Angiotensin Homologs and Analogs as Inhibitors of Rabbit Pulmonary Angiotensin-converting Enzyme*

(Received for publication, November 29, 1976, and in revised form, March 15, 1977)

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The angiotensin-converting enzyme from rabbit lung was purified to a homogeneous protein. The molecular weight of the enzyme or its subunits in sodium dodecyl sulfate-polyacrylamide gel electrophoresis was established as 180,000. Converting enzyme contained about 8% (w/w) hexoses (based on glucose).

The $K_v$ values for the hydrolysis of angiotensin I and [des-Asp]$^\alpha$angiotensin I by the purified enzyme were 80 and 30 $\mu$M, respectively, at 37°C. Chloride ion appeared to increase the affinity of converting enzyme for angiotensin I and [des-Asp]$^\alpha$angiotensin I. The Bothrops jararaca nonapeptide and angiotensin III were competitive inhibitors of the hydrolysis of angiotensin I or [des-Asp]$^\alpha$angiotensin I. The $K_v$ values obtained for angiotensin III and B. jararaca nonapeptide did not change significantly as different enzyme substrates were employed.

Several angiotensin II receptor blockers were found to be potent competitive inhibitors of converting enzyme. The affinity of angiotensin II analogs for converting enzyme was influenced strongly by the charge of the NH$_2$-terminal amino acid residue. Inhibitory activity was enhanced by neutral or basic substituents and attenuated by acidic NH$_2$-terminal residues. Position 8 of angiotensin II is important for the interaction with the converting enzyme. The affinity of the enzyme for angiotensin II analogs was decreased by substituting analogs with branched aliphatic side chains at the COOH terminus of the inhibitor molecule.

These results indicate that [des-Asp]$^\alpha$angiotensin I is a substrate for rabbit pulmonary converting enzyme. The data are consistent with the postulated alternative pathway for the formation of angiotensin III from [des-Asp]$^\alpha$angiotensin I, a product of the hydrolysis of angiotensin I by aminopeptidase. Some angiotensin receptor blockers may act in vitro or in vivo as modulators of converting enzyme activity.

Angiotensin-converting enzyme, a dipeptidyl hydrolase, converts the vasoactive decapeptide, angiotensin I, to the vasoactive octapeptide, angiotensin II (1). Although the importance of converting enzyme in the renin-angiotensin system was first demonstrated by Skeggs et al. (2) and described as a plasma enzyme, the activity of the circulating enzyme was too low to account for the rapid conversion of the decapeptide which occurred in vivo (3, 4). Ng and Vane (3-6) established that the pulmonary converting enzyme was a principal enzyme which catalyzed the in vivo formation of angiotensin II from angiotensin I. The physiologic importance of lung converting enzyme in the production of the angiotensin II has been well established in various species (3, 6, 7).

The converting enzyme also inactivates bradykinin, by cleaving the COOH-terminal dipeptide (Phe-Arg) (6, 9). The converting enzyme is inhibited by peptides isolated from the venom of Bothrops jararaca (1). Inhibition of converting enzyme attenuates responses to angiotensin I and potentiates responses to bradykinin; thus, the converting enzyme may be involved in the maintenance of blood pressure via production of a pressor peptide, angiotensin II, and destruction of a depressor peptide, bradykinin.

The renin-angiotensin system also influences blood pressure by enhancing aldosterone biosynthesis by the adrenal glomerulosa. Angiotensin II and the COOH-terminal heptapeptide of angiotensin II (angiotensin III) are active peptides of the renin-angiotensin system affecting the adrenal cortex. In vivo and in vitro systems, the steroidogenic effect of the heptapeptide was found to be equal to or more potent than that of angiotensin II (10-12). The potent steroidogenic effect of angiotensin III has promoted the hypothesis that this peptide may mediate the steroidogenic effect of the renin-angiotensin system on the adrenal zona glomerulosa (10, 12, 13).

Blair-West et al. (14) hypothesized that angiotensin III may be formed in vivo by two pathways. The heptapeptide may be generated from angiotensin II by aminopeptidase, subsequent to the formation of the octapeptide from angiotensin I by converting enzyme. The second pathway by which angiotensin III may be produced from angiotensin I is through initial hydrolysis of angiotensin I by aminopeptidase followed by the converting enzyme, with [des-Asp]$^\alpha$angiotensin I (nonapeptide), as an intermediate.

