Angiotensin I Converting Enzyme of Calf Lung. Method of Assay and Partial Purification†

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ABSTRACT: A continuous ultraviolet spectrophotometric assay for angiotension I converting enzyme has been developed utilizing amino-terminal-blocked tripeptides as substrates. Z-Phe(NO_2)-His-Leu, Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly have been synthesized as effective substrates for angiotensin I converting enzyme. These substrates have facilitated the partial purification and characterization of angiotensin I converting enzyme from calf lung. This enzyme was prepared from an acetone powder extract of calf lungs by chromatography on CM-cellulose, DEAE-cellulose, Sephadex G-200, and isoelectric focusing to a 700-fold increase in purity. The enzyme was free of detectable contaminating proteolytic enzymes except for the possibility of a dipeptidase. The molecular weight has been estimated as near 300,000 by gel filtration on a Sephadex G-200 column. V_{max}, K_m, and pH_{max} have been established for the substrate Z-Phe(NO_2)-His-Leu as 13.9 sec^{-1}, 0.23 mM, and pH 8.0, respectively. The same constants for Z-Phe(NO_2)-Gly-Gly and Hip(NO_2)-Gly-Gly were 56.7 sec^{-1}, 1.5 mM, pH 7.0 and 99.7 sec^{-1}, 4.8 mM, pH 7.0, respectively. The metal chelators EDTA (1×10^{-6} M) and *o*-phenanthroline (2×10^{-5} M) completely inhibited converting enzyme hydrolysis of Z-Phe(NO_2)-Gly-Gly. The following divalent metal ions, in their order of activity, reversed the inhibition by EDTA or *o*-phenanthroline: $Zn^{2+} > Co^{2+} > Mn^{2+} >>> Mg^{2+}$ and Ca^{2+}.

Angiotensin I converting enzyme, which catalyzes the following reaction to produce the vasoactive peptide angiotensin II, was first discovered by Skeggs *et al.* (1954) in horse plasma.

angiotensin I
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

↓

angiotensin II
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe + His-Leu

A richer source of converting enzyme[1] in the vascular bed of the lung was recently discovered by Ng and Vane (1967). The physiologic importance of the lung enzyme (Hodge *et al.*, 1967; Biron and Huggins, 1968; Bakhle *et al.*, 1969; Oparil *et al.*, 1970) has led many investigators to attempt to isolate the converting enzyme from this tissue (Bakhle, 1968; Hollemans *et al.*, 1969; Huggins *et al.*, 1970; Yang *et al.*, 1971; Cushman and Cheung, 1971). The conversion of angiotensin I to angiotensin II has been traditionally evaluated by measuring the contraction of isolated rabbit aorta strips (Helmer, 1957, 1964). The process of isolating and purifying an enzyme requires a rapid, accurate assay. This necessity has spurred the discovery of several different substrates and chemical assays for measuring converting enzyme activity.

Boucher *et al.* (1970) have measured angiotensin II by a bioassay in nephrectomized rats after separating angiotensin I from angiotensin II on Dowex resin 50W-X2. Several investigators have reported radioimmunological assays which measure angiotensin II in the presence of angiotensin I (Boyd *et al.*, 1967; Catt *et al.*, 1967; Page *et al.*, 1969). Huggins and Thampi (1968) have developed a radiometric assay using [Ile^5,[^{14}C]Leu^{10}] angiotensin I as a substrate and Lee *et al.* (1971b) have modified this procedure by using high-voltage paper electrophoresis to separate His-Leu from the substrate. Piquilloud *et al.* (1970) have described a fluorometric assay in which the formation of His-Leu split from Z-Phe-His-Leu is measured after its reaction with *o*-phthalaldehyde reagent. Cushman and Cheung (1969) have assayed converting enzyme activity by measuring via the ninhydrin procedure the His-Leu released from the substrate Hip-His-Leu or more recently by measuring the released hippuric acid at 228 nm (Cushman and Cheung, 1971). Dorer *et al.* (1970) have developed an automated chemical method for measuring the hydrolysis of angiotensin I by employing the ninhydrin reaction. A spectrophotometric assay at 310 nm was used by Yang *et al.* (1970, 1971) to follow the hydrolysis of Boc-Phe(NO_2)-Phe-Gly by the converting enzyme.

We have also taken advantage of the chromophoric nitro group and have synthesized Z-Phe(NO_2)-His-Leu, the nitrated analog of the effective substrate Z-Phe-His-Leu (Piquilloud *et al.*, 1970) as well as Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly.[2] We have developed an ultraviolet (uv) spectrophotometric enzyme assay using these nitrated tripeptides as substrates, and report here the utilization of these substrates in the partial purification and characterization of converting enzyme from calf lung.

Materials

Angiotensin I (Asp^1, Ile^5) was obtained from Schwarz BioResearch, and angiotensin II (Asn^1,Ile^5) was obtained from Ciba. Tris(hydroxymethyl)aminomethane, reagent

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[1] Various names have been used in the literature for angiotensin I converting enzyme, such as α-carboxypeptide dipeptidohydrolase (Cushman and Cheung, 1971), carboxycathepsin (Elisseeva *et al.*, 1971), dipeptidyl carboxypeptidase, and kininase II (Yang *et al.*, 1971). For convenience we will use the simplified name "converting enzyme."

[2] Abbreviation used is: Hip, hippuryl.

grade, and Hip-L-Phe (lot no. 29B-9000) were purchased from the Sigma Chemical Co. Gly-Gly-Gly and Gly-Gly were purchased from the Nutritional Biochemicals Corp. L-His-L-Leu (control no. HSLE-4) was obtained from Miles Laboratories, Inc. L-Leucinamide hydrochloride was obtained from Mann Research Laboratories. Bovine carboxypeptidase A (46 units/mg) and Leu aminopeptidase (hog kidney) were obtained from the Worthington Biochemical Corp. Thin-layer chromotography plates (F-254 silica gel, 0.25 mm on glass) were obtained from Brinkman Instruments. Triton X-100 (lot no. 00246, B grade), carboxymethylcellulose (no. 3909), and diethylaminoethylcellulose were obtained from Calbiochem. Sephadex G-200 (40–120 M, lot no. 1517), Sephadex G-50 (superfine, lot no. 5739), and Blue Dextran 2000 (lot no. 2678) were purchased from Pharmacia Fine Chemicals, Inc. The proteins utilized for molecular weight determination were obtained as a kit (Nonenzymatic Protein Molecular Weight Markers, Mann Research Lab, Inc., Kit 20900-8109). All metals employed in the metal-chelator study were obtained as the chloride or sulfate salt from Mallinckrodt. *o*-Phenanthroline (Sigma Chemical Co.) and EDTA (J. T. Baker Chem. Co., 8993) were used as metal chelators.

Methods

Synthesis of Peptides

The following peptides were prepared by known procedures: Pyr-Lys-Trp-Ala-Pro (Stewart *et al.*, 1971), Hip-His-Leu (Cushman and Cheung, 1971), and Z-Phe-His-Leu (Piquilloud *et al.*, 1970).

