Angiotensin-Converting Enzyme Inhibitors from the Venom of *Bothrops jararaca*. Isolation, Elucidation of Structure, and Synthesis*

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ABSTRACT: Fractionation of an alcoholic extract of venom of *Bothrops jararaca* has led to the isolation and characterization of six peptides possessing inhibitory activity against angiotensin-converting enzyme. The amino acid sequences proposed for these inhibitors are: *V-2:* Pyr-Trp-Pro-Arg-Pro-Thr-Pro-Gln-Ile-Pro-Pro; *V-6-I:* Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro; *V-6-II:* Pyr-Asn-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro; *V-7:* Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro; *V-8:* Pyr-Ser-Trp-Pro-Gly-Pro-Asn-Ile-Pro-Pro; *V-9:* Pyr-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro. These amino acid sequences have been synthesized by the solid-phase procedure, and the synthetic peptides were found to be identical with the natural inhibitors in their physical, chemical, and biological properties. These peptides are similar to or identical with the bradykinin-potentiating peptides isolated by Ferreira *et al.* ((1970a), *Biochemistry 9*, 2583) from the same venom.

The bradykinin-potentiating activity of extracts of venom of *Bothrops jararaca* was described for the first time by Ferreira in 1965. Similar extracts were later shown to inhibit the conversion of angiotensin I to angiotensin II *in vitro* (Bakhle, 1968) and *in vivo* (Ng and Vane, 1970).

Fractionation of these crude extracts (Ferreira *et al.*, 1970a; Greene *et al.*, 1970) assisted by a bioassay of bradykinin potentiation led to the isolation of nine peptide fractions displaying this activity in different degrees. The amino acid sequence Pyr-Lys-Trp-Ala-Pro[1] was proposed for one of these fractions (V-3-A) and confirmed by synthesis (Stewart *et al.*, 1971; Greene *et al.*, 1970). In a subsequent report (Ferreira *et al.*, 1970b) these fractions were shown to possess also angiotensin-converting enzyme inhibitory activity.

The venom of another snake, *Agkistrodon halys blomhoffii*, has also yielded peptides that potentiate the biological activities of bradykinin and inhibit the angiotensin-converting enzyme (Kato and Suzuki, 1969, 1970; Kimura *et al.*, 1970).

The availability of compounds capable of inhibiting the conversion of angiotensin I to angiotensin II and, thereby, blocking the biological functioning of the renin–angiotensin system, could be of great significance in the elucidation of the highly debated role of angiotensin in normal and pathological states. This possibility and the report that such an inhibitory activity was present in the venom of *B. jararaca* prompted us to undertake, simultaneously and independently from the workers mentioned above, the isolation of the compound or compounds causing this inhibition. This isolation, which was followed with a bioassay for inhibition of angiotensin-converting enzyme *in vitro* (Cushman and Cheung, 1969), led to the characterization, structural elucidation, and synthesis of six peptides capable of inhibiting the conversion of angiotensin I to angiotensin II, *in vitro* and *in vivo*. The amino acid composition of some of these peptides is identical with that of the bradykinin potentiating peptides isolated by Greene *et al.* (1970) from the same venom.

Materials and Methods

Venom of *B. jararaca*, purchased from C. Amaral & Cia. Ltda. (Sao Paulo, Brazil), was extracted according to the procedure of Ferreira (1965) to yield the starting material for further fractionation (crude alcoholic extract).

Column Chromatography. All columns were run at room temperature with gravity feeding. Eluents were monitored for absorbance at 254 mμ; where pyridine-containing buffers were employed, spot tests (Ehrlich, Sakaguchi, and Pauly) were used to detect the peptide peaks. The dimensions of the columns and the eluents used were as follows: *Sephadex G-25*, 5 × 90 cm, 0.2 M acetic acid; *CM-cellulose*, Whatman CM-52 (Reeve Angel, Clifton, N. J.), equilibrated with 0.005 M ammonium acetate, was packed in a column of 2.5 × 19 cm, eluted stepwise with 0.005 M ammonium acetate (fraction II-1) and 0.2 M acetic acid (fraction II-2); *DEAE-Sephadex*, equilibrated with 0.005 M ammonium bicarbonate, was placed in a column of 2.5 × 25 cm, eluted with a linear gradient of ammonium bicarbonate (1500 ml of 0.005 M and 1500 ml of 1 M). *Sephadex G-25–butanol*, Sephadex G-25, 2.5 × 95 cm, equilibrated and eluted with a mixture of 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v); *Sephadex G-25–ethyl acetate*, 2.5 × 100 cm, equilibrated and eluted with a mixture of ethyl acetate–pyridine–water (20:10:11, v/v); *Sephadex LH-20*, 1.5 × 59 cm, equilibrated and eluted with 95% ethanol. When methanol was used instead of ethanol, the dimensions of the column were 2.5 × 80 cm. AG-50W-X2, resin AG-50W-X2, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.), equilibrated with pyridine–acetate buffer (pH 3.1) was packed in a column 1.1 × 45 cm; eluted with a gradient of pyridine–acetate buffer (pH 3.1–5.0) (Schroeder, 1967).