In view of the potential importance of angiotensin III, the present investigation was undertaken to study the kinetics of the hydrolysis of [des-Asp]$^\alpha$angiotensin I and angiotensin I with rabbit lung converting enzyme. Previous studies with the partially purified converting enzyme had suggested that the hydrolysis of angiotensin I and [des-Asp]$^\alpha$angiotensin I might be catalyzed by the same enzyme and at a common active site (15, 16). A modification of the method of Cheung and Cushman (17) for the purification of rabbit converting enzyme from acetone powder extract of lung was developed. The molecular
The effects of chloride ion, B. jararaca nonapeptide, and EDTA on the hydrolysis of [des-Asp']angiotensin I were studied. In addition, some properties of the binding site(s) of the converting enzyme were established by employing a series of angiotensin analogs as enzyme inhibitors.

**EXPERIMENTAL PROCEDURES**

**Purification of Angiotensin-converting Enzyme** — The procedure used for the purification of converting enzyme from rabbit lung acetone powder was a modification of the method reported by Cheung and Cushman (17). After the enzyme was purified through a DEAE-cellulose column, the enzyme fraction was dialyzed extensively against 50 mM potassium phosphate buffer (pH 8.0) to remove chloride. The enzyme activity was then concentrated to a volume of 12 ml by ultrafiltration with an Amicon PM-30 membrane under a N₂ atmosphere. The concentrated enzyme (3 ml) was further purified with a Sepharose 6B column (1.5 × 60 cm). The column flow rate was adjusted to 6 ml/h and each fraction collected contained 3 ml. The enzyme activity was concentrated in Fractions 25 to 37 with a 60% recovery, whereas the majority of the protein was concentrated in Fractions 20 to 38. The method of Lowry et al. (18) was employed for protein determination.

**Standard Disc Gel Electrophoresis of Purified Enzyme** — Standard polycrystalline gel electrophoresis (5% gel) was carried out according to the method of Davis (19) using 5 μg of protein/tube. For detection of protein bands, the gels were stained with Coomassie blue for 2 h. After staining, they were destained with 7.5% acetic acid, 5% methanol at 60°C. The bands of stained protein on gels were located by scanning the gel at 550 nm with a Gilford densitometer. For detection of enzyme activity, the unstained gels were solidified on dry ice. The gels were then sliced into 40 slices, each 2 mm thick. Each slice was eluted by soaking in 200 μl of 50 mM potassium phosphate buffer (pH 8.0) overnight and the eluate was checked for enzyme activity.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis** — Polycrystalline gel electrophoresis in the presence of sodium dodecyl sulfate was performed following the method of Weber et al. (20) using 25 μg of purified enzyme protein. The determination of the number of subunits of the purified enzyme was done with gel electrophoresis. The determination of the subunit molecular weight of the purified enzyme was done with sodium dodecyl sulfate gel electrophoresis. The isolated enzyme yielded a straight line. The molecular weight of the converting enzyme was determined in the segment corresponding to the protein band. The enzyme preparation was homogenous in polyacrylamide-disc gel electrophoresis at pH 9.0. The enzyme activity was eluted from an unstained gel only in the segment corresponding to the protein band. The enzyme eluted from unstained gels hydrolyzed Hip-His-Leu, angiotensin I, and [des-Asp']angiotensin I. The incubation medium was 100 mM potassium phosphate buffer (pH 8.0) containing 300 mM NaCl and 5 mM Hip-His-Leu was employed unless otherwise indicated. When angiotensin I was used as substrate, unless otherwise indicated, the incubation medium was 100 mM potassium phosphate buffer (pH 7.5) containing 90 mM NaCl. With [des-Asp']angiotensin I as substrate, unless otherwise stated, the incubation conditions were identical to that for angiotensin I, except that the incubation buffer contained 30 mM NaCl. All the reactions were initiated by the addition of substrates after preincubation of converting enzyme in the medium for 5 min. At certain intervals, a 50-μl aliquot of the incubation medium was removed and the enzyme reaction was terminated with 50 μl of 10% trichloroacetic acid. The Hip-Leu product in the denatured reaction mixture was estimated following phenylhydrazine treatment. The fluorescence of the phenylhydrazide condensation product of Hip-Leu was determined with a spectrophotofluorometer (Aminco-Bowman) (22). When converting enzyme activity was determined in the presence of inhibitors, the inhibitors were preincubated for 5 min with the enzyme before the addition of the substrate.

