Isolation of Bradykinin-Potentiating Peptides from *Bothrops jararaca* Venom*

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ABSTRACT: Bradykinin-potentiating factor is a pharmacologically active peptide fraction prepared from *Bothrops jararaca* venom (Ferreira, S. H. (1965), *Brit. J. Pharmacol. Chemother. 24*, 163). It inhibits the proteolytic enzymes which inactivate bradykinin and catalyze the conversion of angiotensin I into angiotensin II. We have applied modern methods to the fractionation of the venom and have isolated nine biologically active peptides. The peptides were purified by ultrafiltration, gel filtration on G-25, and ion-exchange chromatography on Dowex 50-X2 and Dowex 1-X2. The amino acid composition and specific activity for the potentiation of the action of bradykinin on the guinea pig ileum are reported for each peptide. The peptides contain from 5 to 13 amino acid residues per molecule. The amino acid sequence of one peptide was shown to be pyrrolidonecarboxyl-Lys-Trp-Ala-Pro. These peptides should provide useful tools for the study and control of the proteolytic enzymes involved in the metabolism of bradykinin and angiotensin.

A peptide fraction from *Bothrops jararaca* venom potentiates the pharmacological actions of bradykinin *in vitro* as well as *in vivo*. Bradykinin-potentiating factor is a dialyzable, trypsin-labile material which has been separated into two components by electrophoresis (Ferreira, 1965). The available evidence indicates that potentiation of bradykinin activity is due to the inhibition of enzymes that normally inactivate bradykinin (Ferreira and Rocha e Silva, 1965; Ferreira, 1966; Ferreira and Vane, 1967). It has recently been shown that bradykinin-potentiating factor also inhibits the conversion of angiotensin I into angiotensin II both *in vitro* (Bakhle, 1968) and *in vivo* (K. K. F. NG and J. R. Vane, unpublished results, cited in Bakhle *et al.*, 1969).

Since limited proteolysis reactions occupy a central role in the release and inactivation of the circulating hormones bradykinin and angiotensin (Erdös, 1966; Page and McCubbin, 1968; Vane, 1969), inhibitors of these reactions may be used to demonstrate the participation of these hormones in various physiological phenomena. Such enzyme inhibitors may also prove to be therapeutically useful in pathological conditions such as inflammation and hypertension. Thus, detailed information about these peptides which will contribute to an understanding of the enzymology of these processes is of both theoretical and practical significance.

We have undertaken the systematic study of the low molecular weight fraction of *B. jararaca* venom using techniques which separate the components on the basis of their physical and chemical properties. After the initial separations based on ultrafiltration and gel filtration on Sephadex G-25 using two different solvent systems, a bioassay for the potentiation of bradykinin activity on the guinea pig ileum was used to select fractions for further study. In this communication the isolations and properties of nine biologically active peptides and the amino acid sequence determination of peptide V-3-A, pyrrolidonecarboxyl-Lys-Trp-Ala-Pro, are reported.

Materials and Methods

B. jararaca venom was supplied by the Butantan Institute, São Paulo, Brazil. Cellulose casing, $^{8}/_{32}$ Nojax Visking Casing, was purchased from Fisher Scientific Co., Fairlawn, N. J., or from Union Carbide Corp., Chicago, Ill. Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Dowex 50-X2 (AG 50W-X2, 200–400 mesh) and Dowex 1-X2 (AG 1-X2, 200–400 mesh) were supplied by Bio-Rad Laboratories, Richmond, Calif. Trypsin (lot TRL-6JA) and porcine carboxypeptidase B (COBC-8J) were purchased from Worthington Biochemical Corp., Freehold, N. J. Trypsin was treated with *p*-toluenesulfonamidophenylethyl chloromethyl ketone (Cyclo Chemical Co., Los Angeles, Calif.) by the procedure of Carpenter (1967). Aminopeptidase M (EL 5-64, Rohm and Haas, GMBH) was obtained from Henley and Co., New York, N. Y. Hippuryl-L-arginine (lot K1228) and L-pyrrolidonecarboxylic acid (lot 1540) were products of Mann Research Laboratories, New York, N. Y. Bradykinin and L-pyrrolidonecarboxylyl-L-alanine (lot 1362) were obtained from Cyclo Chemical Corp. The lyophilized *Pseudomonas* cells used for the preparation of pyrrolidonecarboxylyl peptidase was the gift of Dr. R. F. Doolittle, Department of Chemistry, University of California, San Diego, Calif.

Determination of Bradykinin-Potentiating Activity. Bradykinin activity was determined by measuring isotonic contrac-

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TABLE I: Bradykinin-Potentiating Activity of *B. jararaca* Venom, "Factor," and Fractions or Peptides Derived from Venom.[a]

Fraction or Peptide	Sp Act. Potentiating Units Per μg	Per mμmole	Fraction Or Peptide	Sp Act. Potentiating Units Per μg	Per mμmole
Venom	0.25		IV-1-Bβ	3.29	4.00
BPF[b]	2.50		IV-1-D	31.0	35.0
LMWF[b]	1.90		IV-2	0.23	
II-1	2.80		IV-3	0.008	
II-1-A	5.30	7.25	V-1	19.0	
II-2	0.20		V-1-A	21.0	32.0
III-1	10.0		V-2	0.48	
III-1-A	12.0	16.60	V-3	12.0	
III-2	0.22		V-3-A	14.0	8.50
III-3	0.008		V-3-B	1.50	0.73
III-4	3.25		V-4	0.009	
III-5	0.035		V-5	0.002	
IV-1	18.0		VI	0.075	
IV-1-A	6.25	6.90	VII	0.011	
IV-1-B	14.0		VIII	0.011	
IV-1-Bα	2.20	2.90			

[a] Potentiation of bradykinin was measured in the guinea pig ileum assay. The values are expressed as potentiating units per microgram or millimicromoles per milliliter of tissue bath (*cf.* Methods). Venom and the low molecular weight fraction were heated in a boiling-water bath for 5 min prior to assay. [b] BPF = bradykinin-potentiating factor or simply "factor;" LMWF = low molecular weight fraction.

tion of guinea pig ileum suspended in aerated Tyrode solution containing 1×10^{-4} mg/ml of atropine at 37°. Potentiating activity was estimated in terms of the increase in the response of the assay tissue to a standard dose of synthetic bradykinin (Ferreira, 1965). One "unit" of potentiation is the concentration of peptide, per milliliter of incubation solution, required to increase the effect of a single dose (b) of bradykinin to match that elicited by a double dose (2b) of bradykinin. Specific activity is expressed as potentiating units per microgram or millimicromole of peptide per milliliter of tissue incubation solution. Because of the variation in sensitivity of some ileum preparations the assays reported in this paper were performed on tissues for which one unit of potentiation of peptide III-1-A was achieved by concentrations ranging from 0.05 to 0.07 mμmole per ml. Twofold variations in specific activity have been observed when tissue preparations were not screened by the criterion of peptide III-1-A specific activity. Venom and the low molecular weight fraction were incubated in a boiling-water bath for 5 min prior to assay to inactivate substances which interfered with the bioassay. All other fractions and purified peptides were assayed without this treatment.

