from subsequent fractions. The visible and infrared absorption spectra and, particularly, the NMR chemical shifts of 6-methyl, 8-aromatic, and 2-amino protons are all consistent with the 7-ethoxy chromophore; the material was identical with that obtained by procedure A. Anal. ($C_{26}H_{34}N_4O_5$) C, H, N.

3,10,12-Trimethyl-2*H*,6*H*-oxazino[3,2-*b*]phenoxazin-2-one-5,7-bis[carbonyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-*N*-methylvaline (Threonine Hydroxyl) Lactone] (IVb). Ib (200 mg, 0.16 mmol) was reduced with PtO_2 in 100 ml of methanol. Under nitrogen, the reduction mixture was filtered to remove catalyst, with the filtrate adding directly to 2 ml of pyruvic acid previously placed in the suction flask. After 4.5 hr, the red solution was concentrated under vacuum to ca. 5 ml; ethanol (40 ml) and water (100 ml) were added and the solution was extracted twice with ethyl acetate (200 ml) and washed three times with 100-ml portions of water. The extract was dried over Na_2SO_4 (anhydrous). Filtered and evaporated, the residue was then passed through a Sephadex LH-20 column in 95% ethanol; fractions which contained mainly IVb and were relatively free of starting material, as evidenced by TLC (acidic silica gel, Ciferri solvent), were rechromatographed on Sephadex LH-20. Very careful fractionation and addition of ether to the fractions yielded crystals of IVb·H_2O (190 mg, 90%): mp 238–240°; $\lambda_{max}^{CHCl_3}$ (ϵ) 319 (8700), 397 (6400), 456 nm (8700); $[\alpha]^{20}_{644}$ −101 ± 12° (*c* 0.10, $CHCl_3$). Anal. ($C_{65}H_{88}N_{12}O_{17}\cdot H_2O$) C, H, N.

7-Hydroxyactinomycin D (VIb). IVb (66 mg, 0.05 mmol) in 5 ml of absolute ethanol was stirred with 35 mg (0.154 mmol) of DDQ in 1 ml of absolute ethanol for 2.5 hr under nitrogen and the mixture was evaporated to dryness. The residue was chromatographed on 5 g of acidic alumina and the first eluate, in CH_2Cl_2, and the second eluate, in ethyl acetate, were evaporated to yield 34.2 mg (50%) of Vb. This material, without further purification, was subjected to cleavage with 20 ml of 50% ethanol and 3 g of silica gel, as follows. The mixture was stirred for 1 hr, filtered, and concentrated. The concentrate was extracted with ethyl acetate, the extract evaporated, and the residue was chromatographed in 95% ethanol through Sephadex LH-20. Careful fractionation gave relatively pure VIb·$2H_2O$ (11.5 mg, 20% on the basis of Vb): mp 255–257° dec; $\lambda_{max}^{CHCl_3}$ (ϵ) 469 nm (23000); $[\alpha]^{20}_{644\pm10}$ −214 ± 20° (*c* 0.021, $CHCl_3$). Anal. ($C_{62}H_{86}N_{12}O_{17}\cdot 2H_2O$) C, H, N.

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Synthesis and Evaluation of [Des-Asp^1]angiotensin I as a Precursor for [Des-Asp^1]angiotensin II ("Angiotensin III")

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The nonapeptide [des-Asp^1]angiotensin I (IV), synthesized by Merrifield's solid-phase procedure, was tested as a possible substrate for the converting enzymes from porcine lung and plasma. IV, [des-Asp^1]angiotensin II (III), [des-(Asp^1,Arg^2)]angiotensin II (V), [des-(Asp^1,Arg^2,Val^3)]angiotensin II (VI), [Sar^1,Ile^8]angiotensin II (VII), and [des-Asp^1,Ile^8]angiotensin II (VIII) possessed 0.5, 20, 2, 0, <0.1, and <0.01% of the inotropic activity (rabbit atria), 1, 15, 5, 0, 3, and 0% secretory activity of the cat adrenal medulla, and 0.0, 150, 1, 0.5, 3, and 10% of the adrenal steroidogenic activity of angiotensin II, respectively. When tested for their antagonistic activity in the above tissues, only VII and VIII were found to inhibit responses to angiotensin II. The pA_2 values for VII and VIII were 8.31 and 10.0 in the adrenal cortex and 9.31 and 9.16 in the adrenal medulla, respectively. All these peptides were also tested as product inhibitors for the plasma and lung converting enzymes. With the plasma enzyme, the ID_{50} values were II, 1.6×10^{-4} *M*; III, 5×10^{-5} *M*; V, 1.2×10^{-4} *M*; VI, 5×10^{-4} *M*; VII, 5×10^{-5} *M*; VIII, 5×10^{-4} *M*. Thus, IV is a good substrate for converting enzymes from lung and plasma while all other compounds were inhibitors of these enzymes. The most potent inhibitors of converting enzyme were III followed by VII and VIII. With the exception of II and III, all the other analogs had very low intrinsic activities, per se. These results suggest (a) an alternate pathway for the formation of the heptapeptide III, viz., by the action of converting enzyme on the nonapeptide IV, and (b) that III may also be acting as inhibitor of the converting enzyme by the feedback mechanism.