Hip(NO_2)-Gly-Gly. *p*-Nitrobenzoyl chloride (2.7 g, 14.5 mmoles) in tetrahydrofuran (10 ml) and aqueous sodium hydroxide (13.3 ml of 1 N) were added dropwise simultaneously to a stirred solution of triglycine (2.5 g, 13.2 mmoles) in 0.5 N sodium hydroxide (26.4 ml) at 0°. After standing 2 hr at 0° and 2 hr at room temperature, the solution was washed with ether (two 15-ml portions) and acidified with 1 N sulfuric acid. The resulting precipitate was filtered, washed with ethanol, dried, and crystallized (twice) from a mixture of 2% water in acetic acid and ether to yield 1.52 g of colorless crystals which melted at 260–261° with decomposition. *Anal.* Calcd for $C_{13}H_{14}N_4O_7$: C, 46.16; H, 4.16; N, 16.56. Found: C, 46.08; H, 4.28; N, 16.66.

Z-Phe(NO_2)-Gly-Gly. A solution of benzloxycarbonyl-*p*-nitro-L-phenylalanine (3.44 g, 10 mmoles) (Inouye and Fruton, 1967b), triethylamine (1.54 ml, 11 mmoles), and tetrahydrofuran (25 ml) was cooled to −5° and isobutyl chloroformate (1.3 ml, 10 mmoles) was added. After 5 min, a cold solution of glycylglycine (1.57 g, 12 mmoles), triethylamine (2.52 ml, 18 mmoles), and water (15 ml) was added dropwise. The reaction mixture was stirred 2 hr at room temperature and acidified to pH 3 with 6 N hydrochloric acid. The tetrahydrofuran was evaporated and the resulting precipitate was collected and crystallized from ethanol–petroleum ether (bp 30–60°): yield 2.43 g, mp 144–146°. An analytical sample was prepared by two recrystallizations from methanol–ethyl acetate: mp 145–147°, $[\alpha]_D^{20}$ 18.6° (*c* 0.60, MeOH). *Anal.* Calcd for $C_{21}H_{22}N_4O_8$: C, 55.0; H, 4.8; N, 12.2. Found: C, 55.2; H, 5.07; N, 11.82.

Z-Phe(NO_2)-His-OCH_3. A solution of dicyclohexylcarbodiimide (12.4 g, 0.06 mmole) in chloroform (25 ml) was added dropwise to a stirred, cooled solution of benzyloxycarbonyl-*p*-nitro-L-phenylalanine (17.2 g, 0.05 mmole) in chloroform (400 ml). The resulting thick slurry was cooled and stirred rapidly and a solution of methyl-L-histidinate dihydrochloride (14.2 g, 0.058 mmole), triethylamine (16.8 ml, 0.12 mmole), and chloroform (75 ml) was added dropwise. After 1 hr in an ice bath and 18 hr at room temperature the mixture was filtered, washed with water (four 100-ml portions), 2 M potassium carbonate solution (two 100-ml portions), and water (100 ml), dried over magnesium sulfate, and concentrated to a gum which was suspended in hot ethyl acetate. Methanol was added to dissolve the gum, and the resulting solution was concentrated by boiling to remove most of the methanol yielding two crops of colorless crystals (14.9 g): mp 160.5–162°, $[\alpha]_D^{20}$ −15.6° (*c* 1.04, dimethylformamide). *Anal.* Calcd for $C_{24}H_{25}N_5O_7$: C, 58.2; H, 5.10; N, 14.14. Found: C, 57.94; H, 5.12; N, 13.88.

Z-Phe(NO_2)-His-$NHNH_2$. A solution of the methyl ester (12.75 g, 0.026 mmole), hydrazine (13 ml, 97%), and methanol (400 ml) was prepared and allowed to stand 18 hr at room temperature. The resulting solid was collected by filtration and the mother liquors were evaporated to dryness. The two solids were combined and crystallized from methanol–ethyl acetate to yield three crops of colorless crystals (8.9 g, mp 171° dec). An analytical sample was prepared by two recrystallizations from methanol–ether: mp 171° dec, $[\alpha]_D^{20}$ −13.9° (*c* 0.53, MeOH). *Anal.* Calcd for $C_{23}H_{23}N_7O_6$: C, 55.75; H, 5.09; N, 19.78. Found: C, 55.58; H, 5.25; N, 19.50.

Z-Phe(NO_2)-His-Leu-Ot-Bu. Hydrochloric acid (3.6 ml of 6 N, 20 mmoles) and then sodium nitrite (0.35 g, 5 mmoles in 5 ml water) were added to a solution of the hydrazide (2.5 g, 5 mmoles) in dimethylformamide (40 ml) at −20°. After 5 min, triethylamine (2.8 ml, 20 mmoles) was added, followed by a solution of *tert*-butyl L-leucinate (1.0 g, 5.3 mmoles) (Anderson and Callahan, 1960) in dimethylformamide (5 ml). The mixture was kept at −10° for 18 hr and evaporated. The residue was triturated with ethyl acetate (100 ml) and filtered, and the filtrate was washed with water (three 75-ml portions), dried over magnesium sulfate, and evaporated. The product was crystallized from warm ethyl acetate–petroleum ether (1.5 g). An analytical sample was prepared by filtering a solution of product in 5% methanol–chloroform through a short column of silica gel (25 g) followed by crystallization from ethyl acetate–petroleum ether: mp 137–140°, $[\alpha]_D^{20}$ −31.1° (*c* 0.67, MeOH). *Anal.* Calcd for $C_{32}H_{42}N_6O_8$: C, 60.91; H, 6.51; N, 12.92. Found: C, 60.99; H, 6.74; N, 13.06.

Z-Phe(NO_2)-His-Leu·HCl. A solution of the *tert*-butyl ester (1.2 g, 2.15 mmoles) in 3% HCl–acetic acid (10 ml) was prepared, left at room temperature 2.5 hr, and evaporated. The residue was dissolved in methanol and precipitated by addition of ether to yield a colorless solid (1.0 g). An analytical sample was prepared by a second precipitation from the same solvent: mp 134–135°, $[\alpha]_D^{20}$ −9.1° (*c* 0.63, MeOH). *Anal.* Calcd $C_{29}H_{34}N_6O_8 \cdot HCl$: C, 55.19; H, 5.59; Cl, 5.62; N, 13.32. Found: C, 54.87; H, 5.54; Cl, 5.64; N, 13.23.

Enzyme Units. One unit of converting enzyme activity in this report is defined as 1 nmole of substrate hydrolyzed per min under the assay conditions. Specific activity is expressed as nanomoles of substrate hydrolyzed per minute per milligram of enzyme.

Protein Determination. The protein content was determined by measuring the optical density at 280 nm in a 1-cm cell and by the method of Lowry *et al.* (1951) using bovine serum albumin to construct a standard curve.

Ultraviolet Difference Spectra. Ultraviolet difference spectra were obtained between Z-Phe(NO_2)-His-Leu, Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly, and their respective products obtained by the theoretical hydrolytic action of the converting enzyme. The various substrates and products were

prepared fresh as 5×10^{-4} M solutions in 5% methanol–0.05 M Tris-HCl–0.1 M NaCl (pH 8.0) buffer. All spectra were obtained on a Cary Model 16 double-beam spectrophotometer equipped with a Multipot Accessory zeroed against the appropriate buffer in the wavelength range 350–215 nm. The results were monitored on a 10-in. recorder in conjunction with the Cary recorder interface.

