further substantiating the conclusion that this enzyme has great utility in sequence studies.

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References

Ambler, R. P., and Meadway, R. J. (1968), *Biochem. J. 108*, 893.

Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London) 221*, 337.

Bradshaw, R. A. (1969), *Biochemistry 8*, 3871.

Bradshaw, R. A., Babin, D. R., Nomoto, M., Srinivasin, N. G., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969a), *Biochemistry 8*, 3859.

Bradshaw, R. A., Garner, W. H., and Gurd, F. R. N. (1969b), *J. Biol. Chem. 244*, 2149.

Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1971a), *Biochemistry 10*, 938.

Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1971b), *Biochemistry 10*, 961.

Matsubara, H. (1966), *Biochem. Biophys. Res. Commun. 24*, 427.

Matsubara, H., and Sasaki, R. (1968), *J. Biol Chem. 243*, 1732.

Matsubara, H., Sasaki, R., Singer, A., and Jukes, T. H. (1966), *Arch. Biochem. Biophys. 115*, 324.

Matsubara, H., Singer, A., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 34*, 719.

Nomoto, M., Srinivasin, N. G., Bradshaw, R. A., Wade, R. D., and Neurath, H. (1969), *Biochemistry 8*, 2755.

Ohta, Y. and Ogura, Y. (1965), *J. Biochem. (Tokyo) 58*, 607.

Pétra, P. H., Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 2762.

Amino Acid Sequence of Bovine Carboxypeptidase A. Isolation and Characterization of Selected Peptic and Nagarse Peptides and the Complete Sequence of Fragment F_I*

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ABSTRACT: The isolation and characterization of selected peptides derived from peptic and Nagarse digests of the cyanogen bromide fragment F_I of bovine carboxypeptidase A are described. These data were combined with those obtained from the tryptic, chymotryptic, and thermolytic peptides of this fragment to produce the complete amino acid sequence of this 198-residue fragment. In addition, each of the side chains of Glx and Asx residues was identified as to its acid or amide character.

Attention was directed toward those residues whose identification differed from the assignments made on the basis of X-ray diffraction studies.

In the previous reports (Bradshaw *et al.*, 1971a,b), the isolation and characterization of the soluble tryptic, chymotryptic, and thermolytic peptides of fragment F_I of bovine carboxypeptidase A were described. These data were insufficient to provide an unambiguous sequence for this fragment. Digestion of the whole fragment by pepsin and of the insoluble core remaining after tryptic hydrolysis by Nagarse yielded additional peptides which enabled the completion of the structure. In order to simplify the information that led to the final structure, only those peptides which are necessary for the proof are described. The final assembly indicates that fragment F_I is composed of 198 amino acid residues and that the composition derived from the sequence is in exact agreement with that obtained from acid hydrolysates of fragment F_I.

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Experimental Procedure

Materials. After CNBr treatment of bovine carboxypeptidase A, as described previously (Nomoto *et al.*, 1969), fragment F_I was isolated on Sephadex G-75 which had been equilibrated with 0.1 M propionic acid. Pepsin was purchased from Worthington Biochemical Corporation, and Nagarse from Teikoku Chemical Industry Co., Ltd., Osaka, Japan. Other materials were the same as described previously (Bradshaw *et al.*, 1969a).

Methods. Peptic digestion of F_I was carried out by the addition of pepsin, dissolved in 0.5 M NaCl, to a final concentration

TABLE I: Amino Acid Composition of Selected Peptic Peptides from Fragment F_1.[a]

Amino Acid	Pp-I-6	Pp-I-9	Pp-II-1	Pp-II-3	Pp-III-1	Pp-IV-2	Pp-IV-3	Pp-V-3
Lysine				1.00 (1)	1.21 (1)			
Histidine						0.85 (1)	1.65 (2)	
Arginine						1.06 (1)		
S-Carboxymethyl-cysteine								
Aspartic acid		2.00 (2)		1.12 (1)	0.98 (1)	2.72 (3)	1.00 (1)	
Threonine	0.95 (1)	1.04 (1)	0.98 (1)	2.82 (3)		1.75 (2)	1.70 (2)	
Serine	0.96 (1)			0.88 (1)	0.99 (1)	0.97 (1)	1.21 (1)	1.79 (2)
Glutamic acid	2.21 (2)			2.13 (2)	1.07 (1)	1.91 (2)	4.30 (4)	1.01 (1)
Proline	2.00 (2)	0.96 (1)		1.87 (2)		0.97 (1)	1.80 (2)	
Glycine		1.08 (1)	0.99 (1)	0.98 (1)		1.24 (1)	2.08 (2)	2.11 (2)
Alanine	2.11 (2)	1.02 (1)			4.31 (4)	1.30 (1)	2.00 (2)	1.00 (1)
Valine		1.01 (1)[b]	0.99 (1)		1.95 (2)	0.96 (1)[b]		
Isoleucine	1.85 (2)[b]	0.94 (1)[b]	0.85 (1)	1.03 (1)		0.92 (1)[b]	1.94 (2)[b]	0.98 (1)
Leucine	0.86 (1)		1.07 (1)	2.84 (3)	0.96 (1)	2.03 (2)	3.08 (3)	
Tyrosine				3.03 (3)			0.97 (1)	0.80 (1)
Phenylalanine		0.89 (1)				1.72 (2)	1.60 (2)	
Tryptophan								
Homoserine and lactone			1.05 (1)					
Total	11	9	6	18	11	19	24	8
% Yield	10	17	21	38	7	20	7	10
Purification procedure[d]	D X1	D X1	D X1	D X1	D X1	D X1	D X1	D X1
Residue No.	281–291	109–117	296–301	202–219	220–230	107–125	269–292	247–254

[a] Values are given in residues/mole. The assumed integral values are given in parentheses. [b] Values obtained from 72-hr hy-

which was on a weight basis 1% that of F_1. The concentration of F_1, dissolved in 5% formic acid, was 5%, pH 2.1. After addition of enzyme, the insoluble suspension was stirred at room temperature for 1 hr, at which time the protein was completely solubilized. The reaction mixture was lyophilized and the solid residues redissolved in 0.05 N pyridine–acetic acid, pH 2.4. A small amount of material remained insoluble and was removed by centrifugation.

Nagarse Digestion of Tryptic Core. Tryptic digestion of F_1 (Bradshaw *et al.*, 1971a) and subsequent adjustment to pH 2.0 yielded insoluble material which was removed by centrifugation, then suspended in 10 ml of H_2O. The pH was adjusted to 8.0 with 1.0 N NaOH. Nagarse (1 ml, stock solution, 10 mg in 1 ml of 0.1 M ammonium acetate, pH 8.5) was added and the mixture stirred at pH 8.5 for 1 hr at 25° in the pH-Stat. At this time, an additional 0.5 mg of enzyme was added. The reaction was terminated after an additional 3 hr by the addition of 6 N HCl to pH 2.0. The insoluble material formed was removed by centrifugation.

Purification of Peptides. The soluble peptides obtained from the peptic and Nagarse digests were fractionated as described previously (Bradshaw *et al.*, 1969c) on 2.0 × 25 cm columns of Dowex 50-X8. Soluble tryptic peptides (prepared as described previously by Bradshaw *et al.*, 1971a) were fractionated on a 1.5 × 51 cm column of SE-Sephadex C-25 at 55°. The conditions for this separation were the same as reported previously (Walsh *et al.*, 1970).

Determination of Tryptophan Content of Carboxypeptidase A. Carboxypeptidase A (Anson) was dissolved in 10 M urea, pH 2.7, and treated in triplicate with 2-hydroxy-5-nitrobenzyl bromide by the method of Barman and Koshland (1967). After separating the protein from reagents, each reaction mixture was analyzed in duplicate, both for the content of tryptophan derivative and for protein concentration. The tryptophan derivative was determined spectrophotometrically at 410 nm according to Barman and Koshland, and the protein concentration by amino acid analysis using norleucine as an internal standard, as described by Walsh and Brown (1962).

Other methods employed were the same as those already described (Bradshaw *et al.*, 1969a).

Results

Isolation of the Soluble Peptic Peptides. The elution profile for the separation of the soluble peptic peptides of F_1 on Dowex 50-X8 at 55° is shown in Figure 1. As with the other peptides isolated from F_1 (Bradshaw *et al.*, 1971a,b), purification of the pools was carried out on columns of Dowex 1-X2 and Dowex 50-X2. In view of the fact that several of the peptides isolated gave redundant information, only those peptides which yielded new information concerning the structure were characterized.