It is a widely accepted concept that (a) converting enzyme (dipeptidylcarboxypeptidase), particularly in lung, converts the decapeptide angiotensin I (I) into the octapeptide angiotensin II and (b) angiotensin II acts on vascular smooth muscle to produce vasoconstriction and on the adrenal cortex to stimulate aldosterone biosynthesis. However, recently it has been shown that the C-terminal heptapeptide "angiotensin III" (III) is also responsible for the adrenal cortical stimulation.[1-4] There are two possible pathways by which the heptapeptide III could be formed

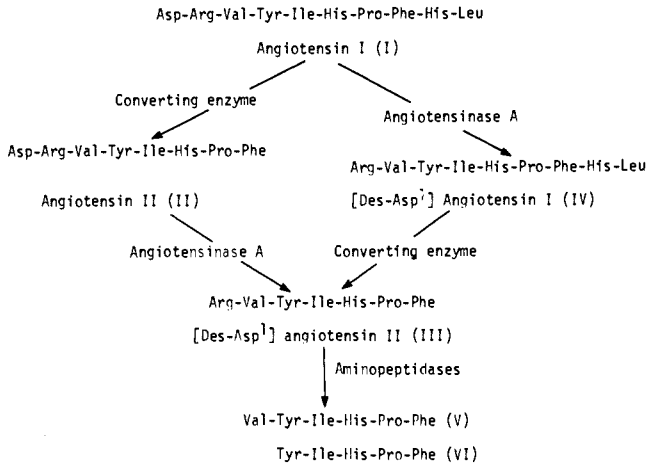

Figure 1. Possible pathways for the formation of [des-Asp¹]angiotensin II.

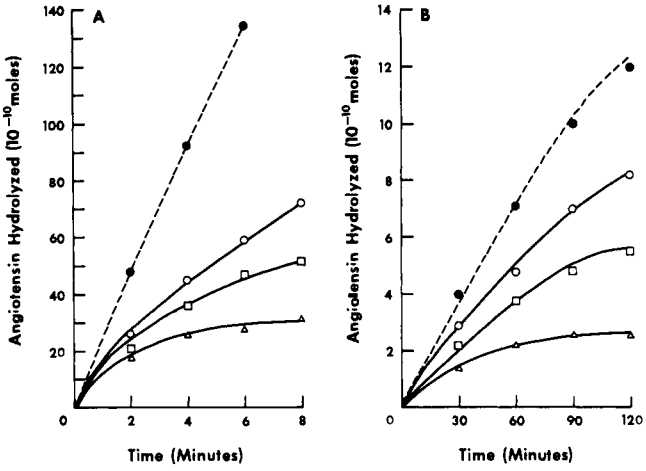

Figure 2. (A) Activity of porcine lung converting enzyme with angiotensin I (1.2×10^{-4} *M*, ●- -●) and [des-Asp¹]angiotensin I as substrate. The concentrations of [des-Asp¹]angiotensin I depicted are 2.7×10^{-5} *M* (▽—▽), 4.25×10^{-5} *M* (□—□), and 8.5×10^{-5} *M* (○—○). (B) Activity of porcine plasma converting enzyme with angiotensin I (6.4×10^{-5} *M*, ●- -●) and [des-Asp¹]angiotensin I as substrate. The concentrations of [des-Asp¹]angiotensin I shown are 1.4×10^{-5} *M* (▽—▽), 3.5×10^{-5} *M* (□—□), and 7.0×10^{-5} *M* (○—○).