Spectrophotometric Assays. Routine converting enzyme assays were determined by monitoring the hydrolysis of the synthetic substrates on the Cary Model 16 spectrophotometer equipped with an interface and recorder. The spectrophotometric assays were conducted in a final volume of 3 ml with substrate at 5×10^{-4} M in 5% methanol–0.05 M Tris-HCl–0.1 M NaCl (pH 8.0). The hydrolysis of each substrate (at 25°) was monitored at its respective wavelength and enzyme rates were calculated from their respective delta extinction coefficients. Carboxypeptidase A activity and aminopeptidase activity were monitored by known uv spectrophotometric methods (Folk and Schirmer, 1963; Mitz and Schlueter, 1958) employing the substrates Hip-Phe at 254 nm and Leu-amide at 250 nm, respectively.

Enzyme Kinetics. All spectrophotometric enzyme kinetic measurements were made at 25° on an assay mixture of 3 ml with substrate at 5×10^{-4} M in 5% methanol–0.05 M Tris-HCl–0.1 M NaCl (pH 8.0). Any nonenzymatic hydrolysis was cancelled out of the measurements by using an appropriate blank cuvet in the double-beam spectrophotometer. The concentration of Z-Phe(NO_2)-His-Leu ranged from 1.25×10^{-4} to 5×10^{-4} M, Z-Phe(NO_2)-Gly-Gly from 2×10^{-3} to 5×10^{-5} M, and Hip(NO_2)-Gly-Gly from 2×10^{-3} to 5×10^{-5} M.

Calculation of Kinetic Constants. The kinetic constants presented in this report were calculated directly from the observed velocities and the initial substrate concentrations. Under conditions in which the same quantity of converting enzyme was employed at all substrate levels, the reaction may have proceeded to 5% of the total hydrolysis by the time the rate measurements were begun. Thus, measurements were usually made between 5 and 15% of the total hydrolysis of the substrate. With all substrates the linear phase of hydrolysis extended further than 15% hydrolysis, so the measurement of initial rates is quite justified. For most assays 5–15 units of enzyme was used per assay; thus, a continual rate was recorded within 10 min on 10-in. chart paper in which full scale was 0.1 absorbance unit.

The constants, K_m and V_{max}, were calculated from the plots of the data according to the method of Lineweaver and Burk (1934). Straight lines that best fit the data for the reciprocal Lineweaver–Burk plots were obtained by linear regression analysis. The inhibition constants (K_i) were determined from the graphical analysis procedure of Dixon (1953) and were obtained by linear regression analysis of the data.

Ninhydrin Assay. The ninhydrin reagent was prepared and stored under nitrogen according to Hirs (1967). The rates of hydrolysis of Hip-His-Leu, Z-Phe-His-Leu, and angiotensin I were measured by reacting the released His-Leu with ninhydrin reagent (Cushman and Cheung, 1969; Skeggs *et al.*, 1956).

Thin-layer Chromatography (tlc). The hydrolytic products of the enzymatic reaction (95% hydrolysis) were determined by tlc employing 20 × 20 cm silica gel plates. The solvent system 1-butanol–acetic acid–ammonia (ammonium hydroxide–water, 1:4) (11:6:3) was utilized for all three substrates and products, whereas 1-butanol–acetic acid–water (6:2:3) was also used for Z-Phe(NO_2)-His-Leu and its products. After 3- to 4-hr development time in sealed glass tanks, the plates were thoroughly dried and the spots developed with either a ninhydrin spray or Pauly Reagent.

Amino Acid Analysis. Converting enzyme (70 units) was incubated at 25° with 2 μmoles of angiotensin I for 42 hr. The products of hydrolysis were subsequently determined on a JEOL-JLC-5AH amino acid analyzer employing a 0.9 × 15 cm Aminex A-7 column with the recommended sodium citrate buffer (pH 5.28). At 55° and a flow rate of 0.8 ml/min, the standard retention times for free His and His-Leu were 62 and 69 min, respectively.

Batchwise CM-cellulose Procedure. The converting enzyme preparation, which had previously been dialyzed for 18 hr against 7 l. of 0.02 M Tris-acetate (pH 5.5) was added to 75 g of CM-cellulose equilibrated with the same buffer. After 45 min the enzyme was separated from the cellulose mixture by filtration on a Büchner funnel. The cellulose was resuspended in 400 ml of buffer and the filtration procedure repeated. The two filtrates were combined and the pH was adjusted to 7.3 with the addition of 1 M Tris base. The proteins were precipitated by adding solid ammonium sulfate to 70% saturation; the solution was stirred for 2 hr and then centrifuged for 60 min at 16,000*g*. The pellet was subsequently dissolved in a minimal amount of cold distilled water, dialyzed for 15 hr against 7 l. of 0.01 M Tris-HCl (pH 8.0), and recentrifuged.

Column Preparation. DEAE-cellulose (0.01 M Tris-HCl, pH 8.0) was packed in a 5 × 100 cm column at an air pressure of 2 psi and equilibrated overnight with buffer at 100 ml/hr. Sephadex G-200 was prepared in 0.01 M Tris-HCl–0.05 M NaCl (pH 8.0) as specified by the manufacturer and packed by gravity in a 5 × 100 cm column. After packing, a reverse flow was employed with the aid of flow adapters (Pharmacia) and a peristaltic pump at 45 ml/hr. Sephadex G-50 was prepared in a similar fashion to a height of 20 cm in a 2.5-cm diameter column and run at 80 ml/hr. Void volumes were determined for the various Sephadex gels by employing a 0.1% solution of Blue Dextran 2000 (lot no. 2078, Pharmacia) in the column buffer.

Isoelectric Focusing. An isoelectric focusing apparatus was set up and run according to the procedure of Haglund (1967). An Ampholine column (LKB 8101, 110 ml) was filled with pH 4–6 Ampholine (1%) and 7.7 mg of converting enzyme in a sucrose density gradient. The temperature of the column was maintained at 4° throughout the 70-hr time period in which a voltage drop of 500 V was applied.

Molecular Weight Estimation. The following marker proteins were employed to standardize a G-200 Sephadex column for molecular weight estimation: apoferritin, γ-globulin, albumin, ovalbumin, and myoglobin. These proteins were dissolved in 0.01 M Tris-HCl–0.05 M NaCl (pH 8.0) column buffer and placed on a G-200 Sephadex column (5 × 79 cm) which was equilibrated at a flow rate of 45 ml/hr (upward flow). Fractions were collected and 280-nm absorbance was determined to detect the protein peaks. Converting enzyme (0.25 mg/3 ml) was also placed on the column in a similar fashion and was monitored for 280-nm absorbance and enzymatic activity.