Characterization of Selected Peptic Peptides. FRACTION I. This fraction was purified on Dowex 1-X2 and yielded 15 pools. Two peptides, Pp-I-6[1] and Pp-I-9, whose compositions are listed in Table I, were characterized. Since, as shown in Table II, both peptides were electrophoretically neutral, the amide assignments for both were established.

[1] Abbreviations used are: Pp-, peptic peptide; Ng-, Nagarse peptide.

Pp-V-4	Pp-VI-5	Pp-VII-3	Pp-VIII-1	Pp-VIII-2	Pp-IX-1	Pp-IX-2	Pp-IX-3	Pp-XIII	Pp-Strip
0.95 (1)	2.31 (2)			1.08 (1)	1.02 (1)	1.00 (1)			1.06 (1)
	1.11 (1)	0.97 (1)	0.82 (1)					0.86 (1)	
	1.09 (1)							1.34 (1)	2.05 (2)
	2.06 (2)								
1.15 (1)	4.83 (5)							1.02 (1)	
	1.02 (1)			2.00 (2)	1.92 (2)	1.00 (1)	0.88 (1)	1.15 (1)	2.06 (2)
0.99 (1)	4.93 (5)	2.68 (3)	2.74 (3)	1.42 (1)	2.89 (3)	1.98 (2)		1.45 (1)	3.86 (4)
1.02 (1)	2.10 (2)	1.08 (1)	1.25 (1)		0.92 (1)			0.94 (1)	
	1.20 (1)								
	4.96 (5)			1.37 (1)	3.20 (3)	1.20 (1)			
	5.22 (5)				1.00 (1)				
2.77 (3.5)[c]	2.10 (2)								0.95 (1)
0.25 (0.5)[c]		0.93 (1)	0.86 (1)	1.89 (2)	3.05 (3)[b]				
		2.05 (2)	2.13 (2)			1.30 (1)	0.94 (1)	1.04 (1)	1.00 (1)
	2.06 (2)	1.10 (1)	1.08 (1)	1.62 (2)	2.71 (3)	0.96 (1)			
	1.04 (1)							0.83 (1)	
	0.40 (1)						1.18 (1)		0.60 (1)
8	36	9	9	9	17	7	3	8	12
20	10	12	10	10	7	6	45	33	12
D X1	D X1	D X1	D X1	D X1	D X1				
174–181	138–173	193–201	194–202	238–246	238–254	231–237	293–295	118–125	126–137

drolysates. [c] See text for discussion. [d] Abbreviations used are: D X1, Dowex 1-X2; D X50, Dowex 50-X2.

FRACTION II. Purification of this fraction on Dowex 1-X2 yielded two peptides, Pp-II-1 and Pp-II-3, whose compositions are listed in Table I. Peptide Pp-II-1 contained one residue of homoserine and thus can be identified as the carboxyl-terminal peptide. Peptide Pp-II-3 contained 17 residues, nine of which were positioned by Edman degradation. The results are summarized in Table II.

FRACTION III. Purification of this pool on Dowex 1-X2 gave several minor and one major peptide, Pp-III-1. Two rounds of Edman degradation allowed specific placement of the peptide. The behavior in high-voltage electrophoresis indicated that the Asx and Glx residues were both present in the amide form (Table III). It is noteworthy that this peptide represents the Ala form of the peptide covering the Glu/Ala allotypic replacement site (Pétra *et al.*, 1969).

FRACTION IV. Although this fraction yielded several peptides, only Pp-IV-2 and Pp-IV-3 supplied new information regarding the structure. The composition of these peptides is given in Table I. One round of Edman degradation was carried out on Pp-IV-2 to position it in the final sequence. Four rounds of Edman degradation on Pp-IV-3 also allowed the unequivocal assignment of this peptide in the final sequence. The partial structure of Pp-IV-3 is given in Table III.

FRACTION V. Chromatography of fraction V on Dowex 1-X2 produced two useful peptides, Pp-V-3 and Pp-V-4. As indicated in Table I, peptide Pp-V-4 contained fractional values of valine and isoleucine because of the Ile/Val replacement present in this segment (Pétra *et al.*, 1969). By the same reasoning used for peptide Th-XI-4 (Bradshaw *et al.*, 1971b), the yields of these residues should be 2.75 of valine and 0.25 of isoleucine as compared to the observed valnes of 2.77 and 0.25. The structural analyses of these peptides are described in Table III.

FRACTION VI. This pool was purified on Dowex 1-X2 and yielded one peptide of value to the sequence analysis. One round of Edman degradation (Table IV) confirmed the positioning of this peptide whose composition showed that it bridged both half-cystinyl residues.

FRACTION VII. One peptide, Pp-VII-3, was derived from the fractionation of this pool on Dowex 1-X2. This nonapeptide was subjected to six successful Edman degradations, as shown in Table IV.

FRACTION VIII. Two peptides were isolated from this fraction after chromatography on Dowex 1-X2. Peptide Pp-VIII-1 contained nine residues, four of which were placed in sequence by Edman degradation. Peptide Pp-VIII-2 also contained nine residues. The initial five were established by Edman degradation. These data are also given in Table IV. The presence of two residues of isoleucine in peptide Pp-VIII-2 was confirmed by 72-hr hydrolysis.

FRACTION IX. Three peptides were isolated from this pool after purification on Dowex 1-X2. Peptide Pp-IX-1 contained 17 amino acids and was positioned by a single Edman degradation. Pp-IX-2, a heptapeptide, was sequenced for three rounds, and Pp-IX-3, a tripeptide, was completely structured by Edman degradations and carboxypeptidase A hydrolysis. These data are summarized in Table V. As with peptide Pp-VIII-2, the content of isoleucine was obtained from a 72-hr hydrolysate.

FRACTION XIII. This pool contained a single peptide and did

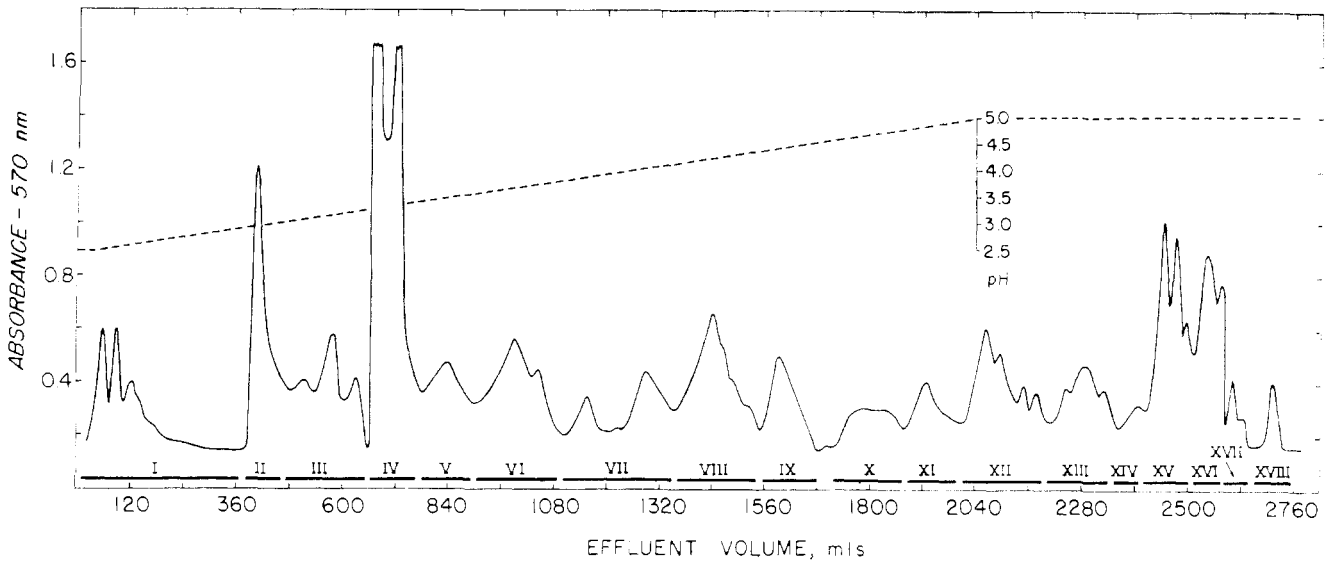


FIGURE 1: Elution profile of the soluble peptic peptides from fragment F_1 of bovine carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. The column was developed at 80 ml/hr with a double linear gradient of pyridine–acetic acid, as described in the text. Fractions of 6.0 ml were collected 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.

not require purification. One residue was released by digestion with carboxypeptidase A and one round of Edman degradation gave amino-terminal phenylalanine. These data are given in Table V.