(Figure 1): first, from the octapeptide angiotensin II by the cleavage of N-terminal aspartic acid by the action of plasma "angiotensinase A", or alternatively from the nonapeptide IV by the cleavage of the C-terminal dipeptide, histidylleucine, by the action of converting enzyme. The nonapeptide IV may be formed by the removal of aspartic acid from the N terminus of angiotensin I.[1] The first pathway, and the existence of the heptapeptide III in the plasma and tissues, is well documented in the literature.[5-8] But the alternate pathway, and the existence of nonapeptide IV in plasma or tissues, has not been reported. We therefore undertook the synthesis of the nonapeptide IV to test as a substrate for the converting enzymes from porcine lung and plasma and for studying its biological activity on isolated rabbit atria and retrogradely perfused cat adrenals.

[Des-Asp¹]angiotensin I (IV) was synthesized by the solid-phase procedure of Merrifield.[9] The protocol used for synthesis was similar to the one previously described by Khosla et al.[10] for the synthesis of analogs of angiotensin II. Precautions were taken to avoid racemization by using 1-hydroxybenzotriazole as an additive during the coupling of *tert*-butyloxycarbonylimidazolylbenzylhistidine.[11] The crude nonapeptide was purified on a column of Sephadex G-25 using 1-butanol–pyridine–water (BPW) (10:2:5, upper phase) by the procedure reported earlier.[12] Fractions giving a Pauly-positive reaction were chromatographed on cellulose TLC plates using BPW as the solvent. Components with the same R_f values were pooled, evaporated to a small volume, and lyophilized; peptides giving correct amino acid analysis for the desired nonapeptide were then rechromatographed on a column of Sephadex G-25 using 1-butanol–acetic acid–water (4:1:5, upper phase). The fractions giving homogeneous spots on TLC were pooled, evaporated, and lyophilized.

Fractions in the column chromatography were cut with emphasis on purity rather than on yield and no attempt was made to rechromatograph the minor fractions for identification purposes. The homogeneity of the peptide was determined by (a) thin-layer chromatography in solvents of different pH, (b) electrophoresis at pH 1.95 and 8.6, and (c) amino acid analysis.

Assay and Action of Converting Enzyme. Converting enzyme activity in porcine lung and plasma was estimated by the *o*-phthaldehyde reaction with His-Leu and the fluorescence of the condensation product was determined (365-nm excitation, 495-nm emission) with an Aminco-Bowman spectrophotofluorometer.[13] This assay was verified by product isolation via high-voltage electrophoresis at pH 3.5 and 8.9 and by rat blood pressure bioassay of angiotensin II.

Results

The amount of His-Leu released from angiotensin I by porcine plasma and lung converting enzymes was linear with time up to 90 (6.4×10^{-5} *M*) and 6 min (1.2×10^{-4} *M*), respectively. However, the enzyme activities were not constant using [des-Asp¹]angiotensin I as substrate at the concentration indicated in Figure 2. As the concentration of [des-Asp¹]angiotensin I was increased, there was a tendency for the enzyme activities to become more linear. This unlinearity of enzyme activities was due to product inhibition. From the Lineweaver–Burk plot, the K_m values for the hydrolysis of angiotensin I and [des-Asp¹]angiotensin I by plasma converting enzyme were 80 and 35 μM, respectively. The K_m values for the hydrolysis of angiotensin I and IV by lung enzyme were 44 and 24 μM, respectively. The V_{max} for the plasma enzyme was 1.4×10^{-9} mol/mg/hr with angiotensin I as substrate and 1.0×10^{-9} mol/mg/hr with [des-Asp¹]angiotensin I. With the lung enzyme, V_{max} with angiotensin I was 1.0×10^{-6} mol/mg/min and with [des-Asp¹]angiotensin I, 6×10^{-7} mol/mg/min. With both enzymes, the hydrolysis of [des-Asp¹]angiotensin I required the presence of Cl^- to achieve maximal activity. In the absence of Cl^-, the activity of plasma enzyme on [des-Asp¹]angiotensin I was decreased to 50% while the activities of the enzyme on angiotensin I were reduced to 16% of the activity obtained in the presence of Cl^-. Similarly, in the absence of Cl^-, the activity of lung enzyme on [des-Asp¹]angiotensin I was decreased to 20% while the activity of the enzyme on angiotensin I was reduced to 7%. It is known that angiotensin II can act as an inhibitor of the plasma converting enzymes.[14] Fitz et al.[15] also reported that metabolites of angiotensin such as pentapeptide VI, hexapeptide V, and heptapeptide III can inhibit human lung converting enzyme. The present study demonstrated that hexapeptide V and heptapeptide III can inhibit porcine plasma converting enzyme. Angiotensin II and hexapeptide V were inhibitory at 5×10^{-5} *M*, but at 1×10^{-5} *M*