The weight of crude fractions was determined on material dried in an evacuated desiccator for 24 hr in the presence

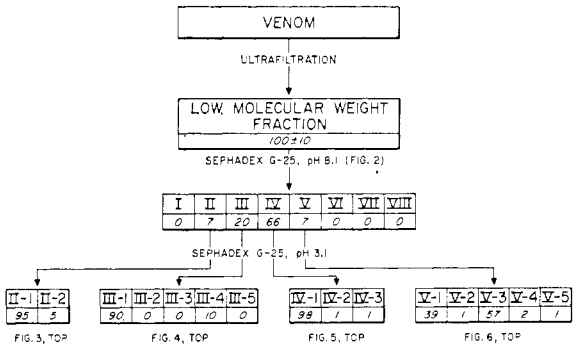

FIGURE 1: Flow diagram for the initial stages in the preparation of bradykinin-potentiating peptides from *B. jararaca* venom. The subsequent steps are described in the Results section. Experimental details are given in the legends to the figures and in the text. The numbers in italics indicate the relative distribution of recovered bradykinin-potentiating activity for each chromatographic stage.

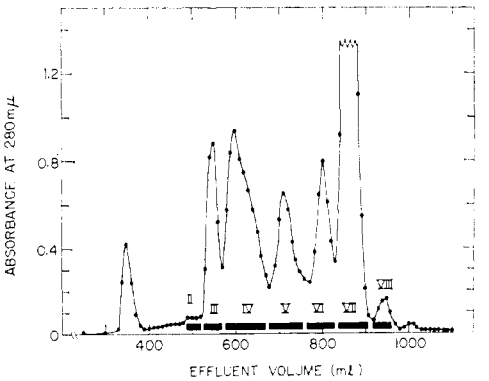

FIGURE 2: Gel filtration on Sephadex G-25 of the low molecular weight fraction of *B. jararaca* venom. The column (2.5 × 250 cm) was equilibrated and developed with 0.1 M ammonium bicarbonate buffer (pH 8.1) at 40 ml/hr, 25°. The fractions indicated by the bars were combined.

of $CaCl_2$. The weight of the purified peptides was determined by amino acid analysis of acid hydrolysates. The minimal chemical molecular weight[1] was used for the calculation of the specific activity (Table I) and the molecular weight reported for each peptide in Table II.

Detection of Peptides. Chromatographic elution patterns were determined by absorbance measurements at 280 mμ for eluates containing ammonium bicarbonate buffer. When pyridine–acetic acid buffers were employed aliquots of the column effluent were subjected to alkaline hydrolysis followed by the ninhydrin reaction (Hirs, 1967). The biological activity of eluates from the column developed with ammonium bicarbonate buffer aliquots was assayed without further treatment. Tube by tube detection of the biological activity was not performed on eluates containing pyridine–acetic acid buffer because the solvent interfered with the biological assay. The distribution of recovered biological activity for both types of columns was based on assay of pools, indicated by the solid bars on the elution diagrams, after solvent was removed by evaporation.

Low Molecular Weight Fraction. In a typical experiment 100 ml of an aqueous solution of dried venom (5–10 mg/ml) was filtered through $^{8}/_{32}$ Visking casing under reduced pressure at room temperature (Berggard, 1961). The solution which passed through the membrane was lyophilized and is denoted low molecular weight fraction (*cf.* Figure 1).

Gel Filtration on Sephadex G-25 at pH 8.1 (cf. Figure 2). The low molecular weight fraction (100–300 mg) was chromatographed on Sephadex G-25 (fine) in a column (2.5 × 250 cm) which was equilibrated with 0.10 M ammonium bicarbonate buffer (pH 8.1). The column was operated at 40 ml/hr and fractions of 10 ml were collected. The effluent corresponding to peaks II–V was lyophilized repeatedly and each pool was individually rechromatographed on Sephadex G-25 equilibrated with pyridine–acetic acid buffer (pH 3.1).

Gel Filtration on Sephadex G-25 at pH 3.1 (cf. Figures 3–6, top). Two columns (0.9 × 200 cm) were connected in series by polyethylene tubing in order to achieve an effective length of 400 cm (King and Norman, 1962). The columns were prepared and developed with pyridine–acetic acid buffer (0.2 M in pyridine, pH 3.1) as described previously (Greene and Giordano, 1969). The effluent corresponding to each peak was assayed for biological activity after being combined and evaporated. The pools with high activity were rechromatographed on Dowex 50-X2.

Dowex 50-X2. Fraction II-1 (Figure 3, top), III-1 (Figure 4, top), V-1, and V-3 (Figure 6, top) were chromatographed on Dowex 50-X2. The columns (0.9 × 60 cm) were equilibrated with pyridine–acetic acid buffer, 0.2 M pyridine (pH 3.1) at 38°. A linear gradient was achieved using 250 ml each of 0.2 M pyridine–acetic acid buffer (pH 3.1) and 2.0 M pyridine–acetic acid buffer (pH 5.0) (Schroeder, 1967). Effluent was collected in 2-ml fractions at 15–20 ml/hr. Fraction IV-1 (Figure 5, top) was chromatographed on Dowex 50-X2 equilibrated and developed with 0.1 M pyridine–acetic acid

[1] The minimal chemical molecular weights are consistent with the elution positions of the peptides on Sephadex G-25 columns developed with pyridine–acetic acid buffer (pH 3.1). The validity of this assumption was demonstrated for peptide V-3-A by amino acid sequence determination.