Disc Gel Electrophoresis. The apparatus employed for analytical disc gel electrophoresis was obtained from the Canalco Co. (Model 6). The procedure described by Davis (1964) was followed employing a large-pore sample gel, a large-pore spacer gel, and a small-pore separating gel. Upon completion of electrophoresis some sample gels were stained for 20 min in 0.25% Coomassie Brilliant Blue, then destained according to Davis at 12.5 mA/column. Unstained sample

TABLE I: Substrates for Converting Enzyme.[d]

Substrate	Wavelength (nm)	ΔOD	Enzyme Act. (Units/mg)	Rel Act.
Z-Phe(NO_2)-His-Leu	306	0.44	1010	1.00
Hip(NO_2)-Gly-Gly	290	0.50	1530	1.5
	291[a]	0.49[a]	795[a]	0.8
Z-Phe(NO_2)-Gly-Gly	301	0.60	1920	1.8
Z-Phe-His-Leu			1340[b]	1.3
Hip-His-Leu			272[b]	0.27
Angiotensin I			105[c]	0.10

[a] These values were obtained with no methanol present in the incubation mixture. [b] These values were obtained by the ninhydrin procedure with a substrate concentration of 5×10^{-4} M. [c] This value was obtained by the ninhydrin procedure with a substrate concentration of 1×10^{-4} M and no methanol present. [d] Assay conditions: 2.0 ml of 7.5×10^{-4} M substrate in 0.1 M NaCl–0.05 M Tris-HCl (pH 8.0) with 5% methanol plus 1.0 ml of enzyme and/or buffer. The assay was determined on a Cary 16 spectrophotometer at 25°, 3.0-mm slit 0.1-OD full scale at the wavelength indicated in the table. Converting enzyme: (300-fold purification).

gels were frozen for about 10 min and sliced into 1.5-mm disks. Each slice was extracted overnight with 1 ml of cold 0.05 M Tris-HCl–0.1 M NaCl (pH 7.0) buffer. The 1 ml of elution buffer was subsequently assayed for enzyme activity with the Z-Phe(NO_2)-Gly-Gly substrate at pH 7.0, 301 nm.

Metal Chelators. Converting enzyme was freed of all extraneous metal ions by dialyzing against 0.01 M Tris-HCl–0.05 M NaCl (pH 8.0) for 48 hr and changing the dialysate three times during this period. The enzyme was preincubated for 5 min with various concentrations of EDTA or *o*-phenanthroline and buffer in a total of 1 ml; then 2 ml of substrate Z-Phe(NO_2)-Gly-Gly (pH 7.0) was added, and the reaction mixture was immediately monitored at 301 nm. The concentration of EDTA and *o*-phenanthroline was varied over a range necessary to obtain a 0–100% inhibition.

Metal Activation of Enzyme. Converting enzyme was prepared for metal activation studies by dialyzing against either EDTA (3×10^{-5} M) or *o*-phenanthroline (3×10^{-5} M) for 18 hr. The converting enzyme containing EDTA was completely inactive when measured against Z-Phe(NO_2)-Gly-Gly (final concentration of EDTA was 1×10^{-6} M). Because the *o*-phenanthroline-treated enzyme was still active, more *o*-phenanthroline was added to the enzyme solution to give a final concentration of 6.8×10^{-5} M, which completely inhibited the enzyme activity. The enzymes were reactivated to various degrees by preincubating for 5 min with various concentrations of different metal ions (Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}).

Results

Ultraviolet Spectra. We have synthesized three new substrates which were employed in a rapid enzyme assay utilizing a uv kinetics spectrophotometer. The basis of this assay depends on the difference in uv absorption of the substrate when compared with the uv absorption of the products formed by the enzymatic reaction. Z-Phe(NO_2)-His-Leu and an equimolar mixture of its enzymatically formed products (Z-Phe(NO_2) and His-Leu) both have appreciable absorption in the uv at concentrations of 1×10^{-4} M. The substrate peaks at 279 nm with an absorption of 1.0, whereas the mixture of products peaks at 282 nm with an absorption of 0.94. Of more importance is the appreciable difference in absorption that occurs between the two curves at wavelengths greater than 290 nm due to a red shift when the substrate is cleaved. This difference is maximized at 306 nm, therefore this is the wavelength chosen to follow the enzymatic hydrolysis of this substrate.

When a difference spectrum of a 5×10^{-4} solution of Z-Phe(NO_2)-His-Leu, in the reference cuvet, and 5×10^{-4} M solution of Z-Phe(NO_2) + His-Leu, in the sample cuvet, was recorded, the Δ absorption at 306 nm was 0.44. Similar uv difference spectra were recorded for Z-Phe(NO_2)-Gly-Gly and Hip(NO_2)-Gly-Gly and their respective products at 5×10^{-4} M concentration. These results are listed in Table I.

In order to evaluate the effect of pH on the activity of converting enzyme, any change in the Δ absorption with variation of pH had to be ascertained. For Z-Phe(NO_2)-His-Leu the difference spectra obtained at pH values between 5 and 10 all resulted in maxima at 306 nm. The Δ absorption value observed at pH 5.0 was 20% greater than the value obtained at pH 10.0. This difference was due to the ionization of the imidazole group on the substrate and not His-Leu since the latter compound did not contribute to the absorption at 306 nm. The experimental points obtained from the Δ absorption values at various pH levels fit the theoretical sigmoid curve obtained by using the pK' of the imidazole group of Z-Phe(NO_2)-His-Leu. By pH titration the pK' was determined to be 6.96. With the other substrates, Z-Phe(NO_2)-Gly-Gly and Hip(NO_2)-Gly-Gly, the Δ absorption did not vary significantly (±2%) with the pH.

Ultraviolet Spectrophotometric Assay. Since various quantities of enzyme from effluent fractions of column chromatographies were monitored for activity by the uv spectrophotometric procedure, it was imperative to establish that the rate of hydrolysis was linear with varying quantities of enzyme. The rate of hydrolysis of 5×10^{-4} M Z-Phe(NO_2)-His-Leu/enzyme unit was found to be constant over a range of 2–30 units of enzyme. Hydrolysis rates were determined from recordings of initial velocities which approximated linearity up to 20% of the total hydrolysis (Figure 1).

Hydrolysis of Substrates. An evaluation of six substrates was conducted with a calf lung enzyme preparation. The rates of hydrolysis of the three nitrated substrates was determined by the uv spectrophotometric assay while the rates of hydrolysis of the remaining three substrates listed in Table I were determined by the ninhydrin procedure. Z-Phe(NO_2)-His-Leu was monitored for hydrolysis by the ninhydrin procedure as well as the uv spectrophotometric method and, as expected, the results correlated. The three substrates assayed by the uv spectrophotometric method were all hydrolyzed at rapid rates with Z-Phe(NO_2)-Gly-Gly having the highest rate. Z-Phe-His-Leu was also a good substrate and was, in fact, hydrolyzed 30% faster than its nitrated analog. Hip-His-Leu and angiotensin I had relative rates of 0.27 and 0.1, respectively, when compared to the rate of hydrolysis of Z-Phe(NO_2)-His-Leu.

Effect of Methanol on the Rate of Hydrolysis. Methanol was required to solubilize Z-Phe(NO_2)-His-Leu and Z-Phe(NO_2)-Gly-Gly; but since Hip(NO_2)-Gly-Gly was soluble with or without methanol, its enzymatic hydrolysis was stud-

TABLE II: Purification Scheme of Converting Enzyme from Calf Lung.

