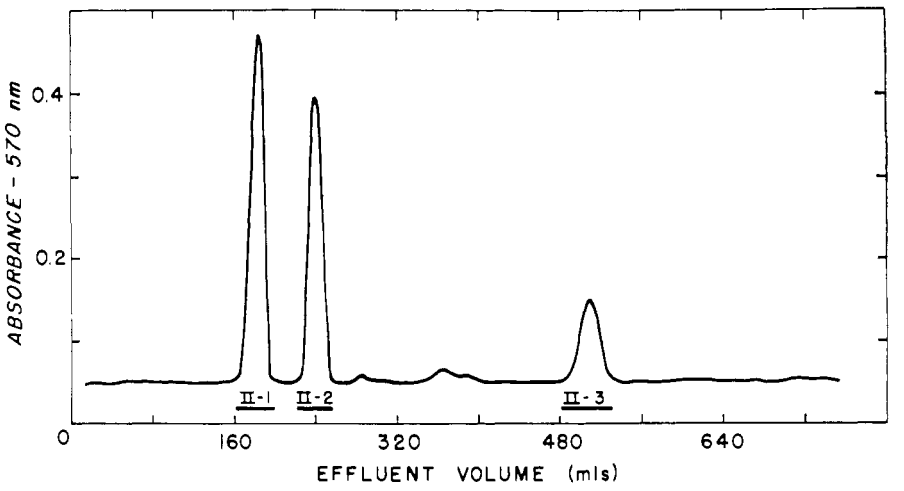

FIGURE 5: Elution profile of the separation of Fraction C-II on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

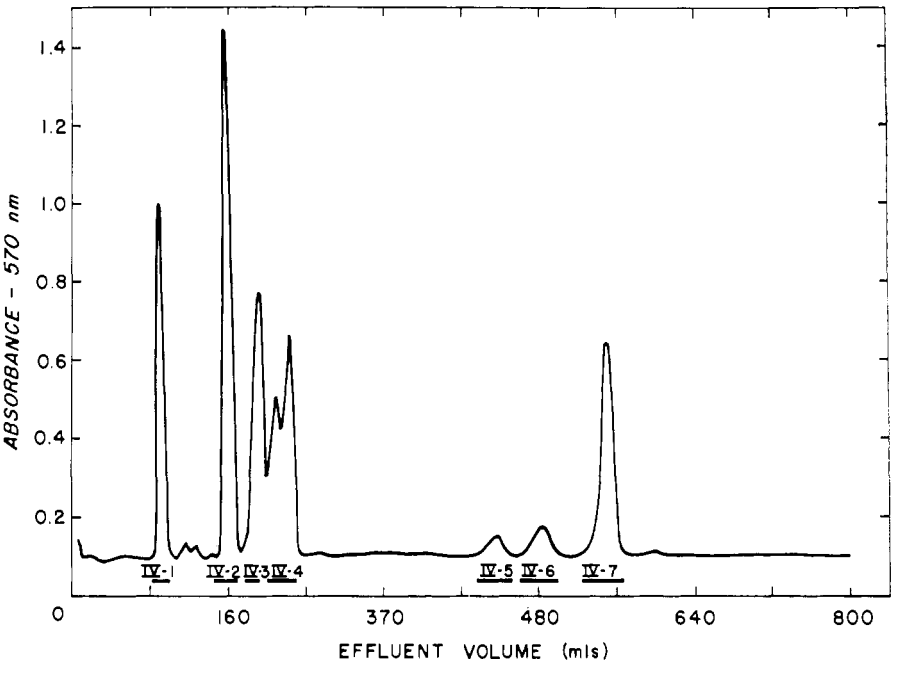

FIGURE 6: Elution profile of the separation of Fraction C-IV on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

procedure used, if any. The last line indicates the residue numbers covered by each peptide as deduced from their position in the final sequence (Bradshaw *et al.*, 1971b).

Characterization of the Soluble Chymotryptic Peptides. FRACTION I. Fractionation of this pool on Dowex 1-X2 resulted in recovery of two peptides in major yield and several minor ones. By purification on Dowex 50-X2, one of the major fractions, C-I-1, was consequently shown to contain two peptides. The other major yield peptide was identical with C-II-3 and was not treated further. The characterization of C-I-1 is given in Table VIII. Peptide C-I-1-2 differs from peptide C-I-1-1 by an additional residue of tryptophan at the carboxyl terminus.

FRACTION II. This pool was purified on Dowex 1-X2, as shown in Figure 5. Three major peaks were observed. Peptide C-II-2 was found to contain only kynurenine, an oxidation product of tryptophan. It was assumed that a portion of the free tryptophan found in pool C-V-6-2 was converted into this compound. The characterization of peptides C-II-1 and C-II-3 is given in Table VIII.

FRACTION III. This pool contained two major peptides after fractionation on Dowex 1-X2. One was identified as belonging to F_{III}; the other, C-III-2, is described in Table IX.