TABLE II: Bradykinin-Potentiating Peptides Isolated from *B. jararaca* Venom.[a]

Peptide	Rel Biol Sp Act. (M)	Amino Acid Composition (Moles/Mole of Peptide)											Amino Acids/ Peptide[b]	Calcd Min Mol Wt	Electrophoretic Mobility
		Trp	Lys	His	Arg	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Ile	Leu			
II-1-A	90				0.97		0.07	0.07	2.00	5.25	2.95		0.94	0.91	13	1352	1.23
III-1-A	200	0.76			0.99				2.00	5.08	2.91		1.00		13	1389	0.98
IV-1-A	80	0.87				0.96		0.87	1.10	4.10	0.95		1.01		10	1094	0.12
IV-1-Bα	34	0.75			0.96	0.08	0.81	0.07	1.90	5.14	0.32		1.00		11	1318	1.20
IV-1-Bβ	47	0.55		0.89		1.11			2.06	3.95	0.23		0.99		10	1216	1.20
IV-1-D	410	0.92			0.93				2.02	4.10		0.07	0.95		9	1120	1.31
V-1-A	380	1.74			0.95				3.04	3.98	0.09	1.06	0.97		12	1506	1.12
V-3-A	100	0.81	1.00						1.01	1.02	0.08	0.97			5	611	1.64
V-3-B	9	0.96						0.92	1.06	2.00	1.02				6	672	0.45

[a] The preparation of the peptides is described in the text. The biological specific activity (molar basis) calculated from the data presented in Table I is reported relative to peptide V-3-A. The amino acid analyses were performed on 22-hr acid hydrolysates. The values for all of the amino acids except tryptophan, threonine, and serine were used as the basis for the calculation of molar ratios of constituent amino acids. Amino acids present at concentrations of 0.05 residue/molecule or less were not reported. No corrections were applied for losses of tryptophan, threonine, or serine caused by decomposition on acid hydrolysis. The values reported for the number of moles of amino acids per mole of peptides and the molecular weight for each peptide have been calculated from amino acid composition data on the basis of the minimum molecular weight. High-voltage paper electrophoresis was carried out in pyridine–acetic acid buffer (pH 3.5) for 3–4.5 hr. The mobility for each peptide was calculated relative to the mobility of glycine except for peptides IV-1-A and V-3-B where glutamic acid and aspartic acid, respectively, were used as reference markers. [b] Moles/mole.

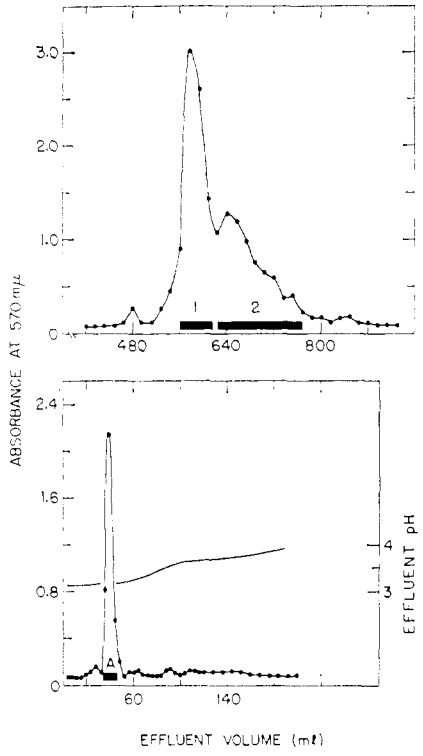

FIGURE 3: Top: gel filtration on Sephadex G-25 on peak II (Figure 2). The column (0.9 × 400 cm) was equilibrated and developed with 0.2 M pyridine–acetic acid buffer (pH 3.1) at 5 ml/hr, 23°. Peptides were located by ninhydrin after alkaline hydrolysis. The fractions indicated by the bars were combined. Bottom: gradient elution chromatography on Dowex 50-X2 of peak 1 (top). The column (0.9 × 60 cm) was operated as described in the text. The fractions indicated by the bars were combined. (O—O) Absorbance at 570 mμ (ninhydrin reaction after alkaline hydrolysis); (——) effluent pH.

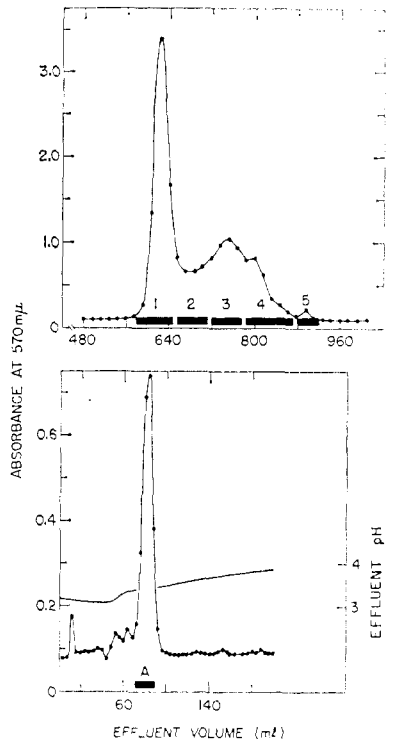

FIGURE 4: Top: gel filtration on Sephadex G-25 of peak III (Figure 2). The experimental conditions were the same as indicated for Figure 3 (top). Bottom: gradient elution chromatography on Dowex 50-X2 of peak 1 (top). The experimental conditions are the same as given for Figure 3 (bottom).

(pH 2.9; 8.1 ml of pyridine and 278 ml of acetic acid per l.) by equilibrium chromatography.

Dowex 1. Fraction IV-1-B (Figure 5, bottom) was rechromatographed on a column of Dowex 1-X2 (0.9 × 30 cm) equilibrated with *N*-ethylmorpholine–picoline–pyridine–acetic acid buffer (pH 9.4) at 38° (Schroeder, 1967). The column was developed with starting buffer (pH 9.4) at 40 ml/hr at 38°. After 30 1-ml fractions had been collected the column was developed with 2.0 N acetic acid. Peptide IV-1-B-α was eluted in tubes 12–16 and IV-1-B-β was eluted with the acetic acid in tubes 46–52.

High-Voltage Electrophoresis. Peptides were subjected to electrophoresis on Whatman No. 3MM paper at 44 V/cm for periods up to 4.5 hr using pH 3.5 buffer (1 ml of pyridine, 10 ml of acetic acid, and 189 ml of H_2O) and pH 6.5 buffer (25 ml of pyridine, 1 ml of acetic acid, and 225 ml of H_2O). The peptides were detected by fluorescence when irradiated with ultraviolet light, by reaction with ninhydrin (0.5% in ethanol), and by the chlorination reaction of Reindel and Hoppe (1954). Peptides were eluted from the paper with 0.1 M NH_4OH.

Amino Acid Analyses. Samples containing 0.1–0.2 μmole of peptide were hydrolyzed in evacuated sealed tubes with 1 ml of twice-distilled constant-boiling HCl containing 1 mg/ml each of 2-mercaptoethanol and phenol (Dr. J. M. Stewart, University of Colorado Medical Center, unpublished procedure). Amino acid analysis of hydrolysates was performed by the method of Spackman *et al.* (1958) on an automatic instrument with provisions for multiple sample application (Alonzo and Hirs, 1968). The sample buffer, 0.2 M sodium citrate (pH 2.2) contained 15% polyethylene glycol. The length of the short column was extended to 15 cm in order to facilitate the estimation of tryptophan. The integration constants (concentration per area) used for tryptophan and leucine were 0.772 and 0.593, respectively. No corrections were applied for the losses of serine, threonine, and tryptophan caused by decomposition on acid hydrolysis.