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[1] The abbreviations recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochemistry 5*, 2485 (1965); *6*, 362 (1966)) are used in this paper. L-Pyroglutamic acid (L-2-pyrrolidone-5-carboxylic acid) is abbreviated Pyr, using the first three letters of either chemical or the trivial name. We feel that this abbreviation should be used instead of PCA because it is more in line with the nomenclature of the other natural amino acids.

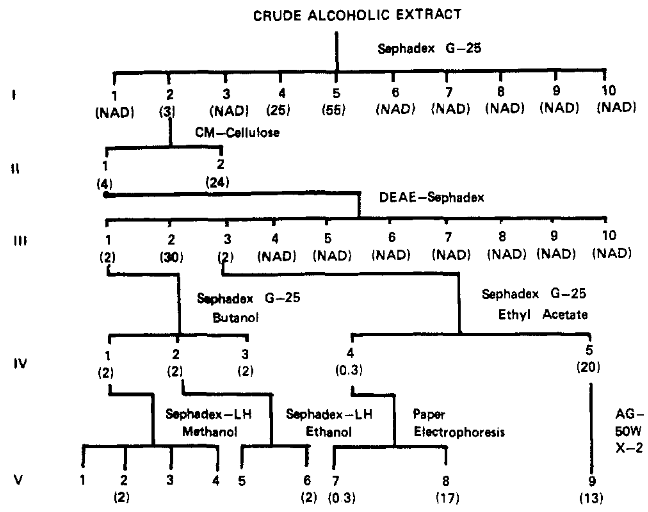

FIGURE 1: Diagram of the fractionation of the alcoholic extract of venom of *B. jararaca*. The number in parentheses under each fraction indicates the angiotensin-converting enzyme inhibitory activity expressed as I_{50}. NAD: no activity detected at 100 μg/ml.

N-Bromosuccinimide oxidations were carried out as described by Ramachandran and Witkop (1967).

Hydrolysis with Papain. The peptide (3 mg) was dissolved in 3 ml of pH 7.5 buffer (1 ml of 0.1 M ammonium bicarbonate, 20 ml of 0.1 M ammonium acetate), 0.3 ml of papain solution (0.1 ml of papain suspension, Worthington Biochemical Corp., Freehold, N. J., and 10 mg of sodium cyanide diluted to 1 ml with pH 7.5 buffer) was added, and the mixture was diluted to 6 ml with the same buffer. After a 20-hr incubation at 37°, 1.5 ml of glacial acetic acid was added and the solution was freeze-dried.

Hydrolysis with trypsin was carried out as described by Car negie (1969); crystalline trypsin (Worthington Biochemical Corp., Freehold, N. J.) was used.

Quantitative Amino Acid Analyses. The samples were hydrolyzed at 110° for 20 hr with constant-boiling hydrochloric acid in glass ampoules sealed *in vacuo*. After drying in a desiccator *in vacuo* over potassium hydroxide, the residue was applied to a single column Technicon amino acid analyzer (Morris and Piez, 1960). The amount of tryptophan was determined by comparing the optical density at 278 mμ of the peptide solution in 0.2 M acetic acid with that of a standard solution of tryptophan in the same solvent. The acid hydrolysates of the synthetic peptides were analyzed in the Jeolco amino acid analyzer (Jeolco Inc., Medford, Mass.) that utilizes the method developed by Spackman *et al.* (1958).

Paper Electrophoresis. For preparative separations, electrophoresis was carried out at approximately 60 V/cm for a period of 50–80 min, using a mixture of formic acid–acetic acid–water (26:120:1000, v/v). Peptide bands were detected by uv absorption or by cutting strips (lengthwise) and spraying them with the following reagents: ninhydrin (Barrollier, 1961), Sakaguchi (Block *et al.*, 1958), Pauly (Block *et al.*, 1958), Rydon (Wolff, 1969), and Ehrlich (Block *et al.*, 1958). The desired bands were cut out and eluted with 0.2 M acetic acid. Aliquots were hydrolyzed for quantitative amino acid analyses and the remainder, if the band was ninhydrin positive, was used for Edman degradation. Analytical electrophoresis was performed following the technique described by Werum *et al.* (1960). Mobilities are measured relative to two reference dyes, Apolon and Amaranth; the distance between the two dyes is defined as 100 Am units. The mobility of a peptide, in Am units, is obtained by dividing the distance traveled by the peptide with respect to the Apolon spot by the Amaranth–Apolon distance and multiplying by 100.