**Identification of Reaction Products** — Purified converting enzyme (0.8 μg) was incubated for 30 min with 100 μg of angiotensin I or [des-Asp']angiotensin I in a final volume of 75 μl. The incubation was terminated by boiling the solution for 5 min. In comparable experiments, the converting enzyme was preincubated with 1 mM EDTA for 5 min before initiating the reaction by adding angiotensin I or [des-Asp']angiotensin I. After the incubation, 50 μl of the reaction mixtures was spotted on Whatman No. 3MM chromatography paper. For identification of the reaction products (His-Leu and angiotensin II) from angiotensin I, high voltage electrophoresis was run at pH 3.5, 4 kV, 50 mA for 1.5 h. When [des-Asp']angiotensin I was used as substrate, His-Leu was also identified by high voltage electrophoresis but at pH 5.6, 6 kV, 70 mA for 1.1 h. Standard angiotensin peptides, His-Leu, arginine, and aspartic acid were also applied to the papers as markers and reference standards and the electrophoreograms were developed by spraying with a mixture of 1% ninhydrin in acetone and 1% cadmium acetate in 50% acetic acid and water (85:15, v/v).


**RESULTS**

The purified converting enzyme preparation contained 1.64 mg of protein with a specific activity of 56 units (a unit is equivalent to 1 μmol of Hip-His-Leu hydrolyzed/min) which represented a purification of more than 1300-fold. The over-all steps for purification are summarized in Table I. The isolated enzyme protein was tested for purity with disc gel electrophoresis. The enzyme preparation was homogeneous in polyacrylamide-disc gel electrophoresis at pH 9.0. The enzyme activity was eluted from an unstained gel only in the segment corresponding to the protein band. The enzyme eluted from unstained gels hydrolyzed Hip-His-Leu, angiotensin I, and [des-Asp']angiotensin I. When the enzyme was subjected to sodium dodecyl sulfate-acrylamide gel electrophoresis under reduced condition, a major band of protein was obtained at the top of the gel. The purified converting enzyme contained no smaller dissociable subunits. Plotting the log of the molecular weight of the standard proteins against their electrophoretic mobilities yielded a straight line. The molecular weight of the converting enzyme estimated from the plot was 180,000.

When the converting enzyme was reacted with an anthrone reagent, a blue-green color developed, indicating that the enzyme was a glycoprotein. Based on a glucose standard, the enzyme content of hexose was 8% (w/w). It is known, however, that the color yield per mol of morosacharide varies. On a molar basis, the color developed by glucose under these reaction conditions is higher than that for other hexoses (21); therefore, it is highly possible that the 8% hexose content of the enzyme determined in this study was an underestimate.

1 The amino acid sequence of B. jararaca nonapeptide is Pro-Pro-Arg-Pro-Glu-Ile-Pro-Pro-OH.

2 The abbreviation used is: Phn, the amide of phenylalanine.
Inhibition of Angiotensin-converting Enzyme

TABLE I

Preparation of purified angiotensin-converting enzyme of rabbit lung

A unit of angiotensin-converting enzyme hydrolyzes 1 µmol of Hip-His-Leu/min as assayed fluorometrically. Converting enzyme was incubated with 100 mM potassium phosphate buffer containing 5 mM Hip-His-Leu and 300 mM NaCl at pH 8.0 in a final volume of 250 µL.

<table>
<thead>
<tr>
<th>Total units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td></td>
<td></td>
<td>-fold</td>
</tr>
<tr>
<td>Acetone powder</td>
<td>520</td>
<td>12000</td>
<td>0.043</td>
<td>100</td>
</tr>
<tr>
<td>Acidification</td>
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<td>3570</td>
<td>0.084</td>
<td>57</td>
</tr>
<tr>
<td>Calcium phosphate gel</td>
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<td>259</td>
<td>1.12</td>
<td>55</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>280</td>
<td>37</td>
<td>7.56</td>
<td>54</td>
</tr>
<tr>
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<td>4.13</td>
<td>30</td>
</tr>
<tr>
<td>Sepharose 6B</td>
<td>92</td>
<td>1.64</td>
<td>56</td>
<td>18</td>
</tr>
</tbody>
</table>

![Fig. 1.](image1.png)

**Fig. 1.** High voltage paper electrophoresis for identification of converting enzyme reaction product, His-Leu, from angiotensin I and [des-Asp']angiotensin I. Electrophoresis was run at pH 3.5, 4 kV (Panel A) and pH 8.9, 6 kV (Panel B) for 1 1/2 h. The substances in the strips of Panel A are: 1, arginine; 2, angiotensin II; 3, angiotensin I; 4, His-Leu; 5, reaction mixture of angiotensin I and converting enzyme; 6, reaction mixture of angiotensin I and converting enzyme (pretreated with EDTA). The samples in each strip of Panel B are as follows: 1, arginine; 2, aspartic acid; 3, His-Leu; 4, [des-Asp']angiotensin I or -angiotensin III, or both; 5, reaction mixture of [des-Asp']angiotensin I and converting enzyme; 6, reaction mixture of [des-Asp']angiotensin I and converting enzyme (pretreated with EDTA).