Step	Sample	Total Units[a]	% Yield	Units/OD 280 nm	Purification
1	Lung extract (56.5 g of acetone powder)	35,600	100	2.5	1
2	CM-cellulose	19,700	55.5	18	7
3	DEAE-cellulose	11,200	32	177	71
4	Sephadex G-200	10,520	29.6	1002	405
5	Isoelectric focusing	5,430	15.2	1740	700

[a] Substrate: 5×10^{-4} M Z-Phe(NO_2)-His-Leu monitored at 306 nm at pH 8.0.

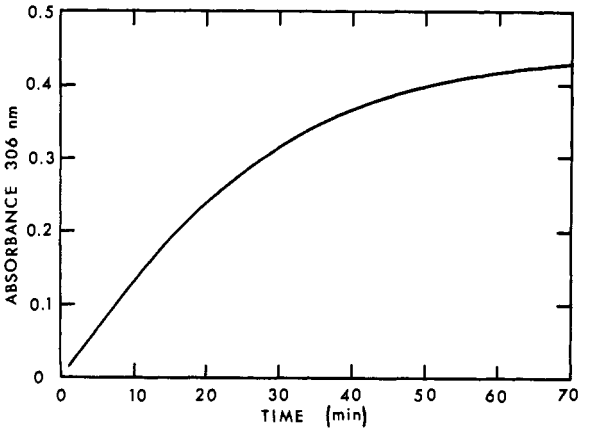

FIGURE 1: Hydrolysis of Z-Phe(NO_2)-His-Leu with time. This is the actual recording of the hydrolysis of 3 ml of Z-Phe(NO_2)-His-Leu 0.0005 M in 0.1 M sodium chloride, 0.05 M Tris-HCl, and 5% methanol at pH 8.0 and 25°) by 48 units of lung enzyme monitored on the Cary Model 16 spectrophotometer at 306 nm, 3.0-mm slit, 0.1 in./min, and 1.0 absorbance unit set to full scale on a 10-in. recorder.

ied under both conditions. The Δ absorption values varied only slightly with or without methanol. Methanol (5–20%) approximately doubled the rate of hydrolysis of Hip(NO_2)-Gly-Gly, but was inhibitory at concentrations greater than 30%. The results with Z-Phe(NO_2)-His-Leu almost paralleled the changes seen with the rates of Hip(NO_2)-Gly-Gly hydrolysis over the range of 5–30% methanol. It is not known whether the large increase in activity would be seen in going from 0 to 5% methanol (Z-Phe(NO_2)-His-Leu is insoluble at <5% methanol), but, by analogy to Hip(NO_2)-Gly-Gly, it would be expected to behave similarly.

Products of Enzymatic Hydrolysis. Angiotensin I converting enzyme is thought to be an α-carboxypeptide dipeptidohydrolase (dipeptidyl carboxypeptidase) that cleaves a dipeptide from the carboxyl terminal of various synthetic substrates, angiotensin I, and bradykinin (Huggins and Thampi, 1968; Dorer *et al.*, 1970; Piquilloud *et al.*, 1970; Yang *et al.*, 1971; Cushman and Cheung, 1971; Elisseeva *et al.*, 1971). To establish that our synthetic compounds were valid substrates for angiotensin I converting enzyme thin-layer chromatographies were performed to demonstrate what products were formed by the enzymatic cleavage of Z-Phe-(NO_2)-His-Leu, Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly. Hydrolysis was permitted to proceed to completion by incubating the enzyme with substrate for periods that were as long as, or ten times longer than, was necessary for 95% hydrolysis. In all instances the respective dipeptide was found with no further breakdown to free amino acid. Under similar conditions of incubation His-Leu, Gly-Gly, Phe-Gly, Hip-Phe, and Phe-Arg were not degraded to free amino acids, but Leu-Leu was partially hydrolyzed. Amino acid analysis of an incubation mixture of converting enzyme with angiotensin I yielded 1.35 μmoles (68% hydrolysis) of His-Leu and only a trace (<1%) of free His. Spectrophotometric assays to detect carboxypeptidase A or aminopeptidase activity were negative, but the possibility of a dipeptidase remains.

Purification of the Converting Enzyme. In order to facilitate prolonged storage and subsequent extraction of the converting enzyme, an acetone powder of calf lung tissue was formed. The converting enzyme was then extracted with water containing 0.1% Triton X-100, a solubilizing agent. By resorting to the acetone powder a total of 30,000–40,000 enzyme units could be routinely obtained upon extraction of 56.5 g of powder (equivalent to 300 g of wet tissue). In this manner the individual variability experienced with previous preparations from frozen tissue could be greatly reduced. The purification scheme outlined in Table II indicates the major steps involved in a typical isolation. The Z-Phe(NO_2)-His-Leu substrate was used to monitor the enzyme activity through the various procedures.

The large loss (~45%) of enzyme units, associated with the batchwise CM-cellulose step, was caused by adjusting the pH of the extract with concentrated acetic acid prior to CM-cellulose. There was minimal loss of activity in subsequent preparations utilizing this procedure with 0.1 N acetic acid. There was no loss of units on the CM-cellulose itself and a 7-fold purification resulted. The sample was next dialyzed and placed on an anion-exchange column (DEAE-cellulose) (5 × 42 cm) and eluted with 0.01 M Tris-HCl (pH 8.0) containing a stepwise gradient of NaCl (0.06, 0.07, 0.09, and 0.12 M). All the converting enzyme activity was located in the protein peak associated with the 0.09 M NaCl step. A majority (57%) of the units placed on this column was recovered in this peak with a 10-fold increase in purity. After concentration the sample was placed on a Sephadex G-200 column (5 × 79 cm) equilibrated with 0.01 M Tris-HCl–0.05 M NaCl (pH 8.0). The converting enzyme eluted at 700 ml, shortly after the void volume (V_0 = 595 ml) and was situated on the leading shoulder of a major protein peak. A 5- to 6-fold increase in purity was achieved with this column concomitant with a 94% recovery of enzyme units.

Isoelectric focusing was used as the final step in the purification scheme. We placed 7.7 mg of protein on a 110-ml column which contained ampholyte in the pH range 4–6. The enzyme activity was spread over a wide area; but the peak activity could be correlated to pH, and the results of 10 isoelectric focusing runs obtained a p*I* 4.68 ± 0.15 SD, ±0.05 SE. After removal of the ampholyte by molecular sieving on Sephadex G-50 the isoelectric focusing technique produced a 1.7-fold increase in purity associated with a 50% recovery of enzyme units. This enzyme preparation was purified 700-fold from the acetone powder extract and was used in all of the investigations described below. The enzyme solution gave a peak absorbance of 278 nm in the uv spectrum and the $E^{1\%}_{280\,nm}$ of 13.1 was determined by measuring the protein content by the folin reaction (Lowry *et al.*, 1951). At the various steps listed in Table I, the enzyme was assayed against the three synthetic substrates: Z-Phe(NO_2)-His-Leu,

TABLE III: Substrates and Kinetic Constants for Converting Enzyme.