Assays for Inhibition of Angiotensin-Converting Enzyme. The inhibitory activity of the peptides was determined by measuring the angiotensin-converting enzyme activity in the presence of different concentrations of peptide from 0.01 to 100 μg per ml. The amount in micrograms per milliliter needed to inhibit 50% of the enzymatic activity is defined as the I_{50}. The enzymatic activity at each peptide concentration was assayed by the method of Cushman and Cheung (1969, 1971). A 5 mM solution of hippuryl-L-histidyl-L-leucine in 100 mM potassium phosphate buffer (pH 8.3) (chloride concentration 300 mM) was incubated with an extract of rabbit lung acetone powder at 37° for 30 min. The amount of hippuric acid liberated was measured spectrophotometrically (228 mμ) after extraction with ethyl acetate. A similar assay was used to follow the fractionation of the venom except that an extract of dog lung was used as a source of enzyme.

Paper and Thin-Layer Chromatography. Paper chromatography was carried out by the descending technique on Whatman No. 3MM paper. The solvent systems employed were 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v) and ethyl acetate–pyridine–water (20:10:11, v/v). Thin-layer chromatograms on silica gel were developed with methanol or dioxane–water (1:1, v/v).

Edman Degradation. The technique of sequential degradation plus dansylation described by Gray (1967) was employed. The dansyl peptides were hydrolyzed with constant-boiling hydrochloric acid for 6 hr at 110°. The dansylamino acids were identified by thin-layer chromatography on silica gel (chloroform–benzyl alcohol–acetic acid, 100:30:5, v/v) (David *et al.*, 1963) and high-voltage electrophoresis.

Synthesis. All the proposed sequences were synthesized by the solid-phase procedure developed by Merrifield (1969). The amino acid derivatives employed were of the L configuration. Starting material was Boc-Pro-resin (5 g; 0.5 mmole/g) and all the manipulations were carried out in a reaction vessel similar to that described by Stewart and Young (1969) using a mechanical shaker (Schwarz-Mann, Orangeburg, N. Y.) modified to rotate the vessel through 360°. The yields of crude peptide varied from 38 to 57%. Crude peptides were purified by countercurrent distribution (1-butanol–pyridine–acetic acid–water, 4:2:1:7, v/v) before removal of the ω-nitro-protecting groups by hydrogenolysis (10% Pd on charcoal; aqueous methanol containing 1 equiv of HCl). The N^{im}-2,4-dinitrophenyl-protecting group of the histidine residue was removed with mercaptoethanol as described by Chillemi and Merrifield (1969). The free peptides were submitted to two more steps of purification: ion-exchange chromatography on DEAE-cellulose (ammonium bicarbonate) and gel permeation chromatography on Sephadex G-25 or Bio-Gel P-2 (0.2 M acetic acid). These chromatographic procedures were performed as described above. The yields of purified peptides varied from 11% for V-9 to 32% for V-8.

Results

Isolation and Characterization of the Active Components. A crude alcoholic extract of *B. jararaca* venom was prepared by the technique described by Ferreira (1965). Figure 1 describes schematically the fractionation of this crude extract by a combination of gel permeation, ion exchange, and parti-

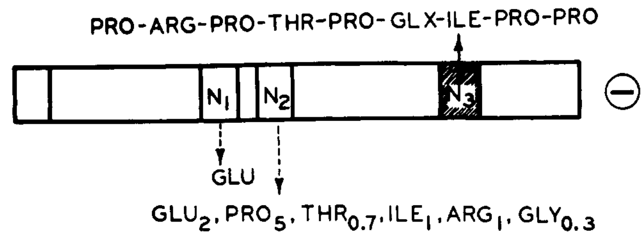

FIGURE 2: Diagram of the electrophoretic separation of the *N*-bromosuccinimide oxidation mixture of V-2. Only the shaded bands gave positive ninhydrin reactions. Solid arrows indicate the sequences obtained by Edman degradation. Broken arrows point to the amino acid composition of the ninhydrin negative bands.

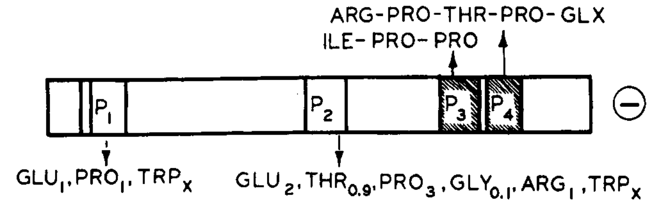

FIGURE 3: Diagram of the electrophoretic separation of the papain hydrolysate of V-2. The presence of tryptophan was ascertained only qualitatively by a positive Ehrlich reaction. See Figure 2 for explanations.