STRIP. This pool, obtained by stripping the column with 2.5 M trimethylamine, contained a single very basic peptide. Two rounds of Edman degradation supplied the information necessary to position the peptide in the sequence.

Isolation of the Soluble Nagarse Peptides from the Tryptic-Insoluble Core. The acid-insoluble precipitate, isolated from the tryptic digest, was digested with Nagarse. This procedure was only partially successful since acid-insoluble material remained at the end of the second digest. Resolution was further complicated by the presence of soluble tryptic peptides in the insoluble core from the tryptic digestion. The soluble Nagarse peptides were fractionated on a column of Dowex 50-X8 with pyridine–acetic acid buffer, as shown in Figure 2.

Characterization of Selected Nagarse Peptides. FRACTION II. Purification of this fraction on Dowex 1-X2 yielded several peptides, three of which were of value for the sequence analysis. Ng-II-5, a heptapeptide whose composition is given in

TABLE II: Characterization of Selected Peptic Peptides from Fractions I and II.[a]

Fraction I:
Peptide Pp-I-6:
Sequence: (Leu,Pro,Ala,Ser,Gln,Ile,Pro,Thr,Ala,Gln)
HVE, pH 6.5: Neutral
Peptide Pp-I-9:
Sequence: Ile-(Val,Thr,Asn,Pro,Asn,Gly)-Phe-Ala
Carboxypeptidase A: 30 min; Ala, 0.40; Phe, 0.31
3 hr; Ala, 0.91; Phe, 0.80
HVE, pH 6.5: Neutral

Fraction II:
Peptide Pp-II-1:
Sequence: Gly-Val-Leu-Thr-Ile-HSer
Peptide Pp-II-3:
Sequence: Leu-Leu-Tyr-Pro-Tyr-Gly-Tyr-Thr-Thr-(Glx,Ser,Ile,Pro,Asx,Lys,Thr,Glx,Leu)
HVE, pH 6.5: Acidic

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

TABLE III: Characterization of Selected Peptic Peptides from Fractions III, IV, and V.

Fraction III:
Peptide Pp-III-1:
Sequence: Asn-Gln-(Val,Ala,Lys,Ser,Ala,Val,Ala,Ala,-Leu)
HVE, pH 6.5: Basic

Fraction IV:
Peptide Pp-IV-2:
Sequence: Leu-(Glx,Ile,Val,Thr,Asx,Pro,Asx,Gly,Phe,-Ala,Phe,Thr,His,Ser,Glx,Asx,Arg,Leu)
Peptide Pp-IV-3:
Sequence: Phe-Glx-Leu-Arg(Asx,Thr,Gly,Arg,Tyr,Gly,-Phe,Leu,Leu,Pro,Ala,Ser,Glx,Ile,Ile,Pro,Thr,-Ala,Glx,Glx)

Fraction V:
Peptide Pp-V-3:
Sequence: Ile-Tyr-Gln-Ala-(Ser,Gly,Gly,Ser)
HVE, pH 6.5: Neutral
Peptide Pp-V-4:
Sequence: Val-Glu-Val-Lys(Ser,Val/Ile,Val,Asp)
HVE, pH 6.5: Acidic

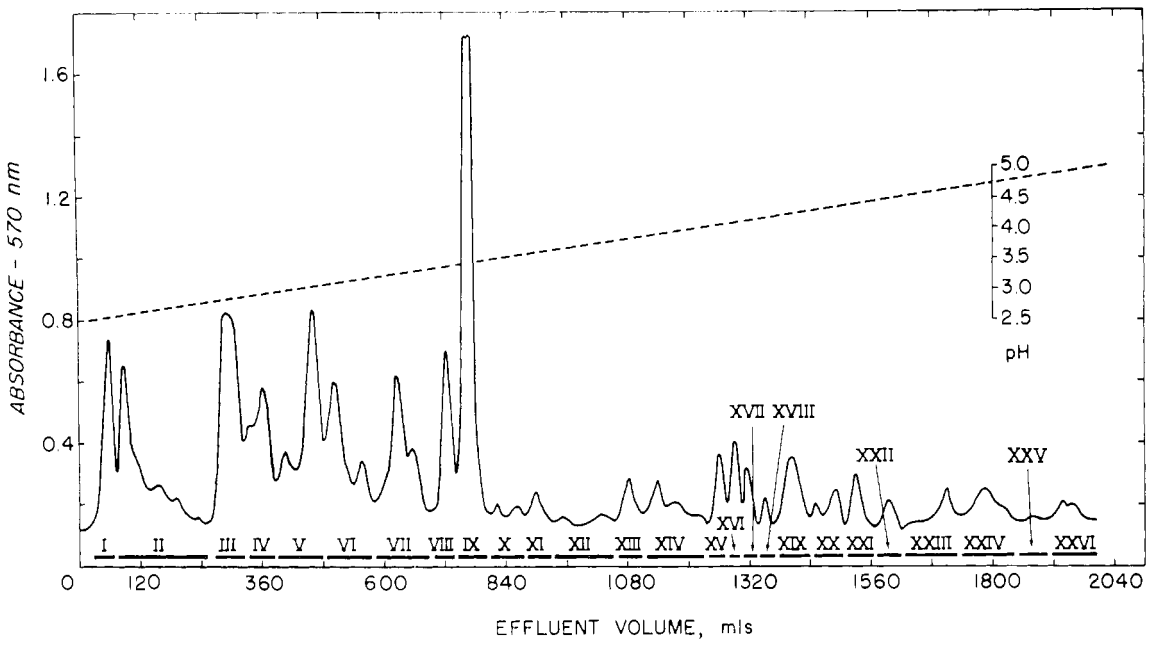


FIGURE 2: Elution profile of the soluble Nagarse peptides derived from the tryptic insoluble peptides of fragment F_I of bovine carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. Details as in Figure 1.

Table VI, was completely structured by Edman degradation. The content of isoleucine was established by 72-hr hydrolysis. Peptides Ng-II-9 and Ng-II-11 were also completely structured, as shown in Table VII. Peptide Ng-II-11 is derived from the region also covered by soluble tryptic peptides.

FRACTION III. This fraction was further treated on Dowex 1-X2 to yield several fragments. One pool, Ng-III-4, was also purified on a column of Dowex 50-X2 to yield a single useful peptide, Ng-III-4-3. The characterization of this peptide as well as that of peptide Ng-III-9 is shown in Table VII.

FRACTION IV. Purification of this pool on Dowex 1-X2 produced one useful peptide, Ng-IV-4. Two rounds of Edman degradation were sufficient to provide the total sequence.

FRACTION IX. This pool contained a single peptide, Ng-IX. The composition of this tetrapeptide is given in Table VI and the sequence analysis in Table VII.

FRACTION XVII. This pool contained a single tetrapeptide, Ng-XVII. Two rounds of Edman degradation were performed, as described in Table VII.

Purification of the Soluble Tryptic Peptides of Fragment F_I on SE-Sephadex. After construction of a tentative sequence from portions of the data described in this and the preceding reports (Bradshaw *et al.*, 1971a,b), it appeared that two tryptic peptides, covering residues 125–127 and 265–272, were not recovered from the Dowex 50-X8 column although they should

TABLE IV: Characterization of Selected Peptic Peptides from Fractions VI, VII, and VIII.

Fraction VI:
Peptide Pp-VI-5:
Sequence: CM-Cys-(Val,Gly,Val,Asx,Ala,Asx,Arg,Asx, Trp,Asx,Ala,Gly,Phe,Gly,Lys,Ala,Gly,Ala,-Ser,Ser,Ser,Pro,CM-Cys,Ser,Glx,Thr,Tyr,-His,Gly,Lys,Tyr,Ala,Asx,Ser,Glx)

Fraction VII:
Peptide Pp-VII-3:
Sequence: Leu-Ser-Ile-His-Ser-Tyr(Ser,Glx,Leu)

Fraction VIII:
Peptide Pp-VIII-1:
Sequence: Ser-Ile-His-Ser-(Tyr,Ser,Glx,Leu,Leu)
Peptide Pp-VIII-2:
Sequence: Tyr-Lys-Tyr-Gly-Ser-(Ile,Ile,Thr,Thr)

TABLE V: Characterization of Selected Peptic Peptides from Fractions IX and XIII.