FRACTION IV. This pool was fractionated into seven peaks, as shown in Figure 6. Pool C-IV-4 was further purified on Dowex 50-X2 but did not show any peptides in sufficient yield to warrant further characterization. Fraction C-IV-5 was found to be a peptide originating from fragment F_{III}, whereas fraction C-IV-6 was of insufficient yield for further analysis. Fraction C-IV-7 was further purified on Dowex 50-X2 and found to contain free phenylalanine and free tyrosine. The peptides found in fractions C-IV-1, -2, and -3 are described in Table IX.

FRACTION V. After subfractionation on a column of Dowex 1-X2, ten pools were obtained, as shown in Figure 7. Fractions C-V-1, -2, and -10 were found to be devoid of peptidic material, while fractions C-V-3 and C-V-4 contained peptides derived from fragment F_{III}. Fraction C-V-5 contained a single tripeptide, as described in Table X. The presence of homoserine and its lactone in this peptide suggest that it is derived

TABLE VII: Amino Acid Composition of the Soluble Chymotryptic Peptides of Fragment F_1.[a]

Amino Acid	C-I-1-1	C-I-1-2	C-II-1	C-II-3	C-III-2	C-IV-1	C-IV-2	C-IV-3	C-V-5	C-V-6-1	C-V-7	C-V-8	C-V-9-1	C-VI-3 PC-1	C-VI-3 PC-2	C-VI-6-1
Lysine				0.94 (1)	0.88 (1)	1.00 (1)						0.77 (1)				1.66 (2)
Histidine																0.96 (1)
Arginine																
S-Carboxymethyl-cysteine				0.88 (1)												
Aspartic acid				1.05 (1)	1.07 (1)	1.00 (1)						1.01 (1)				4.01 (4)
Threonine	2.02 (2)	1.97 (2)		0.88 (1)	2.66 (3)			1.80 (2)	1.02 (1)	1.02 (1)	0.99 (1)	1.01 (1)				
Serine	1.19 (1)	1.08 (1)	0.96 (1)	4.17 (4)	0.95 (1)	1.23 (1)				0.94 (1)	1.02 (1)	1.03 (1)				1.93 (2)
Glutamic acid	3.03 (3)	2.98 (3)		1.19 (1)	2.12 (2)	1.02 (1)		1.02 (1)				0.98 (1)				2.16 (2)
Proline	1.95 (2)	2.00 (2)		0.83 (1)	0.94 (1)							0.96 (1)	1.33 (1)			
Glycine				2.83 (3)			1.16 (1)			0.99 (1)					1.00 (1)	1.21 (1)
Alanine	1.96 (2)	2.02 (2)	2.71 (3)	2.78 (3)		3.69 (4)								0.93 (1)		1.13 (1)
Valine			1.00 (1)			1.81 (2)	1.04 (1)									3.90 (4.5)[b]
Isoleucine	1.76 (2)[b]	1.92 (2)[b]			0.98 (1)				1.00 (1)			0.93 (1)				0.46 (0.5)[b]
Leucine	1.96 (2)	1.86 (2)	1.00 (1)		1.04 (1)	1.00 (1)	2.00 (2)					0.96 (1)	2.11 (2)			
Tyrosine				0.87 (1)						1.01 (1)			1.91 (2)			
Phenylalanine				0.91 (1)							1.80 (2)			1.00 (1)	1.00 (1)	1.93 (2)
Tryptophan		0.99 (1)														
Homoserine and lactone									1.06 (1)							
Total	14	15	6	18	11	11	4	3	3	4	4	8	5	2	2	20
% yield	10	47	41	20	20	16	22	34	60	46	40	54	8	26	18	5
Purification process[c]	D X1; D X50	D X1; D X50	D X1	D X1	D X1	D X1	D X1	D X1	D X1	D X1; D X50	D X1	D X1	D X1; D X50	D X1 PC	D X1; PC	D X1; D X50
Residue No.	280–293	280–294	225–230	148–165	209–219	220–230	295–298	209–211	299–301	235–238	266–269	212–219	202–206	117–118	278–279 or 115–116	170–189

Amino Acid	C-VI-7	C-VI-9	C-VII-1 PC-1	C-VII-1 PC-2	C-VII-2	C-VIII-3-1	C-VIII-4	C-VIII-5-2	C-IX-1	C-IX-3-1	C-IX-4-1	C-IX-4-2	C-X-2	C-X-3	C-XI-1	Total	F_1[d]
Lysine					0.87 (1)	0.65 (1)				1.09 (1)	0.91 (1)		0.85 (1)	0.86 (1)		7	11
Histidine							0.92 (1)	0.95 (1)	0.99 (1)		0.90 (1)					3	4
Arginine		1.08 (1)										2.13 (2)			1.12 (1)	4	6
S-Carboxymethyl-cysteine		1.13 (1)														2	2
Aspartic acid		3.00 (3)			1.03 (1)	0.87 (1)	1.06 (1)				1.93 (2)	1.03 (1)			1.03 (1)	12	17
Threonine							0.88 (1)		1.00 (1)			0.96 (1)				11	16
Serine			0.95 (1)				1.07 (1)	2.38 (2)		1.00 (1)					0.96 (1)	16	24