tion chromatography. The composition of the different active fractions was determined by thin-layer and paper chromatography and by paper electrophoresis. However, these techniques provided necessary, but not sufficient, proof of homogeneity. Thus, fraction IV-1 showed only one component by thin-layer and paper chromatography, but quantitative amino acid analyses (Table I) indicated the presence of a glycine-containing impurity that was subsequently reduced in amount (fraction V-2) by two-stage chromatography on LH-Sephadex. Fraction V-6 was still a mixture of two components (V-6-I and V-6-II) as judged by paper chromatography. One of these components (V-6-II) was identical with IV-3. Fractions IV-3 and V-7 proved to be homogeneous by chromatographic and electrophoretic criteria. However, quantitative amino acid analyses of IV-3 and V-7 indicated a low recovery of aspartic acid that we interpreted, originally, as due to contamination with a component containing no aspartic acid. As will be discussed below, this deviation from the expected value was later shown to be due to an intrinsic property of the amino acid sequence of these peptides and not to inhomogeneity. Fractions V-8 and V-9 behave as homogeneous peptides by all the analytical techniques applied. The rest of the active fractions shown in Figure 1 were not purified further for lack of material.

Determination of the Amino Acid Sequences. The characteristics of all the isolated components of the venom on gel permeation chromatography and their quantitative amino acid compositions indicated that they were peptides ranging in molecular size from nona- to tridecapeptide. The negative reaction with ninhydrin observed in all cases pointed to a blocked N-terminal α-amino group. The presence of at least 1 mole of glutamic acid in every peptide, and the early reports on the isolation of pyroglutamyl peptides from snake venoms (Kato *et al.*, 1966), suggested a similar structural feature here. One of the bradykinin-potentiating peptides isolated from the venom of *B. jararaca* (V-3-A) was also shown to be an N-terminal pyroglutamyl peptide (Greene *et al.*, 1970). Low-resolution mass spectrometry of a methylated (diazomethane) sample of V-2 showed prominent peaks at m/e 84 and 112, confirming the assumption of an N-terminal pyroglutamyl residue (Altamura *et al.*, 1970). The assumption that all other components had the same N-terminal residue was eventually confirmed by synthesis.

Oxidative cleavage of V-2 with *N*-bromosuccinimide yielded a ninhydrin- and Sakaguchi-positive fragment (N_3), albeit in low yield (Figure 2). Edman degradation of this portion yielded the sequence Pro-Arg-Pro-Thr-Pro-Glx-Ile-Pro-Pro. The ninhydrin-negative fragment N_1 was undoubtedly the oxidation product of the N-terminal portion Pyr-Trp. The remaining ninhydrin-negative fragment N_2 was the oxidized, but uncleaved, starting material. When a sample of nonapeptide N_3 was submitted to two cycles of Edman degradation, the dansyl derivative of the remaining heptapeptide showed a charge of -1 on paper electrophoresis. After four more cycles of degradation, the dansyl derivative of the residual tripeptide again showed a charge of -1. These mobilities indicate that glutamine and a C-terminal free carboxylic group were present in this fraction.

Papain was found to be the enzyme of choice for the degradation of these peptides. In the case of V-2, two ninhydrin-positive bands were isolated after electrophoretic fractionation of the papain hydrolysate (Figure 3). Their sequences confirmed the results of the *N*-bromosuccinimide oxidation. Of the two ninhydrin-negative fragments, P_1 is the N-terminal tripeptide Pyr-Trp-Pro, and P_2 the N-terminal octapeptide Pyr-Trp-Pro-Arg-Pro-Thr-Pro-Gln. The structure of V-2 is, therefore: Pyr-Trp-Pro-Arg-Pro-Thr-Pro-Gln-Ile-Pro-Pro.

Electrophoretic fractionation of the *N*-bromosuccinimide oxidation mixture of fraction V-9 (Figure 4) yielded a ninhydrin- and Sakaguchi-positive fragment N_3 that was sequenced by the Edman procedure. The ninhydrin-negative band N_1 corresponds to the oxidized form of the N-terminal tetrapeptide Pyr-Gly-Gly-Trp, and N_2 corresponds to the oxidized starting material. Trypsin digestion of V-9, using the conditions described for cleavage of Arg-Pro bonds (Carnegie, 1969), yielded a mixture from which the C-terminal heptapeptide fragment T_3 was isolated by paper electrophoresis (Figure 5) and sequenced by the Edman technique. The dansyl derivative of this heptapeptide showed an electrophoretic mobility corresponding to a -2 charge. From these data it can be concluded that the amino acid sequence of V-9 is: Pyr-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro.