Fraction IX:
Peptide Pp-IX-1:
Sequence: Tyr(Lys,Tyr,Gly,Ser,Ile,Ile,Thr,Thr,Ile,Tyr,-Glx,Ala,Ser,Gly,Gly,Ser)
Peptide Pp-IX-2:
Sequence: Lys-Ser-Leu(Tyr,Gly,Thr,Ser)
Peptide Pp-IX-3:
Sequence: Thr-Trp-Leu
Carboxypeptidase A: 30 min: Leu, 0.89

Fraction XIII:
Peptide Pp-XIII:
Sequence: Phe-(Thr,His,Ser,Glx,Asx,Arg)-Leu
Carboxypeptidase A: 30 min: Leu, 0.63

Fraction Strip:
Peptide Pp-Strip:
Sequence: Trp-Arg-(Lys,Thr,Arg,Ser,Val,Thr,Ser,Ser,-Ser,Leu)

TABLE VI: Amino Acid Composition of Selected Nagarse Peptides from Fragment F_I.[a]

Amino Acid	Ng-II-5	Ng-II-9	Ng-II-11	Ng-III-4-3	Ng-III-9	Ng-IV-4	Ng-IX	Ng-XVII
Lysine								
Histidine								
Arginine								
S-Carboxymethylcysteine			0.65 (1)					
Aspartic acid		2.08 (2)			1.02 (1)			
Threonine	1.83 (2)	0.98 (1)	1.11 (1)				1.10 (1)	1.00 (1)
Serine	1.05 (1)		2.95 (3)			0.98 (1)	0.85 (1)	
Glutamic acid			1.19 (1)		1.00 (1)	1.03 (1)		
Proline		0.81 (1)	0.86 (1)	0.86 (1)				
Glycine	1.07 (1)	1.02 (1)		1.13 (1)				1.20 (1)
Alanine				1.06 (1)				
Valine								
Isoleucine	1.93 (2)[b]				0.74 (1)			
Leucine				1.98 (2)	0.98 (1)	2.72 (3)		1.00 (1)
Tyrosine	0.69 (1)		0.84 (1)	0.72 (1)			1.01 (1)	
Phenylalanine		1.01 (1)		0.96 (1)	0.74 (1)		0.95 (1)	
Tryptophan								0.60 (1)
Homoserine and lactone								
Total	7	6	8	7	5	5	4	4
% yield[c]	5	20	18	5	47	17	33	8
Purification procedure	D X1	D X1	D X1	D X1; D X50	D X1	D X1		
Residue No.	240–246	111–116	158–165	277–283	104–108	199–203	265–268	293–296

[a] Values are given in residues/mole. The assumed integral values are given in parentheses. [b] Values obtained from 72-hr hydrolysates. [c] Yield calculated on the basis of the amount of material taken for the tryptic digest.

TABLE VII: Characterization of Selected Nagarse Peptides from Fractions II, III, IV, IX, and XVII.

Fraction II:
Peptide Ng-II-5:
Sequence: Tyr-Gly-Ser-Ile-Ile-Thr-Thr
Peptide Ng-II-9:
Sequence: Thr-Asx-Pro-Asx-Gly-Phe
Peptide Ng-II-11:
Sequence: Ser-Ser-Pro-CM-Cys-Ser-Glu-Thr-Tyr
Carboxypeptidase A: 30 min: Tyr, 0.92

Fraction III:
Peptide Ng-III-4-3:
Sequence: Tyr-Gly-Phe-Leu-(Leu,Pro,Ala)
Peptide Ng-III-9:
Sequence: Asx-Ile-Phe-Leu-Glx

Fraction IV:
Peptide Ng-IV-4:
Sequence: Ser-Glx-Leu-Leu-Leu

Fraction IX:
Peptide Ng-IX:
Sequence: Tyr-Ser-Phe-Thr

Fraction XVII:
Peptide Ng-XVII:
Sequence: Thr-Trp-(Leu,Gly)

have been present in the soluble fraction. Consequently, the soluble portion of a tryptic digest, prepared as described previously (Bradshaw *et al.*, 1971a), was applied to a 2.0 × 53 cm column of Sephadex G-25 equilibrated in 0.1 N HAc (Figure 3). The second fraction, II*, was recovered by lyophilization and fractionated on a column of SE-Sephadex employing pyridine acetate buffers (Walsh *et al.*, 1970). The elution profile is shown in Figure 4. In view of the fact that the desired peptides contained tyrosine or tryptophan, each fraction was pooled, examined by high-voltage electrophoresis on paper, and then sprayed with reagents specific for these residues. Fraction II*-9 contained a single tryptophan-positive peptide which proved to be the one that was sought. A single round of Edman degradation and hydrolysis by carboxypeptidase B gave a sequence of Leu-Trp-Arg. The remaining fractions containing tyrosine were fractionated on Dowex 1-X2 and examined by high-voltage electrophoresis and amino acid analysis. However, none of the pools contained the missing tyrosyl peptide.

Tryptophan Content of Carboxypeptidase A_γ. Triplicate analyses with 2-hydroxy-5-nitrobenzyl bromide, as described in Methods, yielded 6.68, 6.50, and 6.67 moles of tryptophan per mole of protein. Although these measurements are in conflict with earlier spectrophotemetric measurements which indicated 7.72–7.95 moles of tryptophan per mole of protein (Bargetzi *et al.*, 1963), they are more nearly in agreement with the seven tryptophans found by sequence analysis (four in F_I and three in F_{III}).

Sequence Analysis of Fragment F_I in the Protein Sequenator. Approximately 200 nmoles of fragment F_I was suspended in 0.5 ml of 50% acetic acid and dried in the cup of a Beckman Sequencer. Eight cycles of the Edman degradation were car-

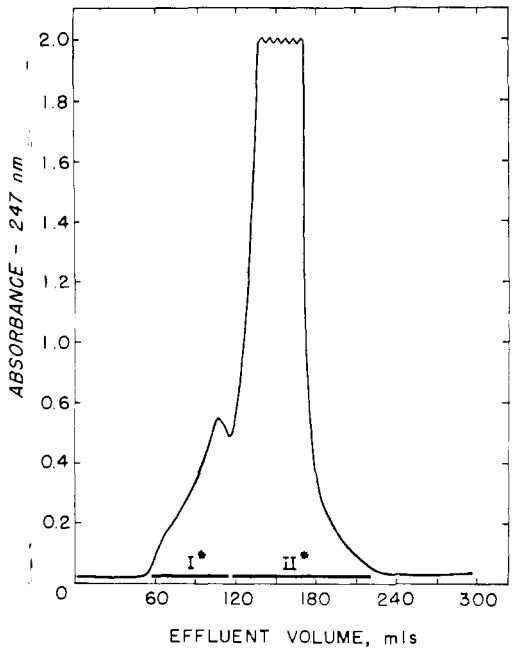


FIGURE 3: Elution profile of the soluble tryptic peptides of fragment F_I on a 2.0 × 53 cm column of Sephadex G-25 equilibrated in 0.1 N acetic acid. The column was developed at 30 ml/hr and 4.6 ml fractions were collected. The tubes were monitored at 247 nm.

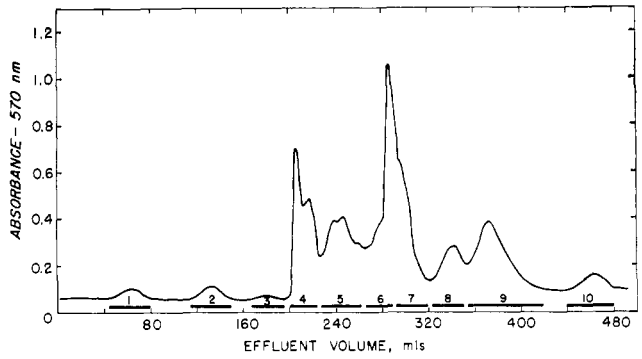


FIGURE 4: Elution profile of the separation of peptide T-II*-2 on a 2.0 × 52 cm column of SE-Sephadex at 55°. The column was developed at 40 ml/hr. Fractions of 2.0 ml were collected. The gradient composition and volumes were the same as in Figure 1.

ried out following the general technique of Edman and Begg (1967). The automatic programming sequence recommended by Beckman Instruments resulted in a 97% stepwise yield. Dithioerythritol was included in the chlorobutane extractant as recommended by Hermodson *et al.* (1970) to improve the PTH-amino acid recoveries. The PTH-amino acids released were identified by gas–liquid chromatography by a method similar to that described by Pisano and Bronzert (1969). The following amino acid sequence was established: Asp-Ile-Phe-Leu-Glu-Ile-Val-Thr-.