Glutamic acid					1.02 (1)	1.00 (1)	1.17 (1)					1.00 (1)						0.95 (1)		11		15.5
Proline	0.78 (1)																			5		6
Glycine		1.15 (1)		1.00 (1)		1.12 (1)					1.06 (1)	1.00 (1)								11		16
Alanine		1.15 (1)			1.14 (1)								1.00 (1)	1.00 (1)						12.5		13.5
Valine		2.20 (2)			0.94 (1)						1.00 (1)									9.5		11.5
Isoleucine						0.73 (1)		1.08 (1)												5.5		12.5
Leucine	1.34 (1)							1.06 (1)		1.00 (1)			0.96 (1)			0.92 (1)	1.00 (1)		12		15
Tyrosine	2.28 (2)		1.00 (1)	0.89 (1)		0.99 (1)		1.00 (1)		0.92 (1)			0.83 (1)						9		13
Phenylalanine											1.02 (1)					0.99 (1)	0.72 (1)		9		10
Tryptophan		0.77 (1)																0.40 (1)	3		4
Homoserine and lactone																			1		1
Total	4	10	2	2	5	6	5	6	2	4	7	8	3	4	6				143.5		198	
% yield	63	70	40	80	80	8	6	5	14	58	12	57	30	10	6							
Purification process[a]	D X1	D X1	D X1; PC	D X1; PC	D X1	D X1; D X50	D X1	D X1; D X50	D X50	F X50; D X1	D X50; D X1	D X50; D X1	D X50	D X50	D X50							
Residue No.	203–206	138–147	258–259	207–208	220–224	260–265	119–123	193–198	119–120	231–234	183–189	270–277	190–192	190–193	121–126							

[a] Values are given in residues/mole. The assumed integral values are given in parentheses. [b] Values obtained from 72-hr hydrolysates. [c] Abbreviations used: D X1, Dowex 1-X2; D X50, Dowex 50-X2. [d] Taken from Nomoto *et al.* (1969).

TABLE VIII: Characterization of Peptides from Chymotryptic Fractions I and II.

Fraction I:
- Peptide C-I-1-1-:
  - Sequence: Leu-(Leu,Pro,Ala,Ser,Glx,Ile,Ile, Pro,Thr,-Ala,Glx,Glx,Thr)
- Peptide C-I-1-2-:
  - Sequence: Leu-Leu-Pro-Ala-Ser-Glx-Ile-Ile-Pro-Thr-(Ala,Glx,Glx,Thr)-Trp
  - Carboxypeptidase A: 3 hr; Trp, 0.40
  - HVE, pH 6.5: Acidic

Fraction II:
- Peptide C-II-1:
  - Sequence: Ser-Ala-Val-Ala-Ala-Leu
- Peptide C-II-3:
  - Sequence: Asx-Ala-Gly-Phe-Gly-Lys-Ala-Gly-(Ala,Ser,-Ser,Pro,CMCys,Ser,Glx,Thr,Tyr)

from the carboxyl terminus of F_I. Fraction C-V-6 was further purified on a column of Dowex 50-X2. In addition to a single peptide, C-V-6-1, free tryptophan was found in pool C-V-6-2. Pool C-V-7 and C-V-8 each contained a single peptide, as described in Table X. Pool C-V-9 was further treated on Dowex 50-X2 but only a single peptide was recovered.

FRACTION VI. This fraction was purified on Dowex 1-X2 and, as shown in Figure 8, nine fractions were obtained. Fractions C-VI-1, -2, and -8 were devoid of sufficient material for further analysis, and fraction C-VI-4 contained peptides derived from fragment F_{III}. Fraction C-VI-3 contained two dipeptides that were separated by paper chromatography. Fraction C-VI-5 contained a single peptide identical with C-V-7. Fraction C-VI-6, after further purification, was found to contain one major peptide, C-VI-6-1. The fractional values of valine and isoleucine (given in Table VII) reflect the allotypic

TABLE IX: Characterization of Peptides from Chymotryptic Fractions III and IV.

Fraction III:
- Peptide C-III-2:
  - Sequence: Thr-Thr-Glx-Ser-Ile-Pro-(Asx,Lys,Thr,Glx)-Leu
  - Carboxypeptidase A: 30 min; Leu, 0.38

Fraction IV:
- Peptide C-IV-1:
  - Sequence: Asn-Gln-Val-Ala-Lys-Ser-Ala(Val,Ala,Ala,-Leu)
  - Leucineaminopeptidase: 3 hr; Asn + Gln, 1.50
- Peptide C-IV-2:
  - Sequence: Leu-Gly-Val-Leu
- Peptide C-IV-3:
  - Sequence: Thr-Thr-Gln
  - HVE, pH 6.5: Neutral

TABLE X: Characterization of Peptides from Chymotryptic Fraction V.