Oxidation of fraction V-7 with NBS was not useful for the isolation of ninhydrin-positive fragments for further degradation. However, application of the technique of sequential oxidation at room temperature and then at 100° (Shaltiel

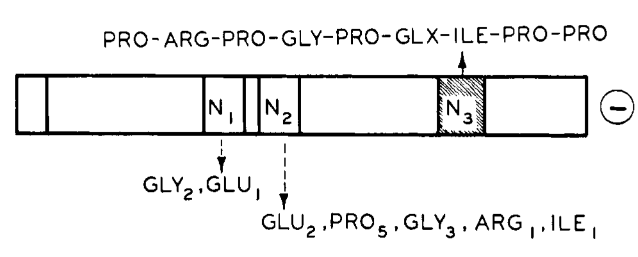

FIGURE 4: Diagram for electrophoretic separation of the *N*-bromosuccinimide oxidation mixture of V-9. See Figure 2 for explanations.

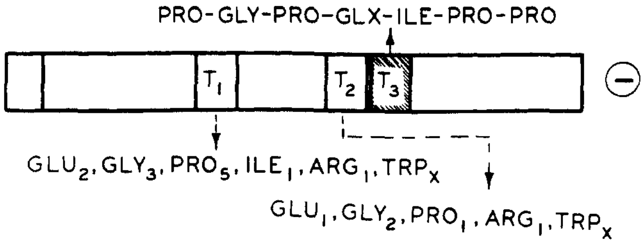

FIGURE 5: Diagram of the electrophoretic separation of the trypsin hydrolysate of V-9. See Figures 2 and 3 for explanations.

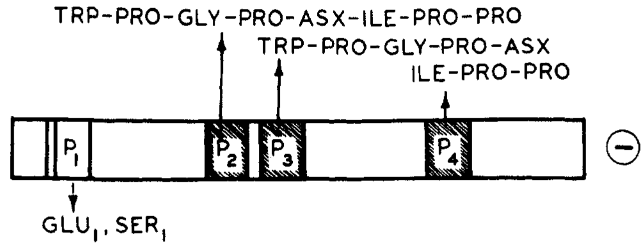

FIGURE 7: Diagram of the electrophoretic separation of the papain hydrolysate of V-8. See Figures 2 and 3 for explanations.

and Patchornik, 1963), followed by dansylation, showed that the amino acid residue following tryptophan and histidine was proline. Papain digestion of V-7 followed by preparative paper electrophoresis (Figure 6) yielded two ninhydrin-positive fragments P_3 and P_4. P_3 was sequenced by the Edman technique without difficulty. However, in the case of P_4, two dansylamino acids of unequal intensity were detected after the first, second, and third cycles of the Edman degradation. These were DNS-Pro and DNS-His, DNS-His and DNS-Pro, and DNS-Pro and DNS-Glu. The first component was always the strongest. After the fourth cycle of the Edman degradation only DNS-Glu was observed. We interpreted these results to indicate the cleavage of the Pro–His bond during the first cycle of the Edman degradation. Identical results were obtained on Edman degradation of the similar fragment obtained after papain hydrolysis of a synthetic sample of V-7. The electrophoretic mobility of V-7 at pH 9.3 indicates a charge of −1, and since the C-terminal tripeptide Ile-Pro-Pro (P_3) has an electrophoretic mobility corresponding to a free carboxyl group, both side chain carboxyls are undoubtedly amidated. The remaining ninhydrin-negative band P_1 is the N-terminal dipeptide Pyr-Asn. The amino acid sequence of this decapeptide V-7 is, then: Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro.

Electrophoretic fractionation of the papain hydrolysate of V-8 showed the pattern described schematically in Figure 7. The three ninhydrin-positive bands were sequenced by the Edman technique. The peptides P_1 and P_3, containing N-terminal tryptophan, displayed the same behavior on Edman degradation as described above for fragment P_3 on V-7. The ninhydrin-negative band P_1 constitutes the N-terminal dipeptide Pyr-Ser. The electrophoretic mobility of V-8 indicates a net charge of −1 that undoubtedly corresponds to the C-terminal carboxyl group of proline. The amino acid sequence of V-8 can, therefore, be written as: Pyr-Ser-Trp-Pro-Gly-Pro-Asn-Ile-Pro-Pro.

