Biochemical Pharmacology, Vol. 20, pp. 1637–1648. Pergamon Press, 1971. Printed in Great Britain

SPECTROPHOTOMETRIC ASSAY AND PROPERTIES OF THE ANGIOTENSIN-CONVERTING ENZYME OF RABBIT LUNG

D. W. CUSHMAN and H. S. CHEUNG

Department of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, N.J. 08903, U.S.A.

(*Received* 17 *April* 1970; *accepted* 21 *August* 1970)

Abstract—A sensitive, fixed-time, spectrophotometric assay for angiotensin-converting enzyme measures the rate of production of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL). The angiotensin-converting enzyme from rabbit lung acetone powder extract, when assayed by this method, is optimally active at pH 8·1 to 8·3 at a chloride ion concentration of 300 mM and an HHL concentration of 5–10 mM; the K_m for HHL is 2·6 mM. The enzyme was inhibited by metal-chelating agents, heavy metal salts and certain peptides. The most effective inhibitors were EDTA; $CdBr_2$; angiotensin II; bradykinin; and a pentapeptide, L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline, a component of *Bothrops jararaca* venom. Enzyme inhibited by 0·1 mM EDTA was completely reactivated after removal of EDTA by dialysis but, after prolonged dialysis of the enzyme against 1 mM EDTA, reactivation could only be achieved by addition of metal ions: $MnCl_2$ (40%), $ZnCl_2$ (100%) or $Co(NO_3)_2$ (160%). The angiotensin-converting enzyme of rabbit lung is a stable, chloride ion-activated metalloenzyme, similar to both the angiotensin-converting enzyme and kininase II of plasma.

IN 1954, SKEGGS *et al.*[1,2] isolated from horse plasma a halide-activated peptidase that converted the decapeptide, angiotensin I, to the pressor octapeptide, angiotensin II, by hydrolytic removal of the C-terminal dipeptide, histidylleucine (Fig. 1). Ng and Vane[3,4] later demonstrated that the enzyme primarily responsible for the conversion *in vivo* of circulating angiotensin I to angiotensin II was localized in the lung; this result was verified by a number of workers.[5–8]

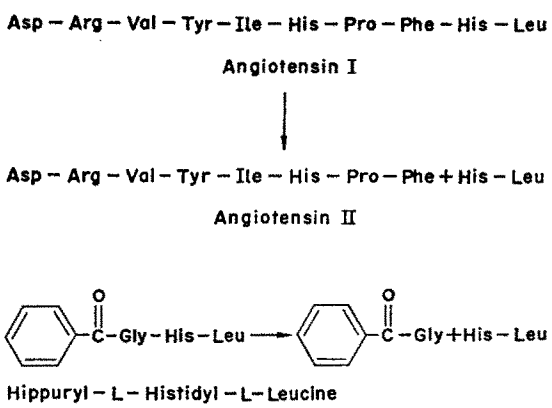

FIG. 1. Reactions catalyzed by the angiotensin-converting enzyme of lung.

Existing biological methods for determining the amount of angiotensin I converted to angiotensin II are inadequate for assay of the angiotensin-converting enzyme in lung extracts or during the early stages of purification. Angiotensin I is active to some extent in all biological assays *in vitro* and *in vivo* for angiotensin II; and "angiotensinases" present in all tissue homogenates destroy both the octapeptide and the decapeptide. For this reason, Huggins and Thampi[9] developed a radiochemical assay that measured the amount of L-histidyl-[U-^{14}C]-L-leucine formed by action of the angiotensin-converting enzyme on synthetic [U-^{14}C-Leu^{10}], [Ile^{5}]-angiotensin I[10], and Dorer *et al.*[11] developed an automated ninhydrin method that measures the chloride ion-dependent increase in ninhydrin-reactive material upon incubation of the enzyme with synthetic [Ile^{5}]-angiotensin I. Hollemans *et al.*[12] have demonstrated the possibility of using a specific radioimmunoassay for angiotensin II to determine angiotensin-converting enzyme activity. These assay methods, however, are still subject to errors, due to hydrolysis of both substrates and products by other tissue peptidases.