Fraction V:
- Peptide C-V-5:
  - Sequence: (Thr,Ile,HSer)
- Peptide C-V-6-1:
  - Sequence: Gly-Thr-Ser-Tyr
- Peptide C-V-6-2:
  - Sequence: (Trp)
- Peptide C-V-7:
  - Sequence: (Ser,Phe,Thr,Phe)
- Peptide C-V-8:
  - Sequence: Ser-Ile-Pro-Asp(Lys,Thr,Glu,Leu)
  - HVE, pH 6.5: Acidic
- Peptide C-V-9-1:
  - Sequence: Leu-Leu-Tyr(Pro,Tyr)

site present in this peptide as well as the slow release of the Val-Val and Ile-Val bonds. Fractions C-VI-7 and C-VI-9 each contained a single peptide. The pure peptides isolated from this pool are described in Table XI. Partial sequence data were obtained for C-VI-6-1, whereas the complete structures of C-VI-7 and C-VI-9 were elucidated.

FRACTION VII. As shown in Figure 9, this pool was purified on Dowex 1-X2. Three fractions were obtained and characterized. Fraction C-VII-1 was found to contain two dipeptides that were purified on paper chromatography. The placement of these peptides, as those found in fraction C-VI-3, is only tentative since the rigorous assignment of dipeptides is usually not possible. Fraction C-VII-2 contained a single peptide, as described in Table XII, while fraction C-VII-3 was found to possess only a single peptide from fragment F_{III}.

TABLE XI: Characterization of Peptides from Chymotryptic Fraction VI.

Fraction VI:
- Peptide C-VI-3 PC-1:
  - Sequence: (Ala,Phe)
- Peptide C-VI-3 PC-2:
  - Sequence: (Gly,Phe)
- Peptide C-VI-6-1:
  - Sequence: Ala-Asx-Ser-(Glx,Val,Glx,Val,Lys,Ser,Val/Ile,Val,Asx,Phe,Val,Lys,Asx,His,Gly,Asx,Phe)
- Peptide C-VI-7:
  - Sequence: Leu-Tyr-Pro-Tyr
- Peptide C-VI-9:
  - Sequence: CMCys-Val-Gly-Val-Asp-Ala-Asn-Arg-Asn-Trp
  - Carboxypeptidase A: 1 hr; Trp, 1.19; Asn, 0.72
  - Carboxypeptidases A and B: 90 min; Trp, 1.17; Asn, 1.09; Arg, 0.72; Ala, 0.17
    3 hr; Trp, 1.16; Asn, 1.67; Arg, 1.00; Ala, 0.60
  - HVE, pH 6.5: Acidic

TABLE XII: Characterization of Peptides from Chymotryptic Fractions VII and VIII.

Fraction VII:
- Peptide C-VII-1 PC-1:
  - Sequence: (Ser,Tyr)
- Peptide C-VII-1 PC-2:
  - Sequence: (Gly,Tyr)
- Peptide C-VII-2:
  - Sequence: Asx-Glx-Val-Ala-Lys
  - Carboxypeptidase B: 30 min; Lys, 0.37

Fraction VIII:
- Peptide C-VIII-3-1:
  - Sequence: (Asx,Glx,Gly,Ile,Lys,Tyr)
- Peptide C-VIII-4:
  - Sequence: Thr-His-Ser-Glx-Asx
  - HVE, pH 6.5: Neutral
- Peptide C-VIII-5-2:
  - Sequence: Leu-Ser-Ile(His,Ser,Tyr)

FRACTION VIII. This pool was purified on Dowex 1-X2, as shown in Figure 10. Fraction C-VIII-1 contained only free arginine, the position of which could not be assigned with certainty. Subfractionation of fraction C-VIII-2 on Dowex 50-X2 was unsuccessful in that no peptides of sufficient yield were obtained. Fraction C-VIII-3 was also purified on Dowex 50-X2 and yielded two peptides, one of which was identified by composition. The other fraction was not purified further. Fraction C-VIII-4 contained a single peptide with the structure shown in Table XII. Fraction C-VIII-5 was further purified on Dowex 50-X2 and yielded two peptides, one of which was derived from fragment F_{III}. The structure of the other, C-VIII-5-2, is given in Table XII.

FRACTION IX. In view of its more basic character, the purification of this pool was carried out with a column of Dowex 50-X2. The elution profile, shown in Figure 11, indicated five main fractions. The last, fraction C-IX-5, was found to contain only peptides derived from fragment F_{III}. Fraction C-

TABLE XIII: Characterization of Peptides from Chymotryptic Fraction IX.