As mentioned above, fraction V-6 was known to be a mixture of two components, but the small amount available did not allow further fractionation. One of these components was identical with IV-3, but the amounts of this fraction available did not permit structural studies. However, it was felt that with the structural information gathered from the other fractions, and the pattern of papain cleavage observed so far, it was possible to interpret the papain hydrolysate of V-6, and to propose amino acid sequences for the two components V-6-I and V-6-II. The electrophoretic separation of the papain hydrolysate of V-6 is depicted in Figure 8. The three ninhydrin-positive bands were sequenced by the Edman procedure. The ninhydrin-negative band P_1 behaved, on paper chromatography, as a mixture of two components, one Ehrlich positive and the other Ehrlich negative. These two components were designated P_{1a} and P_{1b} and assigned the sequences Pyr-Trp-Pro and Pyr-Asx, respectively, on the basis of the amino acid composition and the patterns of papain digestion observed in the preceding cases. Since the uv absorption of V-6 indicated the presence of one Trp residue per molecule, we concluded that the fragments P_{1a}, P_5, and P_3 originated from V-6-I and that fragments P_{1b}, P_4, and P_3 originated from V-6-II. The electrophoretic mobility of these two components pointed out a net charge of 0 from pH 4.7 to 9.3. Therefore, the amino acid sequences of these peptides can be written as follows: V-6-I: Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro; V-6-II: Pyr-Asn-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro. The ninhydrin-negative band P_2 is probably also a mixture of two components originated from the incomplete hydrolysis of the Pro–Arg and Asn–Trp bonds.

Synthesis. The synthesis of the proposed structures was carried out by the solid-phase procedure developed by Merrifield (1969). The technique followed was essentially that described by Stewart and Young (1969). The deprotection of the growing peptide chain was successfully carried out with 1 N hydrochloric acid in acetic acid, even after the introduction of the glutamine residue. However, the comparatively mediocre yields of crude peptide would seem to indicate that a small amount of peptide was removed from the resin

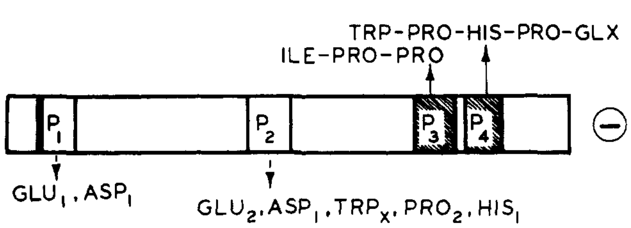

FIGURE 6: Diagram of the electrophoretic separation of the papain hydrolysate of V-7. See Figures 2 and 3 for explanations.

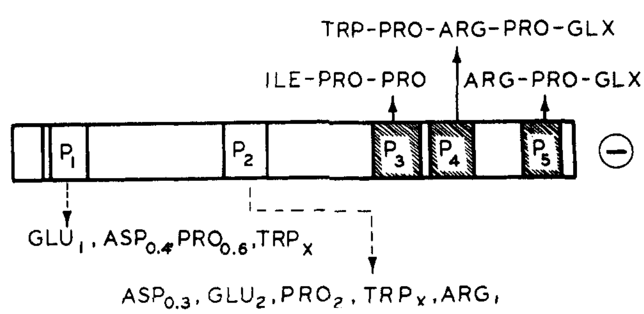

FIGURE 8: Diagram of the electrophoretic separation of the papain hydrolysate of V-6. See Figures 2 and 3 for explanations.

TABLE I: Amino Acid Composition of Peptides Isolated from *B. jararaca* Venom and Their Synthetic Counterparts.[a]

Amino Acid	V-2 N	V-2 S	V-6-I N[b]	V-6-I S	V-6-II N[c]	V-6-II S	V-7 N	V-7 S	V-8 N	V-8 S	V-9 N	V-9 S
Aspartic acid			0.29		0.60	0.59	0.66	0.60	0.99	0.96		
Threonine	0.89	0.92										
Serine									0.89	0.79		
Glutamic acid	2.24	1.85	2.02	2.02	2.01	2.03	2.01	1.98	1.05	1.00	2.00	2.03
Proline	5.58	5.35	4.27	4.08	3.90	4.05	4.50	4.18	4.10	4.57	5.00	5.46
Glycine	0.16								1.00	0.96	2.76	2.71
Isoleucine	0.86	0.93	0.92	0.93	1.00	0.91	1.00	0.85	0.91	0.92	0.94	0.95
Histidine							0.90	1.00				
Arginine	1.00	0.91	1.00	1.01	0.99	0.95					1.00	0.94
Tryptophan	0.84	1.05	0.82	0.93	0.82	0.95	0.80	1.04	0.71	1.03	0.91	1.07

[a] Expressed in moles per mole of peptide, based on the minimum molecular weight. Tryptophan was determined spectrophotometrically. N: natural; S: synthetic. [b] V-6 was used for comparison. [c] IV-3 was used for comparison.