Trypsin Hydrolysis of Peptide V-3-A. The pentapeptide (4.0 μmoles) was incubated with 0.2 mg L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (325 μmoles/min per mg, *p*-tosyl-L-arginine methyl ester hydrolysis; Greene *et al.*, 1966) in 4 ml of 0.06 M sodium phosphate buffer (pH 7.65) for 20 hr at 23°. The enzymatic reaction was stopped by the addition of 0.4 ml of 1 N HCl followed by lyophilization. The product was dissolved in 1.0 ml of H_2O plus 0.1 ml of 0.1 N HCl and chromatographed on Dowex 50-X2 (Figure 8).

Hydrolysis with Carboxypeptidase B. Peptide T-1, 0.1 μmole in 0.10 ml of 0.2 M *N*-ethylmorpholine–acetic acid buffer (pH 8.5), was incubated with chromatographically purified porcine carboxypeptidase B (0.015 mg) at 37° for 1–8 hr. The course of the reaction was routinely monitored by determining the release of lysine by amino acid analysis. The reaction was stopped by the addition of 0.10 ml of 0.10 N HCl and 1.00 ml of 0.2 M sodium citrate buffer (pH

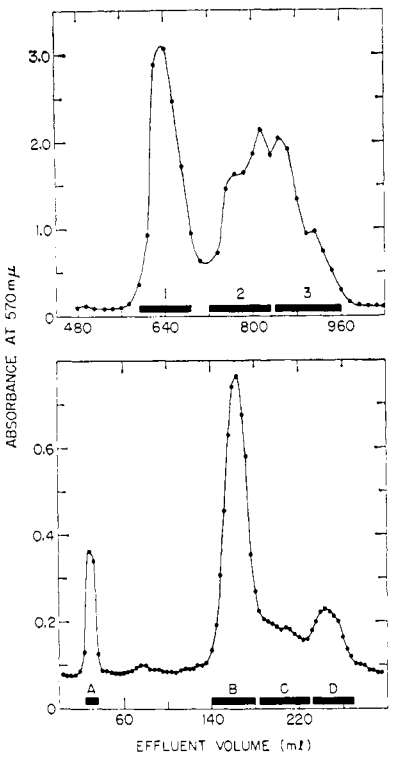

FIGURE 5: Top: gel filtration on Sephadex G-25 of peak IV (Figure 2). The experimental conditions were the same as given for Figure 3 (top). Bottom: equilibrium chromatography of peak 1 (top) on Dowex 50-X2 equilibrated and developed with 0.1 M pyridine-acetic acid buffer (pH 2.9). Peptides were located by ninhydrin analysis after alkaline hydrolysis. The fractions indicated by the bars were combined.

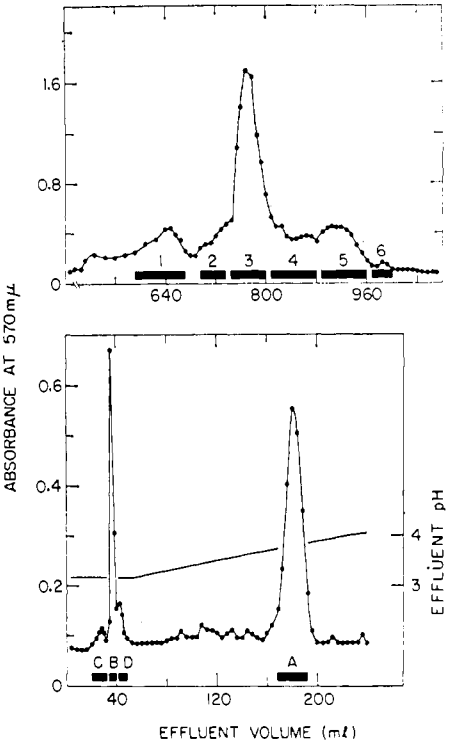

FIGURE 6: Top: gel filtration on Sephadex G-25 of peak V (Figure 2). The experimental conditions were the same as indicated for Figure 3 (top). Bottom: gradient elution chromatography on Dowex 50-X2 of peak 3 (top). The experimental conditions are the same as indicated for Figure 3 (bottom).

2.2) containing 15% polyethylene glycol. This solution was applied to the amino acid analyzer without further treatment.

Pyrrolidonecarboxylic acid was demonstrated to be a reaction product by high-voltage paper electrophoresis in pyridine–acetic acid buffer (pH 3.5) for 30 min at 44 V/cm. Pyrrolidonecarboxylic acid, located by the Reindel–Hoppe peptide detection method (Reindel and Hoppe, 1954), had a mobility of 2.0 relative to aspartic acid. It was estimated quantitatively as glutamic acid after elution from the paper with 0.1 M NH_3 and acid hydrolysis in 6 N HCl, 110° for 22 hr.

The rates of hydrolysis of pyrrolidonecarboxyl-Lys (1×10^{-3} M) and hippuryl-L-arginine (1×10^{-3} M) by carboxypeptidase B in 0.025 M Tris-HCl buffer (pH 8.0), 23°, were determined in order to facilitate comparison of the turnover rates of these substrates with data reported by Wolff *et al.* (1962). The release of lysine from pyrrolidonecarboxyl-Lys was monitored with the amino acid analyzer, and a spectrophotometric procedure was used for hippuryl-L-arginine (Wolff *et al.*, 1962).

Hydrolysis with Pyrrolidonecarboxylyl Peptidase. The enzyme, prepared from lyophilized *Pseudomonas*, was purified to the "G-200 enzyme" stage as recommended by Armentrout and Doolittle (1969) for use in structural studies. The enzyme (total $A_{280\,m\mu}$ 7.5) prepared from 3 g of dry cells had a specific activity of 516 units/$A_{280\,m\mu}$ when assayed with pyrrolidonecarboxyl-Ala. The specific activity of this preparation compares favorably with the value of 475 reported for the enzyme at a comparable level of purification (*cf.* Armentrout and Doolittle, 1969, Table I).

The dipeptide T-1 (0.1 μmole) was incubated at 30° for 18 hr with 0.2 ml of the enzyme ($A_{280\,m\mu}$ 0.960) which had been dialyzed against 0.05 M potassium phosphate buffer (pH 7.3) containing 0.001 M EDTA and 0.01 M 2-mercaptoethanol. The reaction was stopped by the addition of 1.0 ml of 0.2 M sodium citrate buffer (pH 2.2) containing 15% polyethylene glycol. The extent of hydrolysis was determined by measuring the released lysine with the amino acid analyzer. Pyrrolidonecarboxylic acid was extracted with ethyl acetate from a duplicate enzymatic digestion mixture which had been adjusted to pH 2.0. The ethyl acetate soluble material was examined by high-voltage electrophoresis (pH 3.5).