Discussion

Assembly of the Complete Sequence. From the data described in this and the preceding papers (Bradshaw *et al.*, 1971a,b), it is possible to construct the complete amino acid sequence of fragment F_I of bovine carboxypeptidase A. All of the data used in this calculation are summarized in Figure 5. The peptides corresponding to each segment are listed below the designated residues in the order of hydrolysis by trypsin, chymotrypsin, thermolysin, pepsin, and Nagarse, respectively.

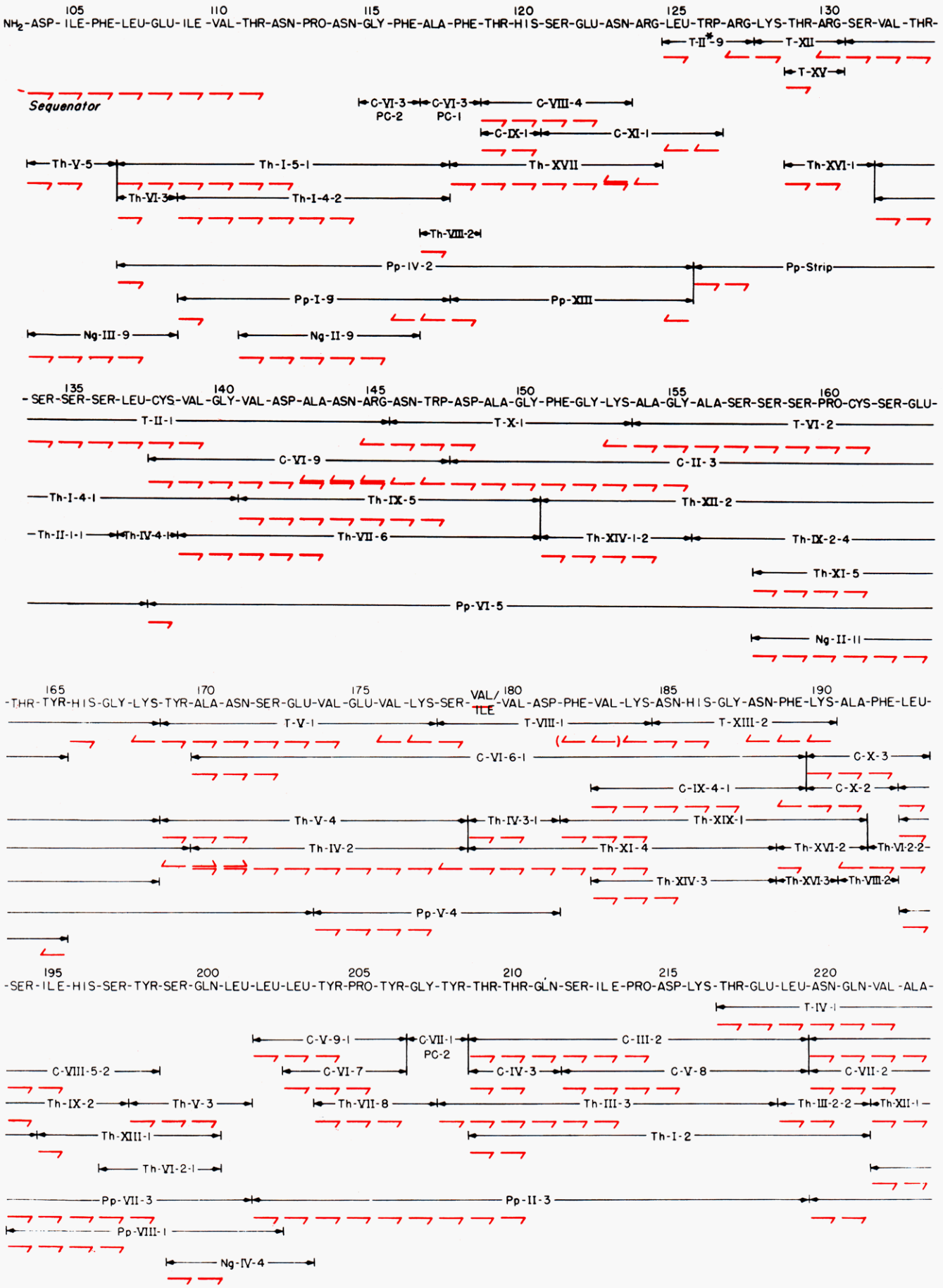
The logic for assembling this structure is summarized below. Independent analyses have indicated that the amino-terminal residue of this fragment is aspartic acid (Nomoto *et al.*, 1969) and that its position in carboxypeptidase Aα is 104. The assignment of Ng-III-9 and Th-V-5 to the amino terminus of F_I is most readily confirmed by the amino acid sequenator which established the sequence of the first eight residues and obviated the necessity for further proof of the Leu-Glu overlap to Th-I-5-1. This peptide, along with T-I-4-2, extends the sequence to Ala 117 and, by addition of Pp-I-9 and Ng-II-9, provides sequence data for all of the intervening residues.

The overlap to Pp-XIII and Th-XVII was proven by the composition of Pp-IV-2. This peptide was anchored at the amino terminus by residue 107 and the exact fit to the overlapping peptides secures this region. The growing segment can be extended to Trp 126 by C-XI-1 to provide a two-residue overlap to T-II*-9. The reliability of this overlap rests in part on the small number of tryptophan residues which can be uniquely positioned throughout the structure (*vide infra*). The adjacent region can be extended by the Pp strip which overlaps the Trp-Arg sequence of T-II*-9 and accommodates T-XII and part of T-II-1.

The segment from residue 131 to 161 can be completely assembled by using only the tryptic and chymotryptic peptides, as described previously (Bradshaw *et al.*, 1971a). The information to complete the order of residues in T-VI-2, extending to position 168, is provided by Ng-II-11. The correctness of this structure is further proven by Pp-VI-5, which extends from 138 to 173. This latter peptide also provides the overlap to T-V-1, C-VI-6-1, Th-V-4, and Th-IV-2, all of which contribute to the next segment. The overlap between T-V-1 and T-VIII-1 and between Th-IV-2 and Th-XI-4 is provided by both Pp-V-4 and C-VI-6-1. The information in T-XIII-2, C-IX-4-1, Th-XIX-1, and Th-XIV-3 extends the sequence to Lys 190. Ala 191 can be placed by Th-XVI-2 and also by difference in composition between Th-XIX-1 and T-XIII-2. This provides a two-residue overlap to C-X-3 and extends the sequence to Leu 193. Another two-residue overlap to Th-VI-2-2 extends the sequence to Ser 194, which provides the basis for attaching Pp-VII-3, Th-IX-2, and C-VIII-5-2. By sequence, Pp-VII-3 extends the structure to Tyr 198 and, by composition, to Leu 201. Pp-VIII-1 indicates that Leu 201 is followed by Leu 202. Th-V-3 and Ng-IV-4 provide the necessary sequence data to reach Leu 203 and finally prove the Leu-Leu-Leu sequence. The overlap to Ng-IV-4 is provided by Pp-II-3 which brings the structure to Leu 219. Several other peptides provide the internal sequence data beyond Thr 210.

Five peptides (C-III-2, Pp-II-3, C-V-8, Th-III-3, and Th-I-2) provide the overlap to peptide T-IV-1 and thus extend the sequence to Lys 225. The information provided by C-IV-1 and T-VI-1 (or T-VII) establishes sequence proof up to Lys 231. This region includes the allotypic replacement site at residue 228. Th-XIV-1-1 establishes the position of Ser 232 and also furnishes a two-residue overlap to Pp-IX-2. The remainder of the proof, up to Lys 239, is provided by T-XI-3 and Th-XIV-1-3.