Fraction IX:
- Peptide C-IX-1:
  - Sequence: Thr-His
- Peptide C-IX-3-1:
  - Sequence: Lys-Ser-Leu-Tyr
- Peptide C-IX-4-1:
  - Sequence: Val-Lys-Asx-His-Gly-Asx-Phe
  - Carboxypeptidase A: 3 hr: Phe, 1.00
- Peptide C-IX-4-2:
  - Sequence: Glu-Leu-Arg-Asp-Thr-Gly-Arg-Tyr
  - Carboxypeptidase A: 30 min; Tyr, 0.42
  - Carboxypeptidases A and B: 3 hr; Tyr, 1.00; Arg, 1.00; Gly, 0.23; Thr, 0.19
  - HVE, pH 6.5: Neutral

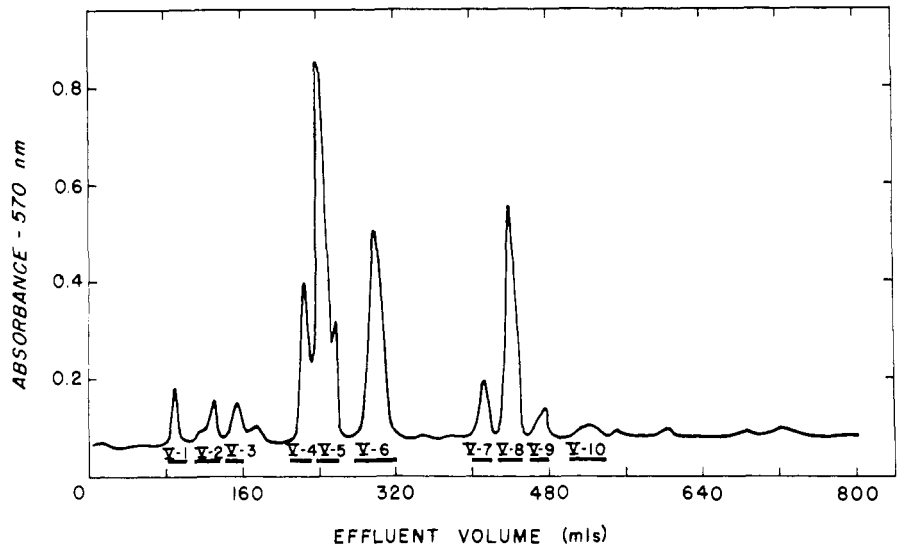

FIGURE 7: Elution profile of the separation of Fraction C-V on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

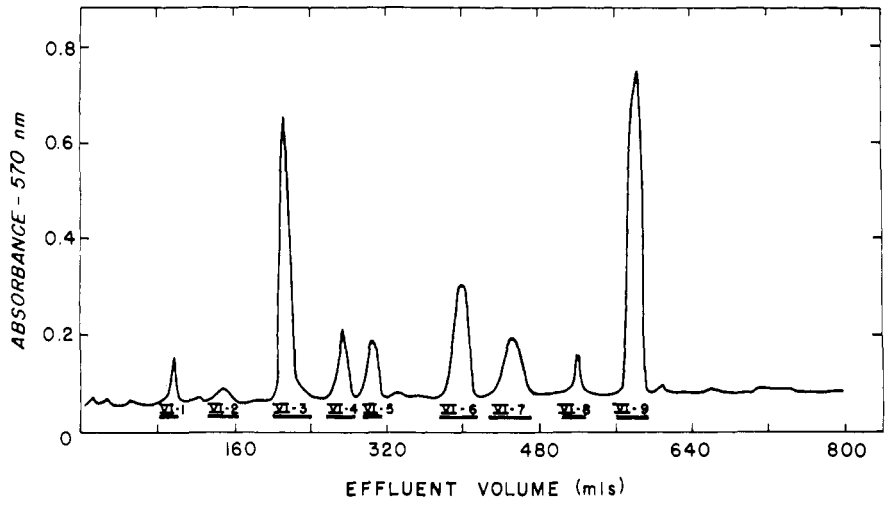

FIGURE 8: Elution profile of the separation of Fraction C-VI on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

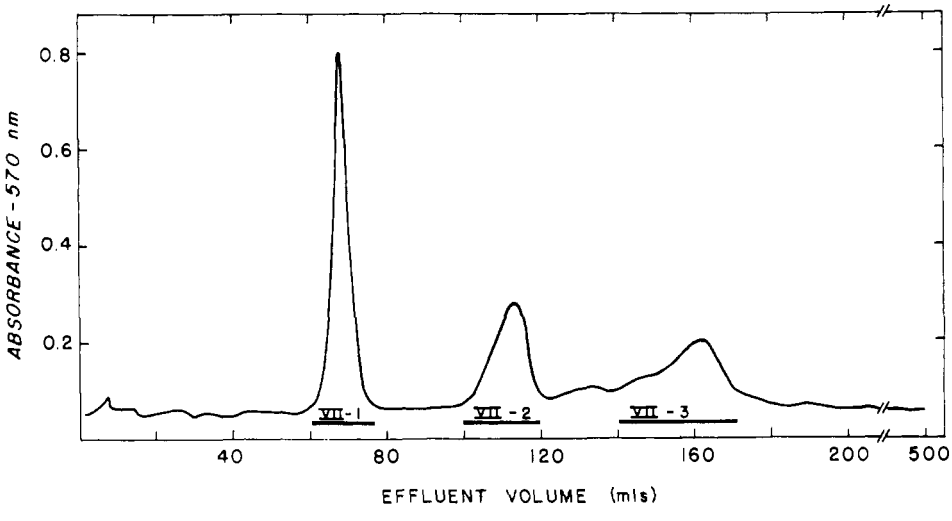

FIGURE 9: Elution profile of the separation of Fraction C-VII on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