The hydrolysis of the pentapeptide V-3-A was examined at both analytical and preparative levels. For analytical experiments 0.1 μmole of the peptide were incubated under the conditions described above for peptide T-1. Pyrrolidonecarboxylic acid was extracted with ethyl acetate and determined by amino acid analysis after acid hydrolysis, in two preparative experiments. The pentapeptide V-3-A (0.1 μmole) was incubated with the same volume and concentration of enzyme for 18 and 72 hr at 25°. The reaction mixture was acidified and chromatographed on Dowex 50-X2. The eluate was analyzed by the ninhydrin reaction after alkaline hydrolysis.

Total Hydrolysis with Aminopeptidase M. A solution containing peptide T-2 (0.1 μmole) in 0.10 ml of 0.067 M

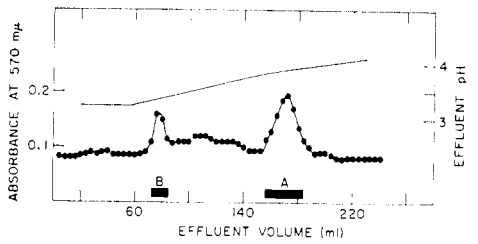

FIGURE 7: Gradient elution chromatography on Dowex 50-X2 of peak I (Figure 6, top). The experimental conditions were the same as indicated for Figure 3 (bottom).

sodium phosphate (pH 7.65) was incubated for 18 hr at 37° with 0.10 ml of 0.1% aqueous solution of aminopeptidase M. The reaction was stopped by the addition of 0.10 ml of 0.1 N HCl and 1.00 ml of 0.2 M sodium citrate buffer (pH 2.2) containing 15% polyethylene glycol. This solution was applied to the amino acid analyzer without further treatment.

Limited Hydrolysis with Aminopeptidase M. The conditions for limited hydrolysis were determined in a series of preliminary experiments on 0.1-μmole aliquots of the peptide. The reaction was stopped with 0.10 ml of 0.1 N HCl and 1.00 ml of 0.2 M sodium citrate buffer (pH 2.2) containing 15% polyethylene glycol. This solution was applied to the amino acid analyzer. Peptide T-2 (Trp-Ala-Pro) was eluted from the short column at 12 min and peptide T-2A (Ala-Pro) was eluted at 182 min from the long column. Lysine and tyrosine were eluted at 34 and 184 min on the short and long columns, respectively.

In a preparative experiment peptide T-2 (2.75 μmoles) was incubated with 0.3 mg of aminopeptidase M in 3.30 ml of 0.060 M sodium phosphate buffer (pH 7.65) for 2 min at 37°. The reaction was stopped by the addition of 0.30 ml of 1 N HCl. An aliquot (0.40 ml) was removed for amino acid analysis and the remaining solution was lyophilized. The product was dissolved in 1.0 ml of H_2O plus 0.10 ml of 0.1 N HCl and chromatographed on Dowex 50-X2.

Edman Degradation. A subtractive procedure was used. The peptides (0.1 μmole) were subjected to one or more cycles of the Edman reaction without purification except for final extraction before acid hydrolysis (Gray, 1967). The details of the procedure used are given in Greene and Giordano (1969) with the exception that the coupling reaction was carried out in 50% pyridine in the absence of trimethylamine. Prior to acid hydrolysis the residue was suspended in 3 ml of 0.1 N HCl and extracted twice with 10 ml of benzene. The organic layer was discarded. After evaporation of the solution the residue was hydrolyzed in 6 N HCl for 22 hr at 110°.

Limited Digestion in NaOH. Aliquots of V-3-A (0.1 μmole) were incubated in 0.10 ml of 1.00 N NaOH in stoppered test tubes at 37° for periods up to 72 hr. The release of glutamic acid was monitored with the amino acid analyzer.

Results

Low Molecular Weight Fraction of B. jararaca Venom. The bradykinin-potentiating activity in the venom was quantitatively separated from enzymes and other high molecular weight substances by ultrafiltration through Visking casing. The recovery of biological activity was markedly reduced when the concentration of the venom solution used for ultrafiltration exceeded 10 mg/ml. The specific activity for bradykinin potentiation in the guinea pig ileum assay of unfractionated venom, the low molecular weight fraction, and of bradykinin-potentiating factor prepared by ethanol extraction of heated venom followed by ether precipitation was 0.25, 1.90, and 2.50 potentiating units per μg, respectively (Table I). On this basis the low molecular weight fraction is eight times more active than venom but less active than the preparation of bradykinin-potentiating factor described previously (Ferreira, 1965).

Purification of Peptides. The flow diagram summarizing the three initial steps in the fractionation of *B. jararaca* venom is presented in Figure 1. The italicized numbers indicate the total recovery of biological activity for the ultrafiltration step and the relative distribution (reported as per cent of recovered activity) of the biological activity present in peaks prepared by the gel filtration steps. The selection of fractions for further purification was based upon the presence of appreciable quantities of biological activity and/or high specific activity (*cf.* Figure 1 and Table I). The material present in the effluent peaks which have been identified in the elution diagrams but not listed in Table II were either heterogeneous by the criterion of amino acid analysis or they were present in too low quantity to permit further purification. Figure 2 shows the elution pattern obtained when the low molecular weight fraction of venom was subjected to gel filtration on Sephadex G-25 equilibrated in ammonium bicarbonate buffer (pH 8.1). Each composite figure (Figures 3–6) illustrates the third step, Sephadex G-25 equilibrated in pyridine–acetic acid buffer (pH 3.1; top of figure) and the fourth step Dowex 50-X2 (bottom of figure) in the purification of peaks II–V (Figure 2). The system of nomenclature used to identify the peptides specifies the route of purification by identifying the elution diagram peaks from which the purified peptides have been prepared. For example, peptide II-1-A was prepared from the low molecular weight fraction by gel filtration on Sephadex G-25 (pH 8.1; peak II, Figure 2) followed by gel filtration of Sephadex G-25 (pH 3.1; peak 1, Figure 3 top) and finally by gradient elution chromatograph on Dowex 50-X2 (peak A, Figure 3 bottom). Peptides III-1-A, V-1-A, V-1-B, V-3-A, and V-3-B were prepared in the same manner. The Dowex 50-X2 elution diagram for V-1-A and V-1-B is illustrated in Figure 7.

Equilibrium chromatography on Dowex 50-X2 using pyridine–acetic acid buffer (pH 2.9; 0.1 M in pyridine) proved to be more effective than gradient elution chromatography for the fractionation of the components present in peak IV-1 (*cf.* Figure 5 bottom). Peptides IV-1-B-α and IV-1-B-β were prepared from peak IV-1-B by chromatography on Dowex 1-X2.