Pp-VIII-2 extends the sequence beyond Lys 239. Since the composition of Pp-IX-1 equals the sum of Pp-VIII-2 and Pp-V-3, the latter peptide must follow Pp-VIII-2 from 247 to 254. Ng-II-5 provides the intermediate sequence data for residues


105 110 115 120 125 130
NH2-ASP-ILE-PHE-LEU-GLU-ILE-VAL-THR-ASN-PRO-ASN-GLY-PHE-ALA-PHE-THR-HIS-SER-GLU-ASN-ARG-LEU-TRP-ARG-LYS-THR-ARG-SER-VAL-THR-
T-II*-9
T-XII
T-XV
Sequenator
C-VI-3
PC-2
C-VI-3
PC-1
C-VIII-4
C-IX-1
C-XI-1
Th-V-5
Th-I-5-1
Th-XVII
Th-XVI-1
Th-VI-3
Th-I-4-2
Th-VIII-2
Pp-IV-2
Pp-Strip
Pp-I-9
Pp-XIII
Ng-III-9
Ng-II-9
135 140 145 150 155 160
-SER-SER-SER-LEU-CYS-VAL-GLY-VAL-ASP-ALA-ASN-ARG-ASN-TRP-ASP-ALA-GLY-PHE-GLY-LYS-ALA-GLY-ALA-SER-SER-SER-PRO-CYS-SER-GLU-
T-II-1
T-X-1
T-VI-2
C-VI-9
C-II-3
Th-I-4-1
Th-IX-5
Th-XII-2
Th-II-1-1
Th-IV-4-1
Th-VII-6
Th-XIV-1-2
Th-IX-2-4
Th-XI-5
Pp-VI-5
Ng-II-11
165 170 175 VAL/ILE 180 185 190
-THR-TYR-HIS-GLY-LYS-TYR-ALA-ASN-SER-GLU-VAL-GLU-VAL-LYS-SER-VAL-ASP-PHE-VAL-LYS-ASN-HIS-GLY-ASN-PHE-LYS-ALA-PHE-LEU-
T-V-1
T-VIII-1
T-XIII-2
C-VI-6-1
C-X-3
C-IX-4-1
C-X-2
Th-V-4
Th-IV-3-1
Th-XIX-1
Th-IV-2
Th-XI-4
Th-XVI-2
Th-VI-2-2
Th-XIV-3
Th-XVI-3
Th-VIII-2
Pp-V-4
195 200 205 210 215 220
-SER-ILE-HIS-SER-TYR-SER-GLN-LEU-LEU-LEU-TYR-PRO-TYR-GLY-TYR-THR-THR-GLN-SER-ILE-PRO-ASP-LYS-THR-GLU-LEU-ASN-GLN-VAL-ALA-
T-IV-1
C-V-9-1
C-VII-1
PC-2
C-III-2
C-VIII-5-2
C-VI-7
C-IV-3
C-V-8
C-VII-2
Th-IX-2
Th-V-3
Th-VII-8
Th-III-3
Th-III-2-2
Th-XII-1
Th-XIII-1
Th-I-2
Th-VI-2-1
Pp-VII-3
Pp-II-3
Pp-VIII-1
Ng-IV-4

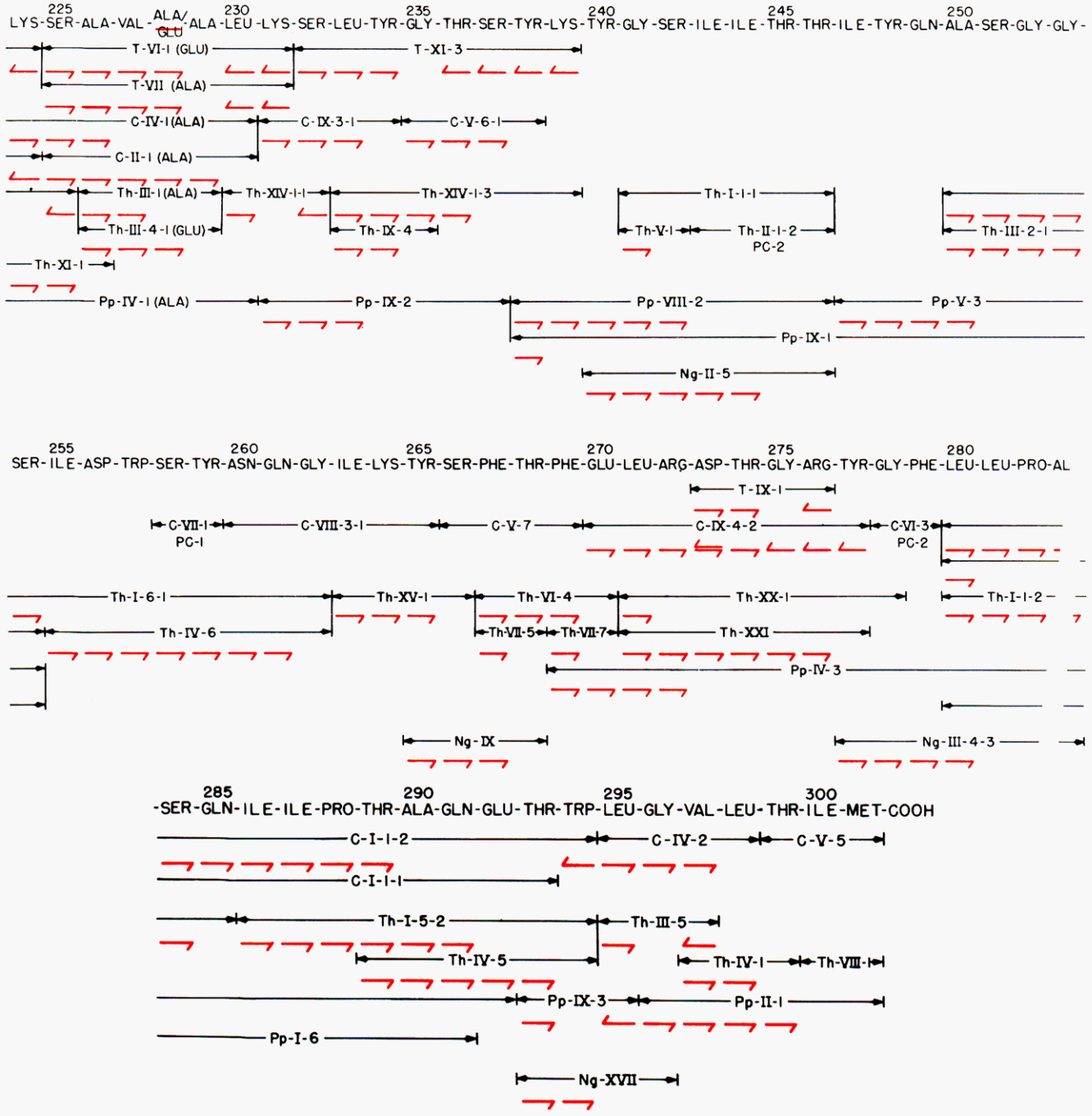


FIGURE 5: The amino acid sequence of fragment F_1 of bovine carboxypeptidase A. The various peptides are indicated by doi e-headed arrows. Residues identified by Edman degradation (→), leucine aminopeptidase hydrolysis (⇀), and carboxypeptidase A and/or ! ıydrolysis (←) are so indicated. Abbreviations used are: T-, tryptic: C-, chymotryptic: Th-, thermolytic: Pp-, peptic: and Ng-, Nagarse.

243 to 246. Th-III-2-1 is coincident with the C terminus of Pp-V-3 and establishes the sequence to Ser 254. The composition of Th-I-6-1 clearly links Th-IV-6 to Th-III-2-1 and defines the structure to Gly 262. C-VIII-3-1 gives a bridge from Th-IV-6 to Th-XV-1.

The terminal region (residues 263–301) can be built from a series of consistent two-residue overlaps provided consecutively by Th-XV-1, Ng-IX, Th-VI-4, and Pp-IV-3. From Pp-IV-3, which actually spans the entire region from 269 to 292, peptides C-IX-4-2, Ng-III-4-3, and C-I-1-2 extend the sequence to Thr 289 and the composition to Trp 294. The sequence data from 290 to 294 are supplied by Th-I-5-2 and Th-IV-5. Pp-IX-3 and Ng-XVII extend the sequence to Gly 296, giving a two-residue overlap to C-IV-2 which provides the final overlap to Pp-II-1, the carboxyl-terminal peptide of the fragment.

The structure produced by this analysis provides an unambiguous sequence of 198 amino acids. The amino acid composition calculated from this sequence is identical with that obtained from acid hydrolysates of the whole F_1 fragment (Nomoto *et al.*, 1969), an absolute requirement of any proposed sequence. Although proof of the structure does not depend on single residue overlaps, several two-residue overlaps were used. The two-residue overlaps occur with residues 107–108, 125–126, 126–127, 190–191, 192–193, 193–194, 202–203, 230–231, 231–232, 238–239, 265–266, 267–268, 269–270,

TABLE VIII: Acid and Amide Assignments of the Aspartic and Glutamic Acid Residues of Fragment F_1.