IX-1 contained a single dipeptide, Thr-His. Fraction C-IX-2 was purified on Dowex 1-X2 and found to contain a single major peptide that was identical with C-IX-4-2. Fraction C-IX-3 was also purified on Dowex 1-X2 to yield peptide C-IX-3-1. The description of this tetrapeptide is found in Table XIII. Fraction C-IX-4 was separated into two fractions on Dowex 1-X2 as shown in Figure 12. Each of these purified peptides was completely structured by Edman degradations and carboxypeptidase hydrolysis, as detailed in Table XIII.

FRACTION X. This fraction was purified on Dowex 50-X2, as

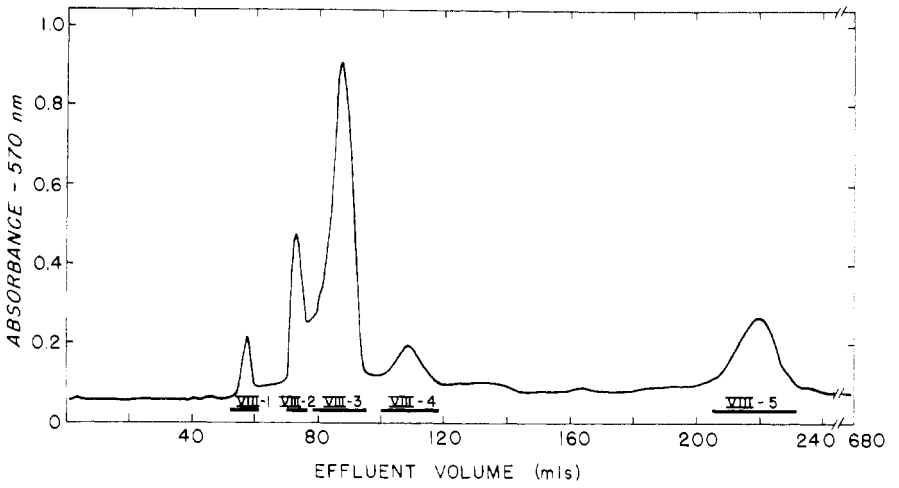

FIGURE 10: Elution profile of the separation of Fraction C-VIII on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

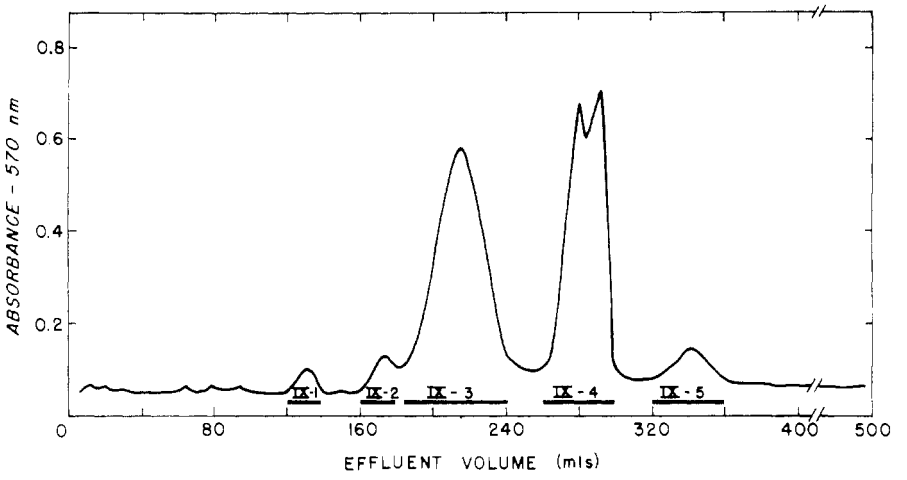

FIGURE 11: Elution profile of the separation of Fraction C-IX on a 0.9 × 50 cm column of Dowex 50-X2 at 55°. The column was developed at 30 ml/hr and 2.0-ml fractions were collected. The gradient was established by 250 ml of 0.2 N pyridine–acetic acid, pH 3.1, and 250 ml of 2.0 N pyridine–acetic acid, pH 5.0.

shown in Figure 13. Fractions C-X-1 and C-X-4 did not contain any peptidic material. The structure of peptides C-X-2 and C-X-3 is shown in Table XIV. By the nature of their structure, it was deduced that these peptides are both derived from the same portion of the molecule.

FRACTION XI. This fraction yielded only a single peptide on purification with Dowex 50-X2. The structure of this peptide is shown in Table XIV.

FRACTIONS XII AND XIII. Purification of fraction C-XII on Dowex 50-X2 yielded a large ninhydrin-positive peak that was devoid of peptidic material. It may be assumed that ammonia or other ninhydrin-positive material gave rise to the material observed in the original fractionation. Similarly, no peptidic material could be recovered from fraction C-XIII.