Amino Acid Composition of Purified Peptides. The amino acid compositions of nine biologically active peptides are presented in Table II. The peptides contain 5–13 residues/molecule. Similarities in amino acid composition are apparent; these include a high proline content as well as the presence of glutamic acid, arginine, and tryptophan in most of the peptides. The peptides are of sufficiently high purity for amino acid sequence determination and with two exceptions

(IV-1-B-α and IV-1-B-β) the integral molar ratios of the constituent amino acids may be taken as strong evidence for homogeneity.

When 2-mercaptoethanol and phenol were not present during acid hydrolysis, the tryptophan content was reduced to 0.4–0.6 residue/molecule. The calculated molecular weight listed in Table II was based on the minimal molecular weight.[1] These values have been used to calculate the specific activities (weight basis) reported in Table I for the purified peptides.

Electrophoresis. The purified peptides were examined by high-voltage paper electrophoresis at pH 3.5 and 6.5. All of the peptides reacted with the Reindel–Hoppe peptide detection reagent and those which contained tryptophan by amino acid analysis were fluorescent when irradiated with ultraviolet light. The peptides, with the exception of V-3-A, which contains lysine, were ninhydrin negative.

Seven of the nine peptides behaved as a single component in both electrophoretic systems. Peptides II-1-A and III-1-A each contained a second minor component. The level of contamination was 7 and 5%, respectively, as determined by amino acid analysis after elution of the major and minor components from the electrophoresis paper.

Distribution and Recovery of Biological Activity. The distribution of recovered activity during the first three steps of the isolation procedure is summarized in Figure 1 (italicized numbers). Most of the activity is present in 5 of the 15 fractions obtained after gel filtration on G-25 operated at pH 3.1. Fraction IV-1 contained 66% of the recovered activity. The recovery of biological activity for the first three steps was approximately 80–90%. When fractions II-1, III-1, V-1, and V-3 were rechromatographed on Dowex 50-X2 by gradient chromatography, the recovery of activity was quite good, 80–90%. The major losses of biological activity occurred in the purification of fraction IV-1. During equilibrium chromatography of fraction IV-1 on Dowex 50-X2 and the stepwise elution of IV-1-B on Dowex 1-X2, recoveries of 60 and 50%, respectively, were achieved.

For the entire isolation procedure approximately 50% of the bradykinin-potentiating activity (guinea pig ileum assay) present in *B. jararaca* venom has been recovered in the nine peptides listed in Table II. The relative distribution of activity reported in this study must be interpreted cautiously because all of the fractionation was done on a single pool of venom. When another sample of venom was examined on Sephadex G-25 (pH 8.1), a similar elution pattern was obtained but the peaks were present in slightly different relative proportions.

Biological Activity of Fractions and Purified Peptides. The specific activities for the potentiation of bradykinin in the guinea pig ileum assay of fractions and purified peptides are given in Table I. With the exception of IV-1-B-α and IV-1-B-β, the purified peptides had higher specific activities than the fractions from which they were prepared.

The molar specific activity of the purified peptides varied over a 50-fold range (Table I). These values are compared in Table II relative to V-3-A whose specific activity has been taken as 100. The peptides are biologically active at extremely low concentrations which are near to the concentration of bradykinin required for biological activity. The most active potentiating peptides acted in concentrations ranging from 3×10^{-8} M (IV-1-D and V-1-A) to 1.2×10^{-7} M (V-3-A,

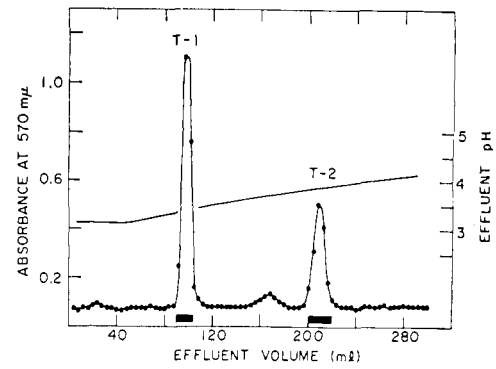

FIGURE 8: Chromatography on Dowex 50-X2 of trypsin digestion of 4.0 μmoles of peptide V-3-A. The experimental conditions are the same as indicated in Figure 3 (bottom). The analytical data are presented in Table III.

pyrrolidonecarboxyl-Lys-Trp-Ala-Pro) to double the response of the assay tissue to a standard dose of bradykinin, 5×10^{-9} M. Thus when the agonist (bradykinin) is present in the assay bath, 6–24 molecules of potentiator peptide produce an effect equivalent to that produced by one molecule of bradykinin.

Sequence Determination of V-3-A. The peptide was not hydrolyzable by pyrrolidonecarboxylyl peptidase, aminopeptidase M, or degradable by the Edman procedure. After digestion with trypsin, a dipeptide (T-1) and a tripeptide (T-2) were isolated in 93 and 85% yield, respectively (Table III), by chromatography on Dowex 50-X2 (Figure 8).

Pyrrolidonecarboxyl-Lys (T-1). The peptide was eluted at pH 3.40 from the Dowex 50-X2 column. It contained equivalent amounts of glutamic acid and lysine after acid hydrolysis. It was slowly hydrolyzed by pyrrolidonecarboxylyl peptidase. After incubation for 18 hr, 0.34 mole of lysine/mole of substrate was released. Pyrrolidonecarboxylic acid was qualitatively identified by high-voltage electrophoresis at pH 3.5. No free glutamic acid was detected in the digestion mixture.

Peptide T-1 was quantitatively hydrolyzed by carboxypeptidase B to pyrrolidonecarboxylic acid and lysine. No free glutamic acid was detected in the enzymatic digestion mixture. Pyrrolidonecarboxyl-Lys and hippuryl-L-arginine were hydrolyzed by carboxypeptidase B at 0.065 and 146 μmoles per l. per min per mg, respectively. On the basis of these determinations and the data reported by Wolff *et al.* (1962), at 1×10^{-3} M substrate concentration pyrrolidonecarboxyl-Lys is hydrolyzed by carboxypeptidase B at $^1/_{700}$ the rate of hippuryl-L-lysine and two times more rapidly than acetyl-L-arginine. In spite of its low rate of hydrolysis, 1–2 μmoles of pyrrolidonecarboxyl-Lys can be completely cleaved with 0.1–1.0 mg/ml of commercial carboxypeptidase B within several hours with negligible corrections required for enzyme autolysis. The hydrolysis of pyrrolidonecarboxyl-Lys by carboxypeptidase B has been reported by Bodnaryk and Levenbook (1968).