Substrate	Units/mg of Enzyme[a]	V_{max} (sec^{-1})	K_m (mmoles)	pH_{max}
Z-Phe(NO_2)-His-Leu	2280	13.9	0.23	8.0
Z-Phe(NO_2)-Gly-Gly	4180	56.7	1.5	7.0
Hip(NO_2)-Gly-Gly	3520	99.7	4.8	7.0
Hip(NO_2)-Gly-Gly (no MeOH)	1780	57.7	3.1	7.5

[a] Routine assays were performed with 5×10^{-4} M substrate in 0.1 M NaCl–0.05 M Tris-HCl (pH 8.0) and 5% MeOH (except where noted) at the proper wavelength. The converting enzyme solution had an absorbance 280 nm of 0.062 and was purified 700-fold.

Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly. The relative rates of enzymatic hydrolysis of these three substrates did not change significantly throughout the purification scheme. There was a variability of ±15% in the relative rates; but there was no trend to an increased or decreased rate, and hence, there was no evidence of a change in substrate specificity with change in enzyme specific activity.

pH Profiles. The pH profiles of the converting enzyme activity toward Z-Phe(NO_2)-His-Leu, Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly were all bell-shaped and gave maximum rates between pH 7 and 8 (Table III). The substrates which had a Gly-Gly cleaved off were hydrolyzed most rapidly at pH 7.0 compared to the pH maximum of 8.0 for Z-Phe(NO_2)-His-Leu.

Kinetic Constants.. Table III lists the kinetic constants, V_{max} and K_m, along with the units of activity by the standard assay procedure for Z-Phe(NO_2)-His-Leu, Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly. The K_m for Z-Phe(NO_2)-His-Leu was calculated from the Lineweaver–Burk plot as 0.23 mM, which makes it the substrate most tightly bound to the enzyme (assuming that $K_m \cong K_s$). However, Z-Phe(NO_2)-His-Leu has the lowest rate of hydrolysis as evidenced by both the standard assay and V_{max}, even though it most resembles the primary structure of the native substrate angiotensin I. Possibly the Gly-Gly portion of the other two substrates presents less of a steric hindrance and thus facilitates the catalytic process. Clearly, no advantage is gained with Gly-Gly substrates through binding, because they have higher K_m values than Z-Phe(NO_2)-His-Leu. At concentrations of Z-Phe(NO_2)-His-Leu higher than 1×10^{-3} M a decrease in activity is evident which suggests some substrate inhibition. The Gly-Gly substrates did not exhibit this phenomenon; but since they both had a higher K_m, this inhibition might have occurred if we had been able to extend our assay procedure to very high concentrations of these substrates. The K_m values for Hip-His-Leu and Z-Phe-His-Leu were 0.22 and 0.23 mM, respectively, which gives these substrates K_m values which are nearly identical with that of Z-Phe(NO_2)-His-Leu.

Z-Phe(NO_2)-His-Leu has been used extensively because it resembles angiotensin I and has the lowest K_m of the nitrated substrates. Z-Phe(NO_2)-Gly-Gly has been used exclusively in the metal-replacement studies (*vide infra*) because of its superior property of remaining soluble in the presence of all metals tested.

Inhibition. The specificity of the converting enzyme hydrolysis of Z-Phe(NO_2)-His-Leu was examined by inhibiting this reaction with the natural substrate angiotensin I (Asn^1,-Ile^5). With Z-Phe(NO_2)-His-Leu at 5×10^{-4} M and pH 8.0 the concentration of angiotensin I necessary to inhibit 50% hydrolysis was 4×10^{-5} M. Product inhibition also occurred; His-Leu and Gly-Gly, both products of the hydrolytic action on synthetic substrates, inhibited the hydrolysis of Z-Phe-(NO_2)-His-Leu and had K_i values of 8.8×10^{-4} and 1.5×10^{-3} M, respectively. The product of the bradykininase activity of this enzyme, Phe-Arg, inhibited the hydrolysis of Z-Phe(NO_2)-His-Leu ($K_i = 8.1 \times 10^{-4}$ M). Pyr-Lys-Trp-Ala-Pro, which had been previously isolated from *Bothrops jaraca* (Ferreira *et al.*, 1970) and subsequently synthesized (Stewart *et al.*, 1971), inhibited the calf lung enzyme hydrolysis of Z-Phe(NO_2)-His-Leu ($K_i = 5 \times 10^{-7}$ M). Low salt concentration in the incubation mixture also inhibited the converting enzyme. The chloride dependence of the calf lung enzyme was not rigorously explored, but the presence 0.1 M chloride ion in the assay was necessary to maintain full activity. When the concentration of chloride ion was decreased to 1 mM, only 11% of the normal activity was retained when we used the Z-Phe(NO_2)-His-Leu substrate.

Properties of Converting Enzyme. MOLECULAR WEIGHT ESTIMATION. Employing the standard proteins of known molecular weight contained in the molecular weight marker kit, a Sephadex G-200 column (5 × 79 cm) was calibrated and a standard graph constructed. A small sample (540 units) of our most purified converting enzyme was then applied, and the enzyme activity peak was found to occur at an elution volume of 700–720 ml, corresponding to a molecular weight of 300,000–330,000.

DISC GEL ELECTROPHORESIS. Four protein bands were observed with our most purified enzyme preparation. The majority of the enzyme activity was situated in the upper portion of the gel (R_F 0.23) in association with one band, and minimal activity (<20%) was detected in a neighboring protein band (R_F 0.27). A majority (60%) of the enzyme activity was recovered when a pre-electrophoretic run of the separating gel in the presence of dithiothreitol was made. Under the standard conditions of gel electrophoresis employing a sample gel, spacer gel, and separating gel with no pre-electrophoretic run, only 20% of the applied enzyme units could be recovered.

METAL CHELATORS. A study of metal activation was conducted by studying the effect of a wide range of metal concentrations (10^{-1}–10^{-7} M) on converting enzyme activity. In these experiments it was necessary to negate the effect of endogenous metal associated with the enzyme by adding a metal chelator, either EDTA or *o*-phenanthroline. The level of metal chelator necessary to effect a 50% inhibition was established as 4.6×10^{-7} M EDTA and 9.1×10^{-6} M *o*-phenanthroline. These were the values obtained in converting enzyme assays in which Z-Phe(NO_2)-Gly-Gly (5×10^{-4} M, pH 7.0) was the substrate and 4.76 μg (10.9 units) of enzyme was present. This substrate was used in all metal studies because Z-Phe(NO_2)-His-Leu precipitated from solution when low concentrations of Zn^{2+}, Ni^{2+}, or Co^{2+} were added.