In the last two columns of Table VII the total number of residues of each amino acid found in the soluble chymotryptic peptides and the complete composition of fragment F_1 are listed (Nomoto *et al.*, 1969). This compilation was made on the basis of the final assignment of the peptides (Bradshaw *et al.*, 1971b), and peptides covering the same region of the fragment were only totalled once.

Examination of Position 151 in Carboxypeptidase A_γ^{Val}. On the basis of the complete amino acid sequence of this enzyme (Bradshaw *et al.*, 1969c; Bradshaw *et al.*, 1971a,b), Lipscomb *et al.* (1969) have reported that a refinement of the X-ray diffraction analysis of the three-dimensional structure indicates a tryptophanyl residue rather than a phenylalanyl residue at position 151. In view of the fact that these workers reported that their studies were performed with the valine allotype (Pétra *et al.*, 1969), a reexamination of chromato-

TABLE XIV: Characterization of Peptides from Chymotryptic Fractions X and XI.

Fraction X:
Peptide C-X-2:
Sequence: Lys-Ala-Phe
Peptide C-X-3:
Sequence: Lys-Ala-Phe-Leu
Fraction XI:
Peptide C-XI-1:
Sequence: Ser-(Glx,Asx,Arg)Leu-Trp
Carboxypeptidase A: 30 min; Trp, 0.81; Leu, 0.53

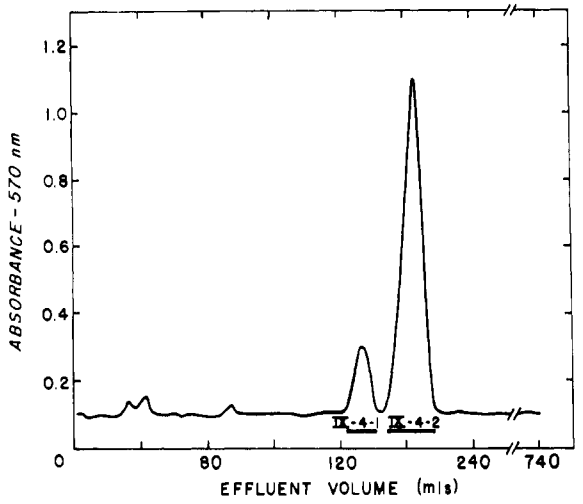

FIGURE 12: Elution profile of the separation of Fraction C-IX-4 on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

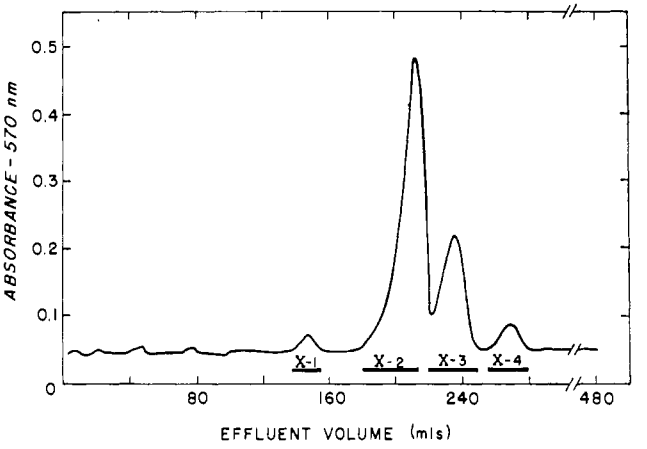

FIGURE 13: Elution profile of the separation of Fraction C-X on a 0.9 × 150 cm column of Dowex 50-X2. Details as in Figure 11.

graphically purified carboxypeptidase $A^{\gamma Val}$ with regard to peptide T-X-1 [which contains residues 146–153 in the final sequence (Bradshaw *et al.*, 1971b)] was carried out. In this study, reduced and *S*-carboxymethylated F_1 was prepared from carboxypeptidase A_γ^{Val} in the same manner as the material prepared from the commercial protein and subjected to tryptic digestion and purification on Dowex 50-X8. Protein concentration was established on a suitable aliquot by use of the automatic amino acid analyzer. Further purification on Dowex 1-X2 was performed as with T-X-1 and the peptide obtained is described in Table XV. As can be seen, only minor contamination was removed on the Dowex 1-X2 column. The tryptophan content was estimated spectrophotometrically at 289 nm in 0.02 M sodium phosphate buffer (pH 6.5) on a sample of peptide that had been exhaustively dried to remove excess pyridine. A slightly high value may still result from pyridine bound to the peptide as counterion, but this contribution was taken to be negligible. Clearly, the composition of this peptide, recovered in 75% yield, is identical with that obtained from the commercial mixture of carboxypeptidase A (Anson) and includes a single residue of tryptophan and phenylalanine. In view of the rigorous assignment of these two residues to positions 147 and 151 (Bradshaw *et al.*, 1971b), it may be safely concluded that residue 151 is phenylalanine in both the valine and leucine forms of the enzyme and that position 151 does not represent an additional site of allotypic replacement.