Trp-Ala-Pro (T-2). The tryptophan content of the peptide was determined quantitatively by amino acid analysis after total enzymatic digestion with aminopeptidase M. The results of subtractive Edman degradation suggested that tryptophan occupied the amino-terminal position and that

TABLE III: Amino Acid Compositions and Subtractive Edman Degradation of V-3-A and Peptides Derived from V-3-A by Trypsin Hydrolysis (Figure 8).[a]

	Peptide (moles of amino acid/mole of peptide)					
	V-3-A	T-1	T-2			
					Edman Degradation	
Amino Acid	6 N HCl, 110°, 22 hr	6 N HCl, 110°, 22 hr	6 N HCl, 110°, 22 hr	Aminopeptidase M	1 Cycle	2 Cycles
Glutamic acid	1.01	1.02				
Lysine	1.00	0.98				
Tryptophan	0.81		0.51	0.93	*0.11*	0.00
Alanine	0.97		0.98	0.99	0.90	0.15
Proline	1.02		1.02	1.01	0.92	0.34
Yield (%)		93	85			
Recovery[b] (%)				94	100	100

[a] The molar ratios and recoveries have been calculated using all of the constituent amino acids except tryptophan. [b] The amount of peptide relative to starting material.

TABLE IV: Amino Acid Composition and Subtractive Edman Degradation of the Products Derived from T-2 by Limited Hydrolysis with Aminopeptidase M.[a]

	T-2A			T-2B	
		Edman Degradation, 1 Cycle[b]			
Amino Acid	6 N HCl, 110°, 22 hr	Acid Hydrolysis 1	No Hydrolysis 2	No Hydrolysis	6 N HCl, 110°, 22 hr
Tryptophan	0.00			1.00	1.00
Alanine	1.03	*0.18*	0.00		
Proline	1.00	0.73	0.39		
Yield (%)				71	58

[a] Units are given in moles of amino acid per mole of peptide. [b] The results for the Edman degradation have been reported on the basis of 100% recovery relative to starting material.

alanine was the second residue (Table III). The dipeptide Ala-Pro was prepared by *limited* aminopeptidase M digestion of T-2 because of the possibility of the destruction of tryptophan during the cyclization step of the Edman degradation by trifluoroacetic acid and because of the low recovery of proline after the second cycle (Table III). When the digestion mixture was chromatographed on Dowex 50-X2, a dipeptide, T-2A (effluent pH 3.43), and tryptophan, T-2B (effluent pH 3.70), were isolated in 63 and 71% yield, respectively (Table IV). The identification of tryptophan was based on its elution position from the amino acid analyzer. The presence of contaminating peptides was excluded by the absence of any other amino acids in the acid hydrolysate of T-2B (Table IV).

The results of subtractive Edman degradation on T-2A were unambiguous (*cf.* Table IV, Edman degradation, column 1). The sequence Ala-Pro was confirmed by the presence of proline and absence of alanine in a sample that had been taken through one cycle of the Edman degradation but had *not* been subjected to acid hydrolysis (Table IV, Edman degradation, column 2). The absence of alanine indicates that T-2A did not contain some Pro-Ala.

The formation of the diketopiperazine of Ala-Pro during trifluoroacetic acid treatment at first seemed to be an appealing explanation for the low recovery of proline after the second cycle of the Edman degradation of peptide T-2 (Trp-Ala-Pro) (Table III; *cf.* Greenstein and Winitz, 1961). However, Ala-Pro was recovered in 84% yield (determined by its elution position and ninhydrin color) on the long column of the amino acid analyzer from a sample of Trp-Ala-Pro which had been subjected to one cycle of Edman degradation and applied to the amino acid analyzer without prior benzene extraction or acid hydrolysis. On this basis it appears that the first cycle of the Edman degradation of Trp-Ala-Pro proceeded without difficulty and Ala-Pro was generated *in situ* in high yield. We have no explanation for the difference in recovery of proline after Edman degradation of Ala-Pro formed *in situ* (Table III) compared with Ala-Pro prepared by enzymatic hydrolysis (Table IV).

The evidence for the amino acid sequence of peptide V-3-A is summarized in Figure 9. The presence of pyrrolidonecarboxylic acid at the amino terminus of the parent peptide was demonstrated by: (1) the release of glutamic acid (0.30 mole/mole of peptide) after treatment with 1 N NaOH for 72 hr at 37°, and (2) the electrophoretic identification of pyrrolidonecarboxylic acid after incubation of the peptide with a mixture of trypsin and carboxypeptidase B. The order of the peptides T-1 → T-2 was assigned on the basis of the specificity of trypsin and the unsuccessful attempts to degrade the peptide by the Edman procedure and aminopeptidase M digestion. The peptide bond between the pyrrolidonecarboxylic acid residue and the α-amino group of lysine was established on the basis of the susceptibility of peptide T-1 to hydrolysis by carboxypeptidase B and the susceptibility of the parent peptide to hydrolysis by trypsin. All of the constituent amino acids have been assigned to the L configuration on the basis of the susceptibility of each peptide bond to enzymatic hydrolysis.

The amino acid sequence of peptides V-3-A, T-1, and T-2 were confirmed by synthesis. The natural and synthetic pentapeptides V-3-A had the same physical and chemical properties and biological activity (L. J. Greene, S. H. Ferreira, and J. M. Stewart, 1970, manuscript in preparation).

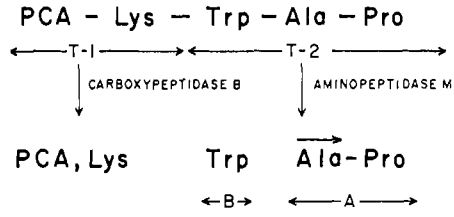

FIGURE 9: Summary of evidence for the derivation of the amino acid sequence of peptide V-3-A. The arrow above alanine indicates the result of Edman degradation.

Discussion

The use of two solvent systems for the development of Sephadex G-25 columns facilitated the purification of the venom peptides. Tryptophan-containing peptides are retarded in their elution from Sephadex G-25 developed with 0.1 M ammonium bicarbonate buffer (pH 8.1). A significant increase in resolution was achieved when fractions corresponding to the peaks shown in Figure 2 were rechromatographed on Sephadex G-25 developed with pyridine–acetic acid buffer (pH 3.1; 1.6% pyridine and 28% acetic acid; *cf.* Figures 3–6, top).[2] Peptide V-1-A, a dodecapeptide containing two residues of tryptophan per molecule, was coeluted with a hexapeptide (V-3-B) and a pentapeptide (V-3-A) in peak V (Figure 2). The dodecapeptide (peak 1) was completely separated from the smaller peptides containing one residue of tryptophan per molecule (peak 3) by rechromatography in the second solvent system (Figure 6, top). The studies of Gelotte (1960), Ruttenberg *et al.* (1965), and Eaker and Porath (1967) illustrate these solute–gel interactions. A purification step using ion exchange was necessary for most of the peptides in order to reach integral values for amino acid ratios. Nevertheless, minor contaminants present in two peptides were removed only by high-voltage electrophoresis of long duration.