Residue No.	Assignment	Method of Determination[a]
104	Asp	HVE, Th-V-5
108	Glu	HVE, Th-I-5-1
112	Asn	HVE, Pp-I-9
114	Asn	HVE, Pp-I-9
122	Glu	HVE, C-VIII-4
123	Asn	CPA, Th-XVII
142	Asp	HVE, C-VI-9
144	Asn	CPA, C-VI-9
146	Asn	CPA, C-VI-9
148	Asp	HVE, T-X-1
163	Glu	HVE, T-VI-2
171	Asn	LAP, Th-IV-2
173	Glu	HVE, Th-IV-2
175	Glu	HVE, Th-IV-2
181	Asp	HVE, Th-IV-3-1
185	Asn	HVE, Th-XI-4
188	Asn	CPA, T-XIII-2
200	Gln	HVE, Th-V-3
211	Gln	HVE, C-IV-3
215	Asp	HVE, C-V-8
218	Glu	HVE, C-V-8
220	Asn	HVE, T-IV-1
221	Gln	HVE, T-IV-1
228	Glu	HVE, T-IV-1
249	Gln	HVE, Pp-V-3
256	Asp	HVE, Th-IV-6
260	Asn	HVE, Th-IV-6 C-2
261	Gln	HVE, Th-IV-6 C-2
270	Glu	HVE, C-IX-4-2
273	Asp	HVE, T-IX-1
285	Gln	HVE, Th-I-1-2
291	Gln	HVE, Pp-I-6
292	Glu	HVE, C-I-1-2

[a] Abbreviations: HVE, high-voltage electrophoresis at pH 6.5; CPA, carboxypeptidase A; LAP, leucine aminopeptidase.

293–294, and 295–296. An analysis of each of these indicates that only the proposed structure can be deduced.

Although no other Leu-Glu sequence occurs in fragment F_1, the overlap at 107–108 is confirmed by direct measurement using the protein sequenator. The overlap at residues 125–126, which involves a tryptophan residue, is easily proven by an examination of the environment of the other tryptophan residues at positions 147, 257, and 294. Trp 294 is also involved in a two-residue overlap but the proof by Pp-IX-3 that it is preceded by threonine and followed by leucine differentiates it from Trp 126, which (as shown by Tp-II*-9) is preceded by leucine and followed by arginine. Thus the two-residue overlaps at 125–126, 126–127, and 293–294 are unique.

The series of two-residue overlaps from 190 to 194 represents one of the more difficult areas in the sequence analysis of fragment F_1. The sequence Lys 190-Ala 191 is given by the combination of T-XIII-2 and Th-XIX and confirmed by Th-XVI-2. Lys 190 may be readily distinguished from all other lysines in the molecule. The only other lysine residue involved in a two-residue overlap is Lys 231 which is preceded by leucine (T-VI-1) and succeeded by serine (Th-XIV-1-1). Thus the three two-residue overlaps involving lysine (190–191, 230–231, and 231–232) can be distinguished in an unequivocal manner.

Following the assignment of C-X-3 to give residues Phe 192–Leu 193, an examination of the remaining phenylalanine residues indicated that none is involved in two-residue overlaps with leucine. The two other Phe-Leu sequences present (106–107 and 279–280) are found in unique segments. This confirmation allows the placement of Th-VI-2-2 to bring the sequence to Leu 193–Ser 194. Since no other Leu-Ser sequence is found in the fragment, this overlap to the adjoining peptides is definite and the region 190–194 is unambiguous.

The two-residue overlap at 202–203 represents the most difficult assignment in the molecule. The extension of Pp-VIII-1 and Th-V-3 by Ng-IV-4 proves a sequence of Leu-Leu-Leu for residues 201–203. The assignment of Pp-II-3 (or C-V-9-1), both of which begin with a Leu-Leu sequence, does not preclude the possibility of an additional leucine residue in this segment. The only argument against a Leu_4 structure is the unambiguous assignment of all of the leucine residues defined by the composition. The only other peptide beginning with a Leu-Leu sequence, C-I-1-1 at position 280–293, can be barred from this region by the overlap of Ng-III-4-3.

The overlap at Tyr 238–Lys 239 involves the only Tyr-Lys sequence in the molecule and is, therefore, unique. The two-residue overlaps from 265 to 270 involve Tyr-Ser, Phe-Thr, and Phe-Glu. The only other Tyr-Ser sequence occurs at 198–199. Whereas the assignment of Th-V-3 to this latter region could be ambiguous, the combination of Th-XIII-1, Pp-VII-3, and Ng-IV-4 proves the proposed structure. An additional Phe-Thr sequence occurs at 118–119 and could cast some doubt on the assignment of Th-XVII and Pp-XIII. The overlap of Pp-IV-2, however, precludes any ambiguity in this region. There is no other Phe-Glu sequence except at residues 269–270.

The establishment of Leu 295–Gly 296 by Ng-XVII creates a two-residue overlap to C-IV-2. The absence of any other Leu-Gly sequence removes the last obstacle to an unambiguous structure.

Assignment of the Amide Residues. Either high-voltage electrophoresis at pH 6.5 or direct identification on the amino acid analyzer of hydrolysates by leucine aminopeptidase or carboxypeptidase A was employed to distinguish aspartic and glutamic acid residues from their respective amide forms. Electrophoretic mobility was only used to distinguish between acidic, neutral, or basic peptides. If more than one positive or negative charge could exist, the identification was made by an additional method. The summary of these assignments is listed in Table VIII. Asp 104 is acidic as evidenced by direct measurement of Th-V-5. Since both 112 and 114 are asparagine, the acidic mobility of Th-I-5-1 proves 108 to be Glu. Th-VI-3, the dipeptide assigned to these residues, is also acidic. Residue 123 was identified as Asn from a carboxypeptidase A digest, thus allowing the identification of Glu 122 from the mobility of C-VIII-4. Both residues 144 and 146 were identified as Asn by hydrolysis with carboxypeptidase A, thus permitting the assignment of 142 as Asp from the acidic mobility of C-VI-9 (the *S*-carboxymethylcysteine residue contributed one negative charge in this peptide). With the identification of 146, 148 could be determined as Asp from the mobility of T-X-1. Residue 163 was identified as Glu from the mobility of T-VI-2, and 171 was assigned as Asn from the direct identification given by the leucine aminopeptidase digest of Th-IV-2. From this latter assignment, both 173 and 175 could

be identified as being in the acid form due to the mobility of Th-IV-2. Residue 181 was shown to be Asp by the mobility of Th-IV-3-1. Residue 188 was identified as Asn from carboxypeptidase A digests, which then allowed the identification of 185 from the mobility of Th-XI-4. Residue 200 was assigned as Gln from high-voltage electrophoresis of Th-V-3, and 211 was Gln from high-voltage electrophoresis of C-IV-3. Both residues 215 and 218 were demonstrated to be in the acidic form from the mobility of C-V-8. From the known nature of 218, the assignment of 220 as Asn and 221 as Gln could be made from the mobility of T-IV-1. Residue 228, one of the allotypic replacement sites, has already been shown to be in the acid form, thus providing a rational explanation for the chromatographic separation of the Val and Leu allotypes of carboxypeptidase A (Pétra *et al.*, 1969). Residue 249 was shown to be Gln from the mobility of Pp-V-3. Residue 256 was identified as acidic from the mobility of Th-IV-6 after demonstration that 260 and 261 were in the amide form in the chymotryptic subpeptide (Asn-Gln-Gly) of Th-IV-6. Residue 273 was identified as Asp from the mobility of T-IX-1, which then allowed the assignment of residue 270 as Glu from the mobility of C-IX-4-2. The assignment of 285 as Gln from the mobility of Th-I-1-2, and 291 as Glu from the mobility of Pp-I-6, allowed the identification of 292 as Glu from the acidic nature of C-I-1-2.

Of the 17 residues of aspartic acid, 10 are found in the amide form, whereas 7 of the 16 glutamic acid residues are present as glutamine. In all, 17 of the 33 residues (or about half) are present as the neutral amide form in this fragment.

Comments on the Complete Sequence of Bovine Carboxypeptidase A. The completion of the sequence analysis of fragment F_I supplies the last link in the determination of the complete amino acid sequence of bovine carboxypeptidase A. Incorporation of these data into the partial structure given by fragments F_N, F_C, and F_{III} (Sampath Kumar *et al.*, 1964; Bargetzi *et al.*, 1964; Bradshaw *et al.*, 1969a; Bradshaw, 1969) yields an uninterrupted array of 307 amino acids for the α form of carboxypeptidase A (Bradshaw *et al.*, 1969b).