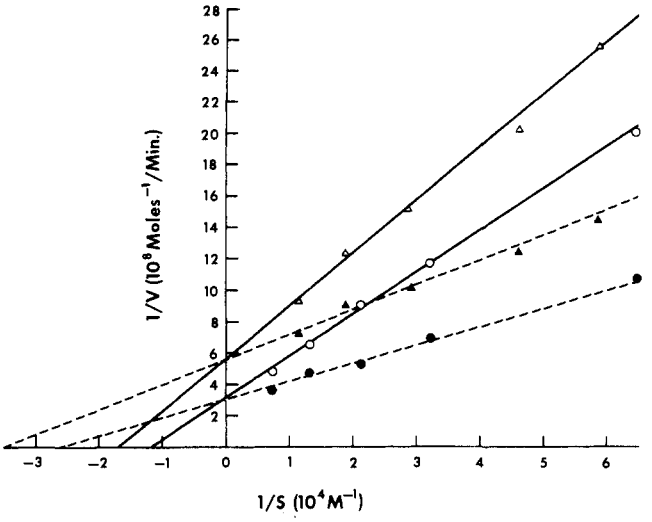

Figure 3. Double reciprocal plots of the effect of [des-Asp[1]]angiotensin II on porcine lung converting enzyme activity. The curves depicted represent angiotensin I as substrate (●- -●); conversion of angiotensin I in the presence of 1×10^{-5} *M* [des-Asp[1]]angiotensin II (○—○); [des-Asp[1]]angiotensin I as substrate (▲- -▲); the hydrolysis of [des-Asp[1]]angiotensin I in the presence of 2×10^{-5} *M* [des-Asp[1]]angiotensin II (△—△).

only the heptapeptide III caused inhibition. At very high concentrations (1×10^{-3} *M*), all three peptides completely inhibited the hydrolysis of substrate. The ID_{50} values for these peptides were 5.0×10^{-5}, 1.2×10^{-4}, and 1.6×10^{-4} *M* for heptapeptide III, hexapeptide V, and angiotensin II, respectively. The inhibitory properties of heptapeptide III were further characterized in porcine lung converting enzyme. The results indicated that heptapeptide III was a competitive inhibitor of angiotensin I and [des-Asp[1]]angiotensin I (Figure 3). With the lung enzyme, angiotensin II and the hexapeptide (2×10^{-5} *M*) inhibited the hydrolysis of angiotensin I by 10–15% without any inhibition on the hydrolysis of [des-Asp[1]]angiotensin I even when the concentration of angiotensin II and the hexapeptide V was increased to 5×10^{-5} *M*. The inhibitory effect of angiotensin II and hexapeptide V on angiotensin I hydrolysis can be overcome by higher concentrations of angiotensin I. The conversion of angiotensin I to angiotensin II or [des-Asp[1]]angiotensin I to heptapeptide III by converting enzyme also required the presence of tightly bound metal or metals on the enzyme itself. This was indicated by the fact that EDTA inhibited the hydrolysis of [des-Asp[1]]angiotensin I. When EDTA (0.1 m*M*) was preincubated with plasma or lung enzyme, the enzyme activities were completely inhibited.

In studies on plasma converting enzyme, *Bothrops jararaca* nonapeptide (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) at a concentration of 1×10^{-5} *M* almost abolished the enzymatic hydrolysis of angiotensin I and [des-Asp[1]]angiotensin I. The ID_{50} values of *B. jararaca* nonapeptide were 2.3×10^{-7} *M* for the hydrolysis of [des-Asp[1]]angiotensin I and 1×10^{-7} *M* for the conversion of angiotensin I. With lung converting enzyme, *B. jararca* nonapeptide at 1×10^{-6} *M* almost completely inhibited the conversion of angiotensin I and [des-Asp[1]]angiotensin I. The ID_{50} values for the *B. jararaca* peptide on angiotensin I and [des-Asp[1]]angiotensin I conversion were 4.5×10^{-8} and 3.5×10^{-8} *M*, respectively.