TABLE II: Comparison of Natural and Synthetic Peptides.[a]

	V-2 Natural	V-2 Synthetic	V-7 Natural	V-7 Synthetic	V-8 Natural	V-8 Synthetic	V-6-I Natural[b]	V-6-I Synthetic	V-6-II Natural[c]	V-6-II Synthetic	V-9 Natural	V-9 Synthetic
Paper electrophoresis												
pH 3.3	+11	+11	+13	+12	−7	−7	+11	+12	+12	+12	+10	+10
4.7	0	0	−4	−4	−20	−20	+1	−1	+1	−1	−13	−13
7.2	−3	−3	−12	−12	−24	−24	−2	−2	−2	−2	−17	−17
7.9	−3	−3	−18	−20	−24	−24	−4	−3	−4	−3	−20	−20
9.3	−4	−4	−24	−24	−24	−24	−4	−3	−4	−4	−20	−20
Thin-layer chromatography												
R_F (methanol)			0.32	0.32	0.35	0.35					0.24	0.24
R_F (dioxane–water)	0.05	0.05										
R_F (1-butanol–HOAc–H_2O							0.18	0.18	0.08	0.08		
Paper chromatography												
R_F (1-BuOH–PyAcOH–H_2O)	0.46	0.46	0.50	0.50	0.62	0.62	0.69	0.69	0.53	0.53		
R_F (EtOAc–Py–H_2O)											0.22	0.22
Rotation												
$[\alpha]^{25}_D$ 0.2 M AcOH		−185°	−193°	−190°		−197°		−190°		−180°	−202°	−184°
c		(0.7)	(1.0)	(0.8)		(1.0)		(0.9)		(0.7)	(0.3)	(0.6)
Inhibitory activity												
I_{50} (µg/ml)	2	5	9	8	39	35	3	1	3	3	13	17

[a] See section on Materials and Methods for details. [b] V-6 was used for comparison. [c] IV-3 was used for comparison.

V-2	Pyr-Trp-Pro-Arg-Pro-Thr-Pro-Gln-Ile-Pro-Pro
V-6-I	Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro
V-6-II	Pyr-Asn-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro
V-7	Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro
V-8	Pyr-Ser-Trp-Pro-Gly-Pro-Asn-Ile-Pro-Pro
V-9	Pyr-Gly-Gly-Trp-Pro-Arg-Pro-Gly-Pro-Glu-Ile-Pro-Pro

FIGURE 9: Amino acid sequences of the angiotensin-converting enzyme inhibitors isolated from the venom of *B. jararaca.*

at each step of deprotection. In an attempt to improve this situation, compound V-9 was resynthesized, using 25% trifluoroacetic acid in methylene chloride for the removal of the *tert*-butyloxycarbonyl protecting group. The crude yields obtained with this procedure were not significantly better than those obtained by the other method. Countercurrent distribution of this crude material showed the presence of significant amounts of trifluoroacetyl peptides. It is possible that, because of the higher basicity of the imino group of proline, the usual washings with triethylamine were not sufficient to remove all the trifluoroacetic acid.

The purified synthetic peptides showed electrophoretic and chromatographic mobilities identical with those of their natural counterparts (Table II). In the two cases in which enough of the natural material was available for measurements, the specific rotation was in agreement with that of the corresponding synthetic peptide, which would confirm the assumption that all the amino acids present in the peptides isolated from the venom of *B. jararaca* are of the L configuration. Paper electropherograms of the papain or trypsin hydrolysates of the synthetic peptides showed patterns identical with those described above for the natural products. Papain hydrolysates of V-6-I and V-6-II, run side by side on paper electrophoresis, reproduced the pattern of the papain hydrolysate of V-6. The papain hydrolysate of synthetic V-6-II was identical with that of fraction IV-3.

The recovery of aspartic acid in the acid hydrolysates (Table I) of the two synthetic peptides containing the Asn-Trp sequence, V-7 and V-6-II, was only 60% of the theoretical value, as it had been in the case of the natural peptides. The recovery of tryptophan in these hydrolysates was also lower than usual. These observations would seem to indicate that a reaction involving the side chain of these two neighboring amino acid takes place under acidic conditions, leading to the formation of a compound that cannot be further degraded into aspartic acid and tryptophan.

The antiotensin-converting enzyme inhibitory activities of natural and synthetic samples are in satisfactory agreement (Table II).

Discussion

Fractionation of the venom of *B. jararaca*, in a search for the compounds responsible for the angiotensin-converting enzyme inhibitory activity, has led to the isolation, characterization, and synthesis of six peptides that have striking similarities to the bradykinin-potentiating peptides isolated by Ferreira *et al.* (1970a) from the same venom. The amino acid composition of some of these peptides is indeed identical with that of the peptides isolated by these workers. Preliminary results indicate that the synthetic peptides described in this paper potentiate very strongly the hypotensive action of bradykinin in rats (S. L. Engel and B. Rubin, 1971, manuscript in preparation). It seems, therefore, that both biological activities are caused by the same compound. In all probability the peptides described in this paper, with the exception of V-6-II, are identical with those isolated by Ferreira *et al.* (1970a) and designated as IV-1-Bα (V-2), IV-1-D (V-6-I), IV-1-Bβ (V-7), IV-1-A (V-8), and III-1-A (V-9). However, direct comparisons will be needed to confirm these identities.