Analysis of the charged groups indicates that 17 of the 29 aspartate groups occur as asparagine while only 11 of the 25 glutamate residues (excluding the allotypic replacement site at 228) occur as glutamine. Thus in the Val form of the enzyme (containing Ala at 228), 28 of the 54 side-chain carboxyl groups are in the amide form and 26 are in the acid form. Interestingly, this is exactly equal to the number of lysyl and arginyl residues present in carboxypeptidase A_α^{Val} and suggests that the isoionic point of this enzyme should be near neutrality.

An analysis of the results obtained from X-ray analysis of this enzyme (Lipscomb *et al.*, 1968) viewed against the completed sequence has been reported (Bradshaw *et al.*, 1969b). In turn, Lipscomb *et al.* (1969) have reported a refinement of their data and have criticized the sequence analysis at the following points. They find that the assignments of Asn, Phe, and Thr at positions 93, 151, and 245 are inconsistent with their interpretations and favor Glx, Trp, and Ser in their places. In addition, they comment that Glu 108 must be in contact with an H_3O^+ molecule if its side-chain assignment is correct. The correctness of the assignments of 151 and 108 has been substantiated by independent analyses using special digests in the first case (Bradshaw *et al.*, 1971a) and data from the protein sequenator in the second. Only residues 93 and 245 have not been independently examined to determine if they represent further sites of allotypic replacements. The unlikely nature of this possibility—at least in the case of position 93—is seen from the yields of the tryptic and chymotryptic peptides covering this region in F_{III} prepared from unchromatographed enzyme (Bradshaw *et al.*, 1969a). The tryptic peptides were found in 49% yield and the chymotryptic peptides in 56% yield, indicating that the Asn form would have to have been recovered in near perfect yield and the Glx form totally lost for this situation to have gone undetected. Thus it would appear that the ambiguities reside in the interpretation of the electron density maps rather than in the chemical analyses.

It is noteworthy, however, that electron density maps are extremely useful in providing alternate kinds of information regarding the alignment of peptides. Such maps can readily remove the type of ambiguity that arises in the assignment of overlaps, *i.e.*, the condition encountered at residues 202–203. Future analyses should profit greatly from the combined use of X-ray diffraction and chemical sequence methods for the expeditious determination of protein structures.

Acknowledgments

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References

Bargetzi, J.-P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Bargetzi J.-P., Thompson, E. O. P., Sampath Kumar, K. S. V., Walsh, K. A., and Neurath, H. (1964), *J. Biol. Chem. 239*, 3767.

Barman, T. E., and Koshland, D. E., Jr. (1967), *J. Biol. Chem. 242*, 5771.

Bradshaw, R. A. (1969), *Biochemistry 8*, 3871.

Bradshaw, R. A., Babin, D. R., Nomoto, M., Srinivasin, N. G., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969a), *Biochemistry 8*, 3859.

Bradshaw, R. A., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969b). *Proc. Nat. Acad. Sci. U. S. 63*, 1389.

Bradshaw, R. A., Garner, W. H., and Gurd, F. R. N. (1969c), *J. Biol. Chem. 244*, 2149.

Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1971a), *Biochemistry 10*, 938.

Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1971b), *Biochemistry 10*, 951.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1*, 80.

Hermodson, M. A., Ericsson, L. H., and Walsh, K. A. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 728.

Lipscomb, W. N., Hartsuck, J. A., Quiocho, F. A., and Reeke, G. N., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 28.

Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Jr., Quiocho, F. A., Bethge, P., Ludwig, M. L., Steitz, T. A., Muirhead, H., and Coppola, J. C. (1968), *Brookhaven Symp. Biol. 21*, 24.

Nomoto, M., Srinivasin, N. G., Bradshaw, R. A., Wade, R. D., and Neurath, H. (1969), *Biochemistry 8*, 2755.

Pétra, P. H., Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 2762.

Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 20*, 5597.

Sampath Kumar, K. S. V., Clegg, J. B., and Walsh, K. A. (1964), *Biochemistry 3*, 1728.

Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58*, 596.

Walsh, K. A., Ericsson, L. H., Bradshaw, R. A., and Neurath, H. (1970), *Biochemistry 9*, 219.

Bradykinin-Potentiating Peptides from the Venom of *Agkistrodon halys blomhoffii*. Isolation of Five Bradykinin Potentiators and the Amino Acid Sequences of Two of Them, Potentiators B and C*

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ABSTRACT: Five bradykinin-potentiating peptides (potentiators A, B, C, D, and E) were isolated from the venom of the Japanese poisonous snake, *Agkistrodon halys blomhoffii*, using gel filtrations on columns of Sephadex G-100, Sephadex G-10, and Sephadex G-25 and ion-exchange chromatography on a column of CM-Sephadex C-50. These five bradykinin-potentiating peptides were characteristic in having a high proline content, 5 or 6 of the 11 amino acid residues being proline residues. The biological activities of these five peptides were measured using isolated guinea pig ileum and rat uterus. Of the five potentiators, B and C had the strongest bradykinin-potentiating activity on guinea pig ileum. On the other hand, only potentiator E showed high bradykinin-potentiating activity on rat uterus, the other four peptides having very slight activity. These peptides did not cause contraction of smooth muscles in the absence of bradykinin. The amino acid sequences of two bradykinin-potentiating peptides, B and C, were determined to be as follows, and these were confirmed by chemical synthesis of the peptides: potentiator B: Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro; potentiator C: Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro.

Bradykinin, which has the amino acid sequence of Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, is a typical plasma kinin, causing contraction of isolated smooth muscles, vasodilatation, and permeability increasing of capillaries. Kinin is liberated into the plasma from its precursor protein, kininogen, by kinin-releasing enzymes, and inactivated by kininase. There is much evidence that plasma kinins are important in various physiological and pathological conditions, although there are still many complex problems regarding their actions.

The contractile action of bradykinin on isolated smooth muscles is known to be potentiated by several types of compounds. Sulfhydryl compounds (Ferreira and Rocha e Silva, 1962; Auerswald and Doleschel, 1967; Cirstea, 1965), such as cysteine, 2,3-dimercaptopropanol (BAL), or thioglycolic acid and reduced glutathione (Edery and Grunfeld, 1969), increase the size of the contraction of isolated guinea pig ileum induced by bradykinin. ϵ-Aminocaproic acid (Doleschel and Auerswald, 1966), a tryptic hydrolysate of denatured human plasma (Aarsen, 1968), an extract of pig liver (Tewksbury and Stahmann, 1965), and products of degradation of fibrinogen by plasmin (Buluk and Malofiejew, 1969) also have bradykinin-potentiating activity. Hamberg *et al.* (1969) attempted to isolate bradykinin-potentiating peptides from a tryptic hydrolysate of human plasma and a plasmic hydrolysate of human fibrinogen, and found that the bradykinin-potentiating activity of the peptides seemed to depend on the presence of C-terminal arginine or lysine. Bovine fibrinopeptide B (Gladner *et al.*, 1963) and human fibrinopeptide A-β (Osbahr *et al.*, 1964) had bradykinin-potentiating activity on rat uterus. Thus, many peptide-like substances were found to have bradykinin-potentiating activity. However, none of their amino acid sequences except those of fibrinopeptides are known yet.

In 1965, Ferreira found that the venom of the snake, *Bothrops jararaca*, contains an alcohol-soluble factor which potentiates the effects of bradykinin both *in vitro* and *in vivo* (Ferreira, 1965). We found that the venom of the Japanese poisonous snake, *Agkistrodon halys blomhoffii* contains two or more bradykinin-potentiating peptides (Suzuki *et al.*, 1966), and later we purified five bradykinin-potentiating peptides from the venom. Preliminary reports have appeared on their amino acid compositions (Kato and Suzuki, 1969) and the amino acid sequence of one of them, potentiator B (Kato and Suzuki, 1970a). This paper describes the purification and biological activities of five bradykinin-potentiating peptides and results of studies on the amino acid sequences of two of them, potentiators B and C. Recently, Ferreira *et al.* (1970a; Greene *et al.*, 1970) purified a bradykinin-potentiating peptide from the venom of *Bothrops jararaca* and determined its amino acid sequence as Pyr[1]-Lys-Trp-Ala-Pro. No similarity was found between this pentapeptide and the undecapeptide potentiator B or C. However, the amino-terminal part of potentiator E (Kato and Suzuki, 1970c), one of the five bradykinin-potentiating peptides described in this paper, is strikingly similar to the above pentapeptide in its amino acid sequence.

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[1] The abbreviation used is: Pyr, pyrrolidonecarboxylic acid.