ACTIVATION BY METAL IONS. EDTA (1×10^{-6} M) and *o*-phenanthroline (6.8×10^{-5} M) completely inhibited the hydrolysis of Z-Phe(NO_2)-Gly-Gly, but the chelator-treated enzyme was returned to an active enzyme by the addition of divalent metal ions. In the study in which the EDTA-in-

hibited enzyme activity was reversed by metals, partial activity was rapidly restored by low concentrations of Zn^{2+}, Co^{2+}, or Mn^{2+} (Figure 2). Full activity was restored by 2×10^{-7} M Zn^{2+}, 1×10^{-5} M Co^{2+}, or 1×10^{-4} M Mn. With Co^{2+} at 2×10^{-5} M the activity reached a maximum of 30% greater than original activity; this was the only metal with which activity greater than 100% was obtained. At concentrations greater than 1×10^{-4} M, Co^{2+} and Zn^{2+} were inhibitory. Mg^{2+} and Ca^{2+} were poor activators and permitted only 50% reactivation at 1×10^{-3} and 1×10^{-2} M, respectively. In analogous experiments with the *o*-phenanthroline-inhibited enzyme, the same general pattern of activation occurred with all metals ($Zn^{2+} > Co^{2+} > Mn^{2+} >>> Mg^{2+}$ and Ca^{2+}) as was shown with the EDTA-treated enzyme.

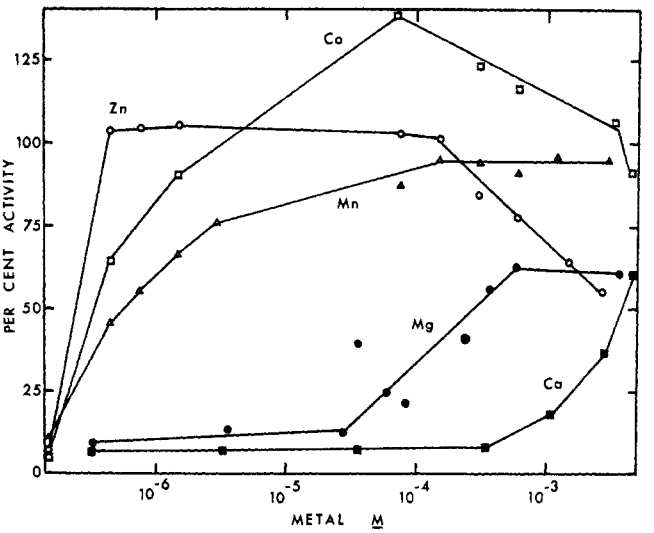

FIGURE 2: Reactivation of the EDTA-treated converting enzyme by the addition of metal ions: Zn^{2+} (○), Co^{2+} (□), Mn^{2+} (△) Mg^{2+} (●), and Ca^{2+} (■). The enzyme (700-fold purity) was dialyzed for 18 hr *vs.* 0.01 M Tris-HCl–0.05 M NaCl buffer (pH 7.0), containing 3×10^{-5} M EDTA. Enzyme activity was monitored with 5×10^{-4} M Z-Phe(NO_2)-Gly-Gly (pH 7.0) at 301 nm and 25°.

Discussion

The use of Z-Phe(NO_2)-His-Leu, Z-Phe(NO_2)-Gly-Gly, and Hip(NO_2)-Gly-Gly as synthetic substrates for converting enzyme is justified by the fact that only carboxyl-terminal dipeptides were generated in the enzymatic reaction. All of these substrates have high Δ extinction coefficients which make them suitable for use at a reasonable concentration (5×10^{-4} M) in routine enzymatic assays. We have also prepared the Z-Phe-His-Leu substrate, initially used by Piquilloud *et al.* (1970), and assaying this substrate by the ninhydrin procedure revealed that the nitrated substrate was hydrolyzed 77% as fast as the nonnitrated substrate. Therefore, the addition of the nitro group can be used as a convenient probe to monitor the enzyme activity in a continuous uv spectrophotometric procedure. The observance of a continual rate insures initial velocity measurements and eliminates the inherent difficulties and interpretation associated with a kinetic rate analysis by a two- or three-point method. In addition, the measurement of enzyme activity at a uv wavelength of 306 nm minimizes the absorption contributed by proteins. Inouye and Fruton (1967a,b) initially exploited the utility of observing a change in absorbance when a bond linking the carbonyl group of a *p*-nitrophenylalanyl residue was cleaved. They used a spectrophotometric assay method at 310 nm to follow the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe by the proteolytic enzyme pepsin. Yang *et al.* (1970, 1971b) were the first investigators to use a nitrated substrate (BOC-Phe(NO_2)-Phe-Gly) for converting enzyme. At 5×10^{-4} concentration this substrate had a Δ absorbance of 0.59 at 310 nm which is nearly identical to the Δ absorbance of Z-Phe(NO_2)-Gly-Gly ($\Delta OD_{310nm} = 0.60$). A comparison of the effectiveness of BOC-Phe(NO_2)-Phe-Gly and our nitrated substrates can be made indirectly through a common substrate, Hip-His-Leu, used by both of us. When all of the substrates are compared at 5×10^{-4} M concentration in sodium chloride and Tris-HCl buffer at near optimum pH values, the relative rates of hydrolysis by converting enzyme are 1:1:3.7:4.5:6.6 for Hip-His-Leu, BOC-Phe(NO_2)-Phe-Gly, Z-Phe(NO_2)-His-Leu, Hip(NO_2)-Gly-Gly, and Z-Phe(NO_2)-Gly-Gly, respectively. (The rates of hydrolyses of Hip-His-Leu and Boc-Phe-(NO_2)-Phe-Gly obtained from Yang *et al.* (1971) were adjusted to comparable rates at 5×10^{-4} M concentration.) It should be pointed out that Yang *et al.* (1971) used a converting enzyme preparation from heparin-treated swine plasma, whereas we used a converting enzyme preparation from calf lung.

The uv spectrophotometric assay was used with both crude and purified converting enzyme samples. The crude enzyme was used only in monitoring column chromatographic procedures, and hence accuracy was not needed. However, in the entire enzyme preparation there was very little carboxypeptidase-like activity which would be the main contributor to any error in the assay. The presence of aminopeptidases or dipeptidases would only effect the free dipeptides which do not contribute any significant absorbance at 290–310 nm. Therefore, even crude preparations of converting enzyme gave representative enzymic rates with this assay procedure. We have not tested tissues other than lung for converting enzyme activity but we believe that this assay is sensitive enought to detect activity in most tissues demonstrated to have converting enzyme activity (Huggins and Thampi, 1968).

In all of our enzyme assays 5% methanol was present in the incubation mixture. The increase in the rate of hydrolysis of the substrates Z-Phe(NO_2)-His-Leu and Hip(NO_2)-Gly-Gly with varying concentrations of methanol has been memtioned. It is difficult to speculate exactly what methanol is doing, but other enzymes such as carboxypeptidase B (Folk *et al.*, 1962) and serum cholinesterase (Main, 1961) can be adversely or favorably affected by alcohols. In these cases it is postulated that the effect of alcohols is limited to modification in the rate of breakdown of enzyme–substrate complex to free enzyme and products and not due to the change of second substrate from water to alcohol (*i.e.*, alcoholysis does not replace hydrolysis).