Discussion

Although the peptides isolated from the soluble fractions of either the tryptic or chymotryptic digests do not nearly account for the whole fragment, it is still possible to deduce the structure of some larger segments from these overlaps alone. In particular, the region surrounding the half-cystinyl residues can be constructed, as shown in Figure 14. Peptide T-II-1, which contains one of the half-cystinyl residues, clearly can be seen to overlap peptide C-VI-9, also containing a half-cystinyl residue. The overlap of C-VI-9 to T-X-1 is based only on two residues but involves a residue (tryptophan) which occurs in only four sites in the fragment, making the overlap more secure. The alignment of C-II-3 to overlap T-X-1 and hence to position T-VI-2 is evident. Thus, on the basis of the data presented, this 38-residue segment can be constructed. Although other shorter segments can also be assembled from these data, their alignment is not sufficiently definite to warrant discussion in this report.

It is significant that this segment also contains residue 151 as deduced from the complete structure (Bradshaw *et al.*, 1971b). On the basis of integrated density levels throughout their electron-density map, Lipscomb *et al.* (1969) have reported a strong preference for tryptophan in place of phenylalanine at this site. Although the relatively high yield of this peptide (48%) from commercial carboxypeptidase A (Anson) seemed to preclude another allotypic replacement site, this point was rigorously checked by isolation of the peptide from the pure allotypic form presumably used by Lipscomb and coworkers in their studies. As expected, this form of the enzyme also yielded the peptide containing phenylalanine 151 and hence conclusively established the correctness of the identification as proposed. These results underscore the apparent pitfalls in interpreting electron-density maps and their subsequent translation into unequivocal protein structures.

The specificity of cleavage displayed by both trypsin and chymotrypsin toward fragment F_1 seems to follow very closely the classical specificities of these enzymes. In fact, no abnormal cleavages by trypsin were noted, while hydrolysis adjacent to

TABLE XV: Amino Acid Composition of Peptide T-X-1 from Carboxypeptidase A_γ^{Val}.

Amino Acid	After Dowex 50-X8 Chromatography	After Dowex 1-X2 Chromatography	Theory
Aspartic acid	2.00	1.97	2
Glycine	2.32	2.12	2
Alanine	0.94	1.03	1
Phenylalanine	1.00	1.00	1
Lysine	1.10	1.10	1
Tryptophan		1.03[a]	1
Total % yield	75	19	

[a] Determined spectrophotometrically by the method of Edelhoch (1967).

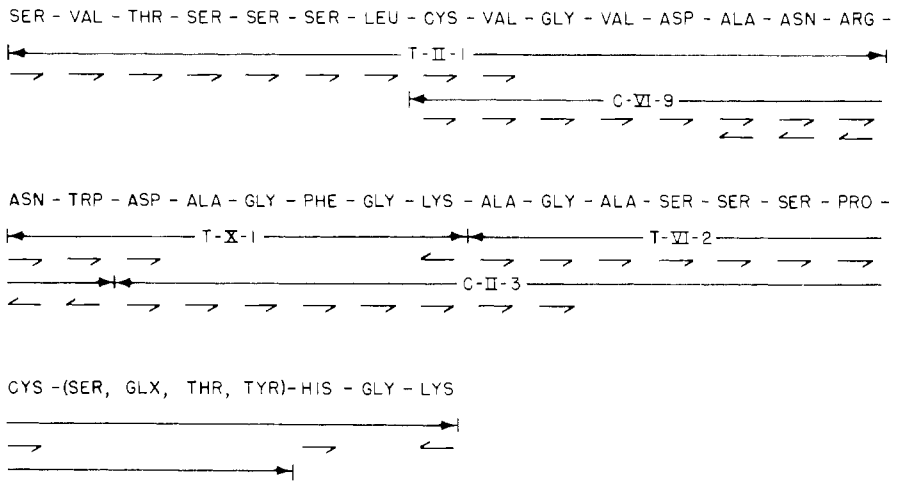

FIGURE 14: Proposed sequence surrounding the half-cystinyl residues of bovine carboxypeptidase A.

Asx (peptide C-VIII-4), Glx (peptide C-IV-3), Lys (peptide C-VII-2), and Thr (peptide C-I-1-1) were the only abnormal sites observed for chymotrypsin.

From a consideration of the number of residues accounted for by the soluble tryptic peptides (90 or 45%) and by the soluble chymotryptic peptides (143.5 or 72%), it is clear that neither enzyme was effective in solubilizing this fragment completely. The problems caused by the large amount of insoluble core remaining in these digests were largely circumvented by the use of thermolysin as the third cleaving enzyme. In this case, almost the entire fragment was recovered in soluble peptides. These data are summarized in the following report (Bradshaw *et al.*, 1971a). The synthesis of the complete sequence from these and additional data obtained from digestion with pepsin and Nagarse is given in the third report (Bradshaw *et al.*, 1971b).

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