[Sar[1],Ile[8]]angiotensin II[10] inhibited porcine lung converting enzyme with 40% inhibition of enzyme activity at 5×10^{-5} *M* peptide concentration. At a concentration 1×10^{-5} *M*, [des-Asp[1],Ile[8]]angiotensin II[16] resulted in only 15% inhibition of the lung enzyme. Therefore, the substituted hepatpeptide was not nearly as active an inhibitor of converting enzyme as was [des-Asp[1]]angiotensin II.

[Sar[1],Ile[8]]angiotensin II and [des-Asp[1],Ile[8]]angiotensin II were also studied for agonist and antagonist activities in the adrenal cortex and medulla and rabbit atria. In adrenal cortical cell suspensions,[17] [Sar[1],Ile[8]]angiotensin II had 2% and [des-Asp[1],Ile[8]]angiotensin II, 10% steroidogenic activity relative to angiotensin II. In the retrogradely perfused cat adrenal, [Sar[1],Ile[8]]angiotensin II had 3% relative activity of angiotensin II with regard to catecholamine secretion[18] while [des-Asp[1],Ile[8]] angiotensin II was devoid of secretory activity. Both analogs were found to be competitive antagonists of angiotensin II in the adrenal cortex ([Sar[1],Ile[8]]angiotensin II, $pA_2 = 8.31$; [des-Asp[1],Ile[8]] angiotensin II, $pA_2 = 10.0$) and in the adrenal medulla ([Sar[1],Ile[8]]angiotensin II, $pA_2 = 9.21$; [des-Asp[1],Ile[8]]angiotensin II, $pA_2 = 9.16$). In paced isolated rabbit left atria,[19] [Sar[1],Ile[8]] angiotensin II had 0.1% and [des-Asp[1],Ile[8]]angiotensin II, 0.01% of the positive inotropic activity of angiotensin II. Only [Sar[1],Ile[8]]angiotensin II was found to block atrial responses to angiotensin II with a pA_2 value of 9.53.

Discussion

The results obtained indicate that, if formed from angiotensin I, the nonapeptide IV will be rapidly converted to the corresponding heptapeptide III which in turn may stimulate the adrenal cortex. Although these data do not prove the existence of this alternate pathway, they do point out that such a mechanism for the generation of the heptapeptide could exist. The present study also indicates another interesting aspect. It appears that the heptapeptide may also function as a product inhibitor of converting enzyme activity.

These studies also suggest that [Sar[1],Ile[8]]angiotensin II may have a dual mechanism of action in vivo. Previous studies[20] have indicated that this compound is a competitive inhibitor of angiotensin II receptors, while the present studies suggest that inhibition of responses to renin and angiotensin I may also be partially due to the blockade of the converting enzyme.

The intrinsic and antagonistic activities (pA_2) of these compounds in different tissues (rabbit aortic strips, rabbit adrenal cortex, and rabbit atria) were found to be different. This indicates that the structural requirements for stimulation or for binding to angiotensin II receptors vary. Therefore, the nature of angiotensin II receptors in various tissues from the same species should be different.

Experimental Section

L-Histidyl-L-leucine was purchased from Calbiochem. [Sar[1],Ile[8]]angiotensin II, [des-Asp[1],Ile[8]]angiotensin II, and [des-(Asp[1],Arg[2]),Ile[8]]angiotensin II were synthesized by Dr. Khosla. Angiotensinase-free porcine plasma converting enzyme was purchased from Miles Laboratories, Ill. Purified porcine lung converting enzyme was generously supplied by Dr. Dorer, Veteran Administration Hospital, Cleveland, Ohio. This enzyme corresponds to fraction G as described previously.[21]