**Absence of Angiotensinases and His-Leu Splitting Enzyme in Purified Converting Enzyme Preparation** — In order to obtain precise kinetic data, the absence of angiotensinase activity which degrades substrates, e.g. angiotensin I and [des-Asp']angiotensin I, Electrophoresis was run at pH 3.5, 4 kV (Panel A) and pH 8.9, 6 kV (Panel B) for 1 1/2 h. The substances in the strips of Panel A are: 1, arginine; 2, angiotensin II; 3, angiotensin I; 4, His-Leu; 5, reaction mixture of angiotensin I and converting enzyme; 6, reaction mixture of angiotensin I and converting enzyme (pretreated with EDTA). The samples in each strip of Panel B are as follows: 1, arginine; 2, aspartic acid; 3, His-Leu; 4, [des-Asp']angiotensin I or -angiotensin III, or both; 5, reaction mixture of [des-Asp']angiotensin I and converting enzyme; 6, reaction mixture of [des-Asp']angiotensin I and converting enzyme (pretreated with EDTA).

Following incubation with the enzyme would demonstrate the hydrolysis or sequestration of His-Leu. We found that all the added His-Leu was recovered.

**Identification of Reaction Product His-Leu** — Histidyl-leucine was identified following isolation with high voltage paper electrophoresis, as shown in Fig. 1. After angiotensin I was incubated with the converting enzyme, a ninhydrin-positive spot corresponding to the migration of standard His-Leu was obtained (Fig. 1, Panel A) on the electrophoreogram. Angiotensin II was also detected. When [des-Asp']angiotensin I was used as the substrate, His-Leu separated easily from the substrate nonapeptide and the heptapeptide product. Under these conditions, nona- and heptapeptides remained at the origin and were not separated. When the enzyme was inhibited by preincubation with EDTA (1 mM) prior to the addition of angiotensin I and [des-Asp']angiotensin I, the spots corresponding to His-Leu and angiotensin II were absent and only the substrates were identified.

**Converting Enzyme Activity as Function of pH and Chloride Ion Concentration** — The pH profile of converting enzyme activity is shown in Fig. 2. Activity was optimal at pH 7.5 to 7.8, with angiotensin I and [des-Asp']angiotensin I as the substrates. When the pH of the incubation medium was increased from pH 7.5 to 8.5, the enzyme activity with angiotensin I as the substrate was decreased to 45% of the optimal activity. In contrast, the enzyme activity was not reduced
significantly using [des-Asp']angiotensin I as the substrate. The effect of variation in NaCl concentration on the activity of the purified enzyme is shown in Fig. 3. The optimal chloride concentration was 30 mM with [des-Asp']angiotensin I as the substrate and 90 mM with angiotensin I as the substrate. In the absence of NaCl, the rate of hydrolysis of angiotensin I was less than 3% of the rate obtained with 90 mM NaCl. With [des-Asp']angiotensin I as the substrate, this value was about 7%. Hydrolysis of [des-Asp']angiotensin I was less dependent than angiotensin I on the presence of chloride.

Kinetic Parameters of Converting Enzyme with Angiotensin I and [des-Asp']Angiotensin I as Substrates—Under standard assay conditions, the reaction velocity was linear with both enzyme concentration and time. The Lineweaver-Burk reciprocal plots for angiotensin I and [des-Asp']angiotensin I as the substrates are shown in Fig. 4. The $K_m$ for angiotensin I was 80 $\mu$M and for [des-Asp']angiotensin I, $30 \mu$M. The major structural difference (other than chain length between [des-Asp']angiotensin I and angiotensin I) is the presence of the aspartic acid residue at the NH$_2$ terminus for angiotensin I and the arginine residue for [des-Asp']angiotensin I. These results suggested that the NH$_2$-terminal amino acid residue might be a determinant of substrate affinity.