A comparison of the rates of hydrolysis of substrates containing a carboxyl-terminal His-Leu at 5×10^{-4} M revealed ratios of 1.00:0.77:0.27 for Z-Phe-His-Leu, Z-Phe(NO_2)-His-Leu, and Hip-His-Leu, respectively. The relative rate of hydrolysis of angiotensin I at 1×10^{-4} M (a value 2–20 times reported K_m values (Huggins *et al.*, 1970; Lee *et al.*, 1971a,b)) was 0.1. We have demonstrated that the synthetic substrates were hydrolyzed more rapidly than angiotensin I, which agrees with the results tabulated by Piquilloud *et al.* (1970). It is also interesting that the substrates Z-Phe(NO_2)-Gly-Gly and Hip(NO_2)-Gly-Gly, which do not resemble angiotensin I, are hydrolyzed more rapidly than Z-Phe(NO_2)-His-Leu, which does resemble angiotensin I. It is not apparent why the His-Leu portion of the molecule would hinder hydrolysis and

release of the dipeptide. Our data also showed that Hip-His-Leu was hydrolyzed at a lower rate than Z-Phe-His-Leu or the nitrated substrates. In agreement with our results concerning substrates with His-Leu and Gly-Gly at the carboxyl terminal, Yang *et al.* (1970, 1971) reported that Hip-Gly-Gly was hydrolyzed more than twice as rapidly as Hip-His-Leu by porcine plasma converting enzyme. In summary, the substrates which have Z-Phe, rather than Hip, containing the carbonyl portion of the hydrolyzed bond and have the carboxyl-terminal dipeptide Gly-Gly, rather than His-Leu, are hydrolyzed most rapidly by the converting enzyme. Several potential substrates of converting enzyme have been studied (Yang *et al.*, 1971; Cushman and Cheung, 1971; Elisseeva *et al.*, 1971) but not extensively investigated; a thorough study of the binding and hydrolysis of various substrates might reveal the structural requirements for the specificity of this enzyme.

After we had established our purification scheme, a preliminary procedure for isolation of converting enzyme from bovine kidney cortex was published by a group from the USSR (Elisseeva *et al.*, 1971). They achieved an enzyme preparation of 1500-fold purity by CM-cellulose, DEAE-cellulose, Sephadex G-200, and hydroxylapatite, but gave no conditions for adsorption or elution of the enzyme with these various chromatographic media.

Isoelectric focusing, which was the final step in our purification procedure, produced a 2-fold purification of the converting enzyme. The effluent fractions from the isoelectric focusing column contained only 10% of the expected enzyme units. Full enzyme activity was recovered after either dialysis or desalting by gel filtration (Sephadex G-50). This observed inhibition was the result of the carrier ampholytes (polyaminopolycarboxylic acids); control assays containing 0.06% of pH 4–6 ampholyte (the same concentration of ampholyte in the assay mixture of column effluent) resulted in 90% inhibition.

With the isoelectric focusing technique we obtained an isoelectric point of 4.7 with the calf lung converting enzyme. These results are in accord with the pH precipitation of horse plasma converting enzyme at pH 5.2 by Skeggs *et al.* (1956) and dog lung converting enzyme at pH 5.2 by Huggins *et al.* (1970) in which the isoelectric point of these proteins is probably close to 5. It is difficult to compare the "same" enzyme from different tissues and species, but our experience with porcine plasma converting enzyme shows similarities between the porcine plasma and calf lung enzymes on ion-exchange chromatography and Sephadex gel filtration. In direct conflict with our molecular weight estimation of 300,000 for calf lung converting enzyme and porcine plasma converting enzyme are the data of Lee *et al.* (1971b) who estimated a molecular weight for porcine plasma converting enzyme of 155,000 when measured by the sucrose density gradient sedimentation technique and Fitz and Overturf (1972) who estimated a molecular weight for human lung converting enzyme of 480,000 on Sephadex G-200. Cushman and Cheung (1971) have suggested that the membrane-bound lung converting enzyme may be the source of the plasma converting enzyme, but more definitive data are needed to establish their similarity or identity.

The enzyme which we isolated from calf lung appeared identical with the angiotensin converting enzyme, because our enzyme preparation converted angiotensin I to angiotensin II and shared many other properties of the converting enzyme. These properties include chloride ion dependence for full activity, inhibition by the metal chelators EDTA and *o*-phenanthroline, and reactivation of the activity with Zn^{2+}, Co^{2+}, and Mn^{2+} as found by others (Dorer *et al.*, 1970; Yang *et al.*, 1971; Huggins *et al.*, 1970; Cushman and Cheung, 1971; Fitz *et al.*, 1971) studying the converting enzyme. Also, the enzyme is inhibited by Pyr-Lys-Trp-Ala-Pro (Stewart *et al.*, 1971; Cushman and Cheung, 1971), Phe-Arg (Yang *et al.*, 1971), His-Leu (Yang *et al.*, 1971), and angiotensin II (Cushman and Cheung, 1971; Lee *et al.*, 1971b), all known inhibitors of the converting enzyme.

The kinetic constant, K_m, has been determined for the converting enzyme from a variety of sources for several different synthetic substrates. The K_m for Hip-His-Leu was determined by us as 0.22 mM for calf lung enzyme, whereas Cushman and Cheung (1971) have found 2.6 mM for the same substrate with the rabbit lung enzyme. Also, we have obtained a K_m of 0.23 mM for Z-Phe-His-Leu with calf lung enzyme, whereas Piquilloud *et al.* (1970) have obtained 0.05 mM for this substrate with human plasma enzyme. Two substrates, which are used in uv spectrophotometric assays, have a nitro group on the amino acid contributing the carbonyl group of the hydrolyzed peptide bond and have K_m values of 0.23 mM (Z-Phe(NO_2)-His-Leu) and 0.90 mM (Boc-Phe-(NO_2)-Phe-Gly) for the calf lung and porcine plasma (Yang *et al.*, 1971) enzymes, respectively. It is apparent now that converting enzyme is a metalloprotein and that a divalent metal ion is necessary for full activity. The converting enzyme isolated from the lung and plasma of many different species can be inhibited by both the metal chelators, EDTA and *o*-phenanthroline. The reactivation of the EDTA-treated enzyme with Zn^{2+}, Co^{2+} or Mn^{2+}, Ca^{2+} and Mg^{2+} has been carried out by others (Dorer *et al.*, 1970; Cushman and Cheung, 1971) over a concentration range of 100-fold with the enzyme from a different source, whereas we have investigated the effects of these metals over a range of 10,000-fold, and have confirmed the results of these investigators.

The metal which is associated with the converting enzyme in the natural state is unknown, but from the metal activation studies of Dorer *et al.* (1970) and Cushman and Cheung (1971) as well as our own data, Zn^{2+}, Co^{2+}, or Mn^{2+} are the most likely candidates. Bovine carboxypeptidase (which is a monopeptidyl carboxypeptidase whereas converting enzyme is a dipeptidyl carboxypeptidase) is a metalloprotein which exists as a Zn-enzyme under physiologic conditions (Vallee *et al.*, 1960). Also it is pertinent to mention that Co^{2+} activates carboxypeptidase A to greater than 100% activity (Vallee *et al.*, 1960) just as it activates the converting enzyme to greater than 100% activity in the present investigation (Figure 2) and the report of Cushman and Cheung (1971). Thus, in analogy to carboxypeptidase A, it is likely that Zn^{2+} could be the metal activating converting enzyme *in vivo* as suggested by Cushman *et al.* (1971); but conclusive evidence such as the ^{65}Zn experiments with carboxypeptidase A (Vallee *et al.*, 1960) must be performed to prove the existence of a particular metalloprotein.

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