[Des-Asp[1]]angiotensin I (IV). TLC (cellulose) showed R_f 0.59 (1-butanol–acetic acid–water, 4:1:5); R_f 0.74 (1-butanol–ethyl acetate–acetic acid–water, 1:1:1:1); R_f 0.78 (1-butanol–pyridine–water, 65:35:65); R_f 0.82 (1-butanol–acetic acid–water–pyridine, 30:6:24:20); R_f 0.76 (1-propanol–H_2O, 1:1). Ionophoresis, carried out on filter paper strips (S & S 2043A) in Beckman electrophoresis cell at 400 V using formic acid–acetic acid buffer [prepared by diluting 60 ml of formic acid and 240 ml of acetic acid to 2 l. with water (pH 1.9)], gave 0.92-fold relative electrophoretic mobility of [des-Asp[1]]angiotensin I, as compared to histidine. The corresponding electrophoretic mobility of the compound in Beckman barbiturate buffer B_2 (pH 8.6) was 1.03 as compared to histidine. Detec-

tion of the compound on TLC (cellulose) plates and on electrophoresis filter papers was carried out by spraying with diazotized sulfanilic acid (Pauly's reagent). The amino acid ratios in the acid hydrolysate (in 6 N HCl at 110° for 24 hr in a sealed tube in the presence of phenol) were Arg, 1.0; Val, 1.08; Tyr, 1.01; Ile and Leu, 2.10; His, 1.98; Pro, 1.05; Phe, 1.06. (It was difficult to separate the isoleucine peak from the leucine peak on the amino acid analyzer, Jeolco 5AH model.)

Assay for the Porcine Plasma Converting Enzyme. Enzyme (1 mg) was incubated in a total volume of 60 μl of 0.05 M sodium phosphate buffer (pH 7.5) containing 0.1 M sodium chloride. The substrates, [Ile⁵]angiotensin I and [des-Asp¹,Ile⁵]angiotensin I, were in a final concentration of 1.28×10^{-5}–20×10^{-5} M. Enzymes were preincubated for 5 min at 37°C and reactions were initiated by the addition of substrate. Each incubation was carried out at 37°C for certain intervals and terminated by adding 50 μl of 10% trichloroacetic acid. The reaction mixture was then diluted to 0.7 ml with distilled water, followed by the addition of 0.4 ml of 2 N NaOH. The formation of histidylleucine was detected by adding 0.1 ml of 1% (w/v) o-phthaldehyde in methanol, followed exactly 4 min later by the addition of 0.2 ml of 6 N HCl. The samples were centrifuged at 15000 rpm (Sorvall) for 10 min and the fluorescence of the supernatant fluid was measured with a fluorometer: excitation, 365 nm; emission, 495 nm. A calibration curve for histidylleucine was prepared by adding increasing concentrations of histidylleucine to 1 mg of enzyme in a final volume of 60 μl of 0.05 M sodium phosphate buffer (pH 7.5) containing 0.1 M NaCl and the fluorescence was measured by following the same procedures as described above. In each case, the fluorescence measured in the presence of substrate was corrected for that in the absence of substrate. The validity of measuring histidylleucine released to reflect the enzyme activity was supported by the finding that this enzyme preparation did not contain any enzyme that splits histidylleucine. The inhibitory effect of *B. jararaca* nonapeptide, angiotensin fragments, and EDTA on the plasma converting enzyme activity was carried out by preincubation of these inhibitors with the enzyme for 5 min prior to the addition of substrate. The concentration of [des-Asp¹]angiotensin I was 7.0×10^{-5} M and the incubation time was for 90 min.

Assay for the Porcine Lung Converting Enzyme. The assay conditions for porcine lung converting enzyme using angiotensin I as substrate were optimized. Enzyme (3 μg) was incubated in a total volume of 250 μl of 0.05 M sodium phosphate buffer (pH 7.5) containing 0.1 M sodium phosphate. The substrate, angiotensin I or [des-Asp¹]angiotensin I, was in final concentrations of 1.5×10^{-5}–12×10^{-5} M. The enzyme was preincubated for 5 min at 37°C. The reaction was initiated by adding substrate. At certain time intervals, 50 μl of the reaction mixture was pipetted out and the reaction was terminated with 50 μl of 10% trichloroacetic acid. The formation of histidylleucine was measured fluorometrically as described above. The fluorescence measured in the presence of substrate was also corrected for that in the absence of substrate. The inhibitory effect of angiotensin fragments, EDTA, and *B. jararaca* nonapeptide on angiotensin converting enzyme was carried out by preincubating these inhibitors with enzyme for 5 min at 37°C. The concentration of angiotensin I and [des-Asp¹]angiotensin I was 1.5×10^{-5} M and the reaction time was 2 min. The activity of angiotensin converting enzyme was assayed as described.

Pressor Assay. The mean pressor activity as determined by pressor assay in vagotomized, ganglion-blocked rats[22] was 8.77 ± 0.2% (n = 5) of angiotensin II.

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