Effect of B. jararaca Nonapeptide on Conversion of Angiotensin I and [des-Asp']Angiotensin I—The inhibition of the converting enzyme by B. jararaca nonapeptide has been studied in detail by Cheung and Cushman (17). This peptide is one of the most potent converting enzyme inhibitors available. The B. jararaca nonapeptide was shown to act as a competitive inhibitor of the converting enzyme with respect to the hydrolysis of angiotensin I or Hip-His-Leu and did not compete with chloride ion for the converting enzyme. Kinetic studies were performed to see if the hydrolysis of [des-Asp']angiotensin I was inhibited by B. jararaca nonapeptide and this inhibitory peptide did compete with [des-Asp']angiotensin I and angiotensin I for the enzyme. The $K_i$ (inhibitor constant) values calculated for B. jararaca nonapeptide were 1.0 $\mu$M when angiotensin I was the substrate and 1.5 $\mu$M when [des-Asp']angiotensin I was the substrate (data not shown).

Effect of Angiotensin II and Metabolites on Converting Enzyme Activity—Angiotensin II, angiotensin III, and the COOH-terminal hexapeptide of angiotensin II have been reported to inhibit converting enzyme (4, 15, 23). In the present study, these angiotensin metabolites were found to be competitive inhibitors of the hydrolysis of angiotensin I and [des-Asp']angiotensin I. As depicted in Fig. 4, angiotensin III at a concentration of 10 $\mu$M did not alter the maximal enzyme activity, while the $K_m$ values were increased. The $K_i$ value for angiotensin III was 16 $\mu$M with angiotensin I as the substrate and 28 $\mu$M with [des-Asp']angiotensin I as the substrate. Angiotensin II and COOH-terminal hexapeptide were weaker inhibitors than angiotensin III (Table II). The effect of all these peptides on converting enzyme activity were overcome...
by increasing the concentration of angiotensin I and \(\text{des-Asp'}\text{langiotensin I}\).

**Studies on Relative Inhibition of Conversion of Angiotensin I and \(\text{des-Asp'}\text{langiotensin I}\) by B. jararaca Nonapeptide and Angiotensin Metabolites** – The \(K_{\text{m}}\) value for \(\text{des-Asp'}\text{langiotensin I}\) was about 3 times less than that of angiotensin I, but a factor of 3 was relatively small. To provide more convincing evidence that \(\text{des-Asp'}\text{langiotensin I}\) is a better substrate than angiotensin I, the hydrolysis of the decapentapeptide was studied in the presence of competitive inhibitors. The converting enzyme activity was determined at a substrate concentration of 50 \(\mu\text{M}\), and the \(\text{per cent inhibition}\) induced by various concentrations of \(\text{B. jararaca nonapeptide, angiotensin II, angiotensin III, and the hexapeptide of angiotensin II}\) are compared in Table II. When the chloride concentration in the incubation media was 90 \(\text{mM}\) for angiotensin I and 30 \(\text{mM}\) for \(\text{des-Asp'}\text{langiotensin I}\), it was found that the \(\text{per cent inhibition}\) of nonapeptide hydrolysis was markedly less than that of the decapentapeptide with all of the inhibitors studied. The \(\text{per cent inhibition}\) of the hydrolysis of angiotensin I and \(\text{des-Asp'}\text{langiotensin I}\) was comparable to that shown in Table II, whether conversion was determined at 30 or 90 \(\text{mM}\) of NaCl. These results clearly demonstrated that the enzyme affinity for \(\text{des-Asp'}\text{langiotensin I}\) exceeds that for angiotensin I.