None of the venom fractions characterized in this study showed an amino acid composition similar to that of the pentapeptide V-3-A of Ferreira *et al.* (1970a). However, there were indications (amino acid composition and molecular size) that a peptide similar to or identical with this pentapeptide was present in the fraction I-4 of the Sephadex fractionation.

Since there is only a superficial resemblance between the structure of V-3-A (Pyr-Lys-Trp-Ala-Pro) and that of the nona- to tridecapeptides described in this paper it was important to compare the angiotensin-converting enzyme inhibitory activities of these two groups of peptides under the same conditions. A synthetic sample of V-3-A (M. A. Ondetti and J. Pluscec, 1971, manuscript in preparation) showed a very high inhibitory activity in the assay *in vitro* described above (I_{50} = 0.05 μg/ml). However, this inhibitory activity is rapidly destroyed if V-3-A is incubated with the crude enzyme in the absence of substrate and chloride ion (D. W. Cushman and H. S. Cheung, 1971, unpublished data). When similar incubations are carried out with any of the synthetic peptides described above the inhibitory activity is unaltered. The results of the inhibition of angiotensin I conversion *in vivo* (Engel *et al.*, 1971) showed a similar pattern. The pentapeptide V-3-A elicits a very transient blocking of the pressor response of angiotensin I in rats, whereas the longer peptides produce a marked and long-lasting inhibition. These differences could be attributed to the higher resistance toward enzymatic degradation exhibited by these peptides.

There is a considerable degree of similarity among all the converting-enzyme inhibitors described in this paper (Figure 9). Schematically, they could be described as being formed by the combination of a C-terminal portion identical for all of them (Ile-Pro-Pro), a middle sequence that can be a tetra- (Pro-X-Pro-Glx) or a hexapeptide (Pro-X-Pro-Y-Pro-Glx), and an N-terminal peptide of the general structure Pyr-M-N-Trp. Comparison of the I_{50} of the two decapeptides V-8 and V-6-II which have identical N- and C-terminal sequences and a middle tetrapeptide sequence seems to indicate that the basic residue in position 5 is capable of greatly enhancing the inhibitory activity.

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Gas Chromatographic Determination of Gangliosides in Mouse Cell Lines and in Virally Transformed Derivative Lines*

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ABSTRACT: The distribution of gangliosides in established mouse cell lines in tissue culture was investigated, before and after transformation of the cells with SV40, a tumorigenic DNA virus. A suitable chemical derivatization and gas–liquid chromatography procedure was developed for the carbohydrate residues of the gangliosides. Epithelial-like cell lines from A AL/N strain mouse and highly contact-inhibited fibroblastic 3T3 cell lines from both Swiss and Balb c strain mouse had drastically reduced content of the disialotetrasaccharide ganglioside (G_{D1a}) after the SV40 virus induced transformation in culture. This finding is in complete agreement with our previous observation on these and similar cell lines employing thin-layer chromatography and colorimetric techniques. The method of derivatization and of gas–liquid chromatography for the carbohydrate residues of the gangliosides allowed definitive identification of the mouse gangliosides, and represents an accurate and internally consistent method suitable for the quantitation of the small amount of various gangliosides present in cell lines.

We have reported that when established mouse cell lines in culture are transformed by the tumorigenic DNA viruses polyoma or SV40, a change occurs in the various gangliosides[1] present in the cells. The amount of the higher gangliosides, especially the disialotetrasaccharide ganglioside G_{D1a}, is drastically reduced in the virally transformed cells (Mora *et al.*, 1969; Brady and Mora, 1970). The ganglioside analysis was carried out on the glycolipid extracts after thin-layer chromatography by standard colorimetric methods.

Higher gangliosides, including G_{D1a} and G_{M1}, were also detected by thin-layer chromatography in various other cultivated cells from different species, including in human fibroblasts (Hakomori, 1970), adult hamster kidney epitheloid cells, monkey kidney, etc. (Klenk and Choppin, 1970), and also in two types of mouse cells (Yogeeswaran *et al.*, 1970), but are apparently absent in a particular baby hamster kidney fibroblast cell line (BHK21) previously employed to study

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[1] The nomenclature used for gangliosides is from Svennerholm (1963); G_{D1a}, N-acetylneuraminylgalactosyl-N-acetylgalactosaminyl[N-acetylneuraminyl]galactosylglucosylceramide; G_{M3}, galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]galactosylglucosylceramide; G_{M2}, N-acetylgalactosaminyl-[N-acetylneuraminyl]galactosylglucosylceramide; hematoside, G_{M3}NAc or G_{M3}NG, N-acetyl- or N-glycolylneuraminylgalactosylglucosylceramide, respectively.