The presence of a family of compositionally related, biologically active peptides in venom might be the result of proteolytic cleavage of a common precursor peptide or protein by enzymes present in venom. On the basis of the amino acid compositions of the active peptides there is no evidence for such a relationship among the peptides, except for IV-1-D, which might be derived from peptide V-1-A in this manner. It is possible, however, that limited regions of the peptides responsible for biological activity are identical.

The amino acid sequence determination of peptide V-3-A, pyrrolidonecarboxyl-Lys-Trp-Ala-Pro, was complicated by the presence of the cyclized form of glutamic acid (pyrrolidonecarboxylic acid) at the amino terminus and proline at the carboxyl terminus of the molecule. It was necessary to demonstrate that the resistance of the peptide to the normal degradative procedures was due to the terminal pyrrolidonecarboxylic acid residue and not to the presence of other blocking groups. Because of the possibility of the cyclization of glutamine and glutamic acid to pyrrolidonecarboxylic acid during isolation, it is not possible to state definitely that pyrrolidonecarboxylic acid is present in the pentapeptide at the time of biosynthesis and secretion. However, the pyrrolidonecarboxylic acid residue does contribute to the biological activity of the peptide. Glu-Lys-Trp-Ala-Pro is only 6% as active as pyrrolidonecarboxyl-Lys-Trp-Ala-Pro for the potentiation of bradykinin activity on the guinea pig ileum (L. J. Greene, S. H. Ferreira, and J. M. Stewart manuscript in preparation). Pyrrolidonecarboxyl-Gln-Trp and pyrrolidonecarboxyl-Asn-Trp are also present in *B. jararaca* venom but these peptides do not have bradykinin-potentiating activity (Kato *et al.*, 1966). The structure of the bradykinin-potentiating peptide (V-3-A), containing three heterocyclic rings, is similar to pyrrolidonecarboxyl-His-Pro-NH_2 which has recently been shown to have the biological properties of thyrotropin releasing factor (Bøler *et al.*, 1969).

None of the biologically active peptides listed in Table II could be degraded by the Edman procedure (D. C. Bartelt, 1970, unpublished result) and with the exception of V-3-A all were ninhydrin negative after electrophoresis. Since the peptides contain at least one residue of glutamic acid per mole, after acid hydrolysis, it seems likely that pyrrolidonecarboxylic acid is a structural feature common to all of the bradykinin-potentiating peptides reported here.

Bradykinin-potentiating activity has been detected in snake venom other than that of *B. jararaca* (Ferreira, 1965; Suzuki *et al.*, 1967) in a mixture of peptides derived from trypsin-treated plasma (Aarsen, 1968; Hamberg *et al.*, 1968), in the peptidic fraction of a porcine liver extract (Tewksbury and Stahman, 1965), in some fibrinopeptides (Gladner *et al.*, 1968), and in the carboxyl-terminal pentapeptide of bradykinin (Garbe, 1967). Of these, the amino acid sequence is known only for the fibrinopeptides and the bradykinin fragment. Recently Kato and Suzuki (1969) described the

[2] The separations achieved with the ammonium bicarbonate solvent systems were not improved by doubling the length of the column. On rechromatography in the same buffer each peak gave a single peak with the expected retention volume.

isolation and amino acid composition of five bradykinin-potentiating peptides from the venom of *Agkistrodon halys blomhoffii*. Although none of the peptides is identical in amino acid composition with the peptides described here, each contains four residues of proline and one residue of glutamic acid per eight to ten amino acid residues. Only one peptide contains tryptophan. In addition to similarities in size and composition, the peptides from *A. halys blomhoffii* venom did not react with ninhydrin. The potentiation elicited by all of these substances depends upon the presence of the potentiator in the bath at the time of the assay, and in this respect differs from the phenomenon discovered by Edery (1964) in which guinea pig ileum is permanently sensitized by treatment with chymotrypsin.

The bradykinin-potentiating action of the "factor" has been demonstrated in a variety of pharmacological tests such as systemic blood pressure (Ferreira, 1965; Amorim *et al.*, 1967), dilation of cerebral vessels (Graeff *et al.*, 1965), capillary permeability, central nervous effects induced by intraventricular injection (Graeff *et al.*, 1967), coronary vasodilation (Antonio, 1967), and on the response of several smooth muscle preparations. Bradykinin-potentiating factor specifically potentiates the action of bradykinin in all these systems without altering the effects of acetylcholine, histamine, substance P, prostraglandins, or eledoisin. The "factor" has been used *in vivo* as a methodological tool to demonstrate the participation of bradykinin-like substances in the pharmacological effects of proteolytic enzymes and of cellulose sulfate (Rothschild, 1967). It has been suggested that the potentiation is due, at least in part, to the inhibition of the hydrolysis of bradykinin by peptidases present in blood and tissues. This suggestion arose from three observations: (1) bradykinin-potentiating factor was able to inhibit bradykinin-inactivating enzymes of plasma and blood; (2) the potentiation *in vivo* was correlated with degree of inhibition of bradykinin-inactivating enzymes of circulating blood; (3) eledoisin, which is much more resistant than bradykinin to inactivation by blood, is not potentiated by bradykinin-potentiating factor (Ferreira, 1965, 1966; Ferreira and Rocha e Silva, 1965).

The fractions and peptides described here also inhibit a partially purified preparation of angiotensin converting enzyme from dog lung. Peptide IV-1-D caused 50% inhibition of the enzyme activity at 2×10^{-8} M (Ferreira *et al.*, 1970). Infusion of synthetic V-3-A, pyrrolidonecarboxyl-Lys-Trp-Ala-Pro inhibits the pulmonary conversion of angiotensin I *in vivo* in the rat (L. J. Greene, S. H. Ferreira, and J. M. Stewart, manuscript in preparation).

The most significant feature of the results obtained thus far is that a group of relatively small polypeptides, containing 5–13 amino acid residues/molecule, is responsible for the pharmacological activity of bradykinin-potentiating factor. They inhibit proteolytic enzymes at concentrations in the range 10^{-6}–10^{-8} M. These venom peptides are much smaller than the proteins (mol wt 6,000–50,000) which inhibit trypsin and trypsin-like enzymes (Vogel *et al.*, 1969; Barker, 1969). Knowledge of their amino acid sequences may permit us to prepare small synthetic peptides which are specific inhibitors of the enzymes responsible for the inactivation and release of circulating hormones such as bradykinin and angiotensin II.

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