**Studies on Binding Capacity of Angiotensin Analogs Modified at \(\text{NH}_2\)-Terminus of Peptide Chain** – From the data presented, converting enzyme affinity for angiotensin clearly does not relate to the length of the peptide chain. In all probability, the \(\text{NH}_2\)-terminal amino acid is a determinant of the substrate affinity and inhibitory potency of these peptides. To establish if the converting enzyme has a recognition site which keys on the \(\text{NH}_2\)-terminal amino acid residue, the affinities of several angiotensin analogs were determined. These analogs were demonstrated to be competitive inhibitors of converting enzyme with respect to the hydrolysis of angiotensin I. The analogs are aligned in Table III to facilitate direct comparison of the \(\text{NH}_2\)-terminal amino acid structures and respective inhibitor constants. \(\text{Arg'}\), \(\text{des-Asp',Gly'}\), and \(\text{des-Asp',\beta-Ala'}\) octapeptides which do not have aspartic acid at the \(\text{NH}_2\)-terminus were the most potent inhibitors, with a potency comparable to that of angiotensin III. Replacement of the aspartyl residue with \(\text{N-methylglycyl (sarcosine)}\) or \(\text{N,N-di-methylglycyl at the NH}_2\)-terminus also increased the inhibitory potency. A similar result was obtained when the carboxyl group of the aspartyl residue was derivatized to an amide, e.g. \(\text{Asn'}\text{angiotensin II}\) and \(\text{Asn',Phn'}\) angiotensin II. When \(\text{Asp',Arg'}\) were removed and valine was exposed as the \(\text{NH}_2\)-terminus (e.g. COOH-terminal angiotensin II hexapeptide), a weak inhibitor of conversion was obtained. These results strongly indicate that a carboxyl group at the \(\text{NH}_2\)-terminus of the inhibitors or substrates, or both, reduces the apparent affinity for a binding site on the enzyme.

**Table III**

*Inhibitor constant of angiotensin-converting enzyme inhibitors that are \(\text{NH}_2\)-terminally modified angiotensins II*

The \(K_{\text{i}}\) values are determined by the method of Dixon (24). The purified converting enzyme of rabbit lung (0.125 \(\mu\text{g}\)) was incubated with angiotensin I (50 \(\mu\text{M}\)) in the absence or presence of four concentrations of inhibitors (10 to 200 \(\mu\text{M}\)) in a final volume of 0.125 ml. The inhibitors were preincubated with the enzyme for 5 min. The initial velocity of the reaction was determined within 4 min.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>(\text{NH}_2)-Terminal Structure</th>
<th>(K_{\text{i}}) ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A II</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>A III</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>A III'</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>(\text{des-Asp',Gly'})</td>
<td>A II</td>
<td>12</td>
</tr>
<tr>
<td>(\text{des-Asp',Glu'})</td>
<td>A II</td>
<td>20</td>
</tr>
<tr>
<td>Hypertension (COOH)</td>
<td>A II</td>
<td>27</td>
</tr>
<tr>
<td>(\text{N,N-dimethyl-Gly'})</td>
<td>A II</td>
<td>43</td>
</tr>
<tr>
<td>(\text{Nmr'})</td>
<td>A II</td>
<td>12</td>
</tr>
<tr>
<td>Hexapeptide</td>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>

*Hypertension is \(\text{[Asn',Val']angiotensin II}\). Phn is the abbreviation for the amide of the COOH-terminal carboxyl of phenylalanine. The hexapeptide is the COOH-terminal hexapeptide of angiotensin II.

**Table IV**

*Comparison of inhibitory potency of angiotensin-converting enzyme inhibitors derived from the COOH terminus of angiotensin*

The \(K_{\text{i}}\) values were determined by the method of Dixon (24). The incubation condition was identical with that described in Table III. The initial velocity of the reaction was also determined within 4 min.

<table>
<thead>
<tr>
<th>Peptide COOH - Terminal Structure</th>
<th>(K_{\text{i}}) ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A III</td>
<td>16</td>
</tr>
<tr>
<td>(\text{des-Asp'})</td>
<td>130</td>
</tr>
<tr>
<td>(\text{des-Asp',Val'})</td>
<td>10</td>
</tr>
<tr>
<td>(\text{des-Asp',Ala'})</td>
<td>10</td>
</tr>
<tr>
<td>(\text{Ala'})</td>
<td>43</td>
</tr>
<tr>
<td>(\text{Asn',Phn'})</td>
<td>140</td>
</tr>
<tr>
<td>(\text{Asn',Phn'})</td>
<td>40</td>
</tr>
<tr>
<td>(\text{Asn',Val'})</td>
<td>180</td>
</tr>
</tbody>
</table>

*No inhibition was observed when the concentration of the angiotensin analog was at 0.1 \(\text{mM}\).*
angiotensin II were not different from the natural homologs which terminate with phenylalanine (e.g. angiotensin II and III). These data suggest that the aromatic ring at the COOH terminus of angiotensin II is not a major determinant of binding to the active site of the enzyme. This suggestion was further supported by the comparable $K_m$ values determined for [Sar', Ala']angiotensin II and [Sar']angiotensin II. However, when the phenylalanyl residue of more potent inhibitors (i.e. angiotensin III) was replaced with isoleucine, the affinity markedly decreased. Both [Val'] and [Ile']angiotensin II were less potent than angiotensin II as inhibitors of converting enzyme. In contrast, [des-Asp', Ala']angiotensin II was a very potent inhibitor of converting enzyme with a potency comparable to that of angiotensin III. These data indicate that a COOH-terminal amino acid residue containing a branched aliphatic side chain will decrease the affinity.

The data with analogs modified at both the NH$_2$ and COOH terminus ([Sar', Ala']-, [des-Asp', Ile']-, and [des-Asp', Ala']-angiotensin II) also indicate that the elimination of the carboxyl group of the aspartyl at the NH$_2$ terminus increases the affinity.

**DISCUSSION**

It is well established that angiotensin III is an active peptide of the renin-angiotensin system in the adrenal zona glomerulosa (10–12). It has been hypothesized that angiotensin III may be formed locally *in vivo* through a metabolic pathway with [des-Asp']angiotensin I as an intermediate (13, 14). If this pathway for the formation of angiotensin III is to be considered to play a significant role in the regulation of aldosterone biosynthesis, [des-Asp']angiotensin I should be approximately as good as angiotensin I as a substrate for the converting enzyme.

The lower $K_m$ value obtained from the nonapeptide as compared to angiotensin I with purified rabbit pulmonary converting enzyme indicates that the nonapeptide has a better enzyme affinity. The observations that the conversion of [des-Asp']angiotensin I is much more resistant than angiotensin I to inhibition with competitive inhibitors, such as *B. jararaca* nonapeptide, angiotensin II, angiotensin III, and the COOH-terminal angiotensin II hexapeptide, is consistent with the $K_m$ values being different. These data are in agreement with previous results obtained with partially purified plasma and lung converting enzymes (15, 16). In addition to the lower $K_m$ value for the nonapeptide, the value of $V_{max}/K_m$ for the deca- and nonapeptides is about the same. The value of $V_{max}/K_m$ is proportional to the percentage rate of hydrolysis of the substrate when the concentration of the substrate is very low in comparison to $K_m$ (25). Since the concentration of angiotensin I in the circulation is about 5 orders of magnitude lower than $K_m$, the percentage rate of hydrolysis is a more adequate index of the rate of hydrolysis under physiologic conditions. The comparable $V_{max}/K_m$ values for the deca- and nonapeptides suggest that the hydrolysis rate of angiotensin I and [des-Asp']angiotensin I will be identical if each peptide is present at the same endogenous concentration. At the present time, the nonapeptide has not been identified or quantified in plasma.

The findings of other investigators clearly indicate that angiotensin I is degraded to form [des-Asp']angiotensin I in *vitro*. Ackerly et al. (26) found that after a single passage through isolated, perfused feline adrenal glands, the major metabolite of [H]angiotensin I (60 to 65%) was [H]-labeled [des-Asp']angiotensin I. Chiu et al. (16) suggested that a metabolite of angiotensin I in the effluent from perfused rat lungs resembled [des-Asp']angiotensin I. Ackerly et al. (26) also found that the nonapeptide had high immuno-cross-reactivity with an angiotensin I antisera (65%). Circulating levels of angiotensin I have been estimated by immunoassay and this result implies that if a significant amount of [des-Asp']angiotensin I is present in the circulation grossly inaccurate estimates have been made of angiotensin I in the blood. Although the present study does not establish the precise metabolic pathway for the formation of angiotensin III, the data clearly support the contention that two pathways may be operative *in vivo*.

A compound which can inhibit selectively the hydrolysis of [des-Asp']angiotensin I or angiotensin I would be a valuable tool to evaluate the pathways of the renin-angiotensin-aldosterone system. The present study determined whether *B. jararaca* nonapeptide and EDTA, which inhibit the conversion of angiotensin I, inhibited the hydrolysis of [des-Asp']-angiotensin I. Both inhibitors of angiotensin I hydrolysis also depressed the hydrolysis of the angiotensin nonapeptide.

Further attempts to distinguish the conversion of the deca- from the nonapeptide revealed that both peptides require the presence of the chloride ion but the dependence on this anion is less for [des-Asp']angiotensin I than for angiotensin I. It has been shown that the chloride concentration required by converting enzyme differs with the substrate. In general, the better the affinity of the substrate, the lower the dependence on chloride. The reported apparent $K_m$ values for SQ 20413 ([H]. *jararaca* pentapeptide), bradykinin, angiotensin I, and Hipp-His-Leu are approximately 0.1 mM, 1 mM, 40 mM, and 2.5 mM, respectively (9, 17, 27). The optimal chloride concentrations required for hydrolysis of these substrates are 0, 10, 90, and 300 mM, respectively. This relationship between $K_m$ and chloride requirement was demonstrated in the present study in that the nonapeptide has higher affinity than angiotensin I for the converting enzyme and requires a lower chloride concentration (30 mM) than angiotensin I (90 mM). The chloride ion is thought to act at an allosteric site of converting enzyme to initiate a stable conformational change. Dorer et al. (9) reported that the porcine enzyme's affinity for bradykinin was increased by 300% in the presence of an optimal concentration of chloride, as compared to the affinity in the absence of chloride. These observations are consistent with allosteric activation of the enzyme by chloride ion. In the present study, it was observed that when the chloride concentration was increased from 30 to 90 mM for angiotensin I and 15 to 30 mM for [des-Asp']angiotensin I, the affinity for both peptides was increased.

After disc gel electrophoresis, the purified rabbit lung enzyme showed the presence of a single protein band. The protein band corresponded to the enzymatic activity eluted from the gels with Hip-His-Leu, [des-Asp']angiotensin I, and angiotensin I as substrates. These data indicate that all three compounds are hydrolyzed by the same enzyme. *B. jararaca* nonapeptide, angiotensin III, II, and the COOH-terminal hexapeptide of angiotensin II are competitive inhibitors of the hydrolysis of the deca- and nonapeptides. Comparable inhibitor constants were calculated for angiotensin III (or *B. jararaca* nonapeptide) with angiotensin I and [des-Asp']angiotensin I as the converting enzyme substrates. Purified converting enzymes have been reported to hydrolyze both angiotensin I and bradykinin (8, 9). Therefore, it seems likely that the hydrolysis of angiotensin I, [des-Asp']angiotensin I, and bradykinin by rabbit lung converting enzyme involves a
common active site on the same enzyme.

Although angiotensin-converting enzyme cleaves dipeptyl residues from its substrate, it is an exopeptidase. The presence of a free COOH-terminal carboxyl group on the substrate is an absolute requirement for hydrolysis (25, 28). The necessity of a COOH-terminal carboxyl also has been reported for the inhibitory effect of B. jararaca peptides or their analogs, or both, on converting enzyme (29). Methylester or amide derivatives of the B. jararaca peptides and their analogs decreased the inhibitory activity of these peptides. In the present study, [Asn',Phe']angiotensin II was a weaker inhibitor of converting enzyme than was [Asn',Phe']angiotensin II.

In addition to the established requirement of a COOH-terminal carboxyl, the present study clearly indicates that an NH2-terminal carboxyl group increases Km and K, values. Elimination of the NH2-terminal aspartyl of angiotensin II, substitution with other amino acid residues, or modification of the carboxyl by amionation enhanced avidity for the enzyme. The data also indicated that N-methylation of the NH2-terminal amino group tended to reduce the inhibitory activity of the peptide (e.g., [Sar']angiotensin II versus [des-Asp',Gly']angiotensin II). These results are consistent with the high affinity of bradykinin, B. jararaca nonapeptide, and pentapeptide (SQ 20475), peptides which do not contain either a terminal amino group tended to reduce the inhibitory activity of the enzyme than was [Asn',Phe']angiotensin II.

The inhibitor constant of Ides-Asp',Ile' and Ides-Asp',Ala' and [Sar',Ala']angiotensin II suggest that the deca- and nonapeptides with these modifications would avidly bind to and perhaps be hydrolyzed rapidly by converting enzyme. These deca- or nonapeptide precursors may be of potential use to study or to control the renin-angiotensin system. An analog such as [Sar',Ala']angiotensin I would compete with angiotensin I for the converting enzyme. Blockade of the receptors(s) for angiotensin and product inhibition of conversion would be mediated by any initial reaction product. The precursor peptide may be converted to an antagonist of angiotensin right at or adjacent to the angiotensin receptor, or both. The excess precursor and product would be degraded further to smaller fragments devoid of any pharmacologic effect.

Acknowledgments—We wish to thank Dr. Carlos Villar-Palasi for his instruction on gel electrophoresis and many indispensable suggestions and Dr. Laura Huang for her valuable advice and discussions.

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Inhibition of Angiotensin-converting Enzyme

Angiotensin homologs and analogs as inhibitors of rabbit pulmonary angiotensin-converting enzyme.

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