We previously reported the activity of a 70-fold purified angiotensin-converting enzyme from dog lung on the N-terminal protected tripeptide, hippuryl-L-histidyl-L-leucine* (HHL, Fig. 1), using a quantitative ninhydrin assay for detection of liberated histidylleucine.[13] Roth *et al.*[14] measured the histidylleucine released from another protected tripeptide, *N*-benzyloxycarbonyl-L-phenylalanyl-L-histidyl-L-leucine. The present paper describes a sensitive spectrophotometric assay for determination of the amount of hippuric acid formed from HHL by action of the angiotensin-converting enzyme. Such properties of the HHL-hydrolyzing enzyme of rabbit lung extract as are necessary for defining optimal assay conditions, and for confirming the identity of this activity with the angiotensin-converting enzyme, have been included.

MATERIALS AND METHODS

Materials. Peptides and amino acid derivatives were obtained from the following commercial sources: *N*-benzyloxycarbonyl-L-histidyl-L-leucine, *N*-benzyloxycarbonyl-L-histidine and *N*-benzyloxycarbonyl-L-leucine from Fox Chemical Company; L-histidyl-L-leucine, hippuryl-L-arginine, hippuryl-L-phenylalanine, hippurylglycine and bradykinin from Cyclo Chemical Corp.; *N*-benzoyl-L-tyrosine ethyl ester, and *p*-toluene sulfonyl-L-arginine methyl ester from CalBiochem; L-leucinamide from Pierce Chemical Company; [Ile^{5}]-angiotensin I and [Ile^{5}]-angiotensin II from Schwarz Bioresearch, Inc. The [Asn^{1}, Val^{5}]-angiotensin II was a gift from Dr. F. C. Kull of Ciba Pharmaceuticals; samples of human fibrinopeptides A and B were supplied by Dr. K. Blombäck of Karolinska Institutet, Stockholm, Sweden.

Chromagram plastic-backed silica gel thin-layer plates were purchased from Eastman–Kodak; bovine carboxypeptidase A, trypsin and chymotrypsin from Worthington Biochemical Corp.; pig heart leucine aminopeptidase from Boehringer–Mannheim; and rabbit lung acetone powders from Pel-Freez Biologicals. Other chemicals were reagent grade and obtained from commercial sources.

Synthesis of hippuryl peptides. Hippuryl derivatives of L-hisitidine, L-leucine and L-histidyl-L-leucine were prepared by reaction of the respective compounds with *p*-nitrophenylhippurate synthesized by the method of Bodanszky and duVigneaud.[15]

* The following abbreviations are used: HHL, hippuryl-L-histidyl-L-leucine; SQ 20,475, L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline.

The *p*-nitrophenylhippurate (0·15 M in dioxane–water, 2:1) was allowed to react with a slight molar excess of L-histidine, L-leucine or L-histidyl-L-leucine at a pH of 8·5, maintained with 1 N NaOH from the syringe of a Radiometer TTT1 pH-Stat. Upon completion of the reaction, each bright yellow mixture was titrated to pH 4·0, extracted thoroughly with ether, and evaporated to dryness; the dry product was desalted by extraction into absolute ethanol and again brought to dryness. The crude products were purified by partition chromatography on a 2 × 40 cm column of silica gel, equilibrated and developed with *n*-butanol–acetic acid–water (7:1:2); hippuryl peptides were monitored by their absorbance at 235 mμ. All three products were homogeneous, as judged by thin-layer chromatography in *n*-butanol–acetic acid–water (7:1:2) and *t*-butanol–4·5% ammonia (4:1); each was quantitatively cleaved by pancreatic carboxypeptidase A to yield hippuric acid and the expected amino acid(s).

Larger quantities of hippuryl-L-histidyl-L-leucine, synthesized from histidylleucine methyl ester, and the pentapeptide, L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline (SQ 20,475) were supplied by Dr. M. Ondetti, Dr. J. Pluscec, Mrs. N. Williams and Mr. E. Weaver of the Organic Chemistry Department, the Squibb Institute.

Rabbit lung acetone powder extract. All experiments on the properties of the angiotensin-converting enzyme described in this paper have utilized an extract of rabbit lung acetone powder prepared by blending 10 g of the powder in 100 ml of 50 mM potassium phosphate buffer, pH 8·3, and centrifuging for 40 min at 40,000 *g*; the clear supernatant is highly active and stable for months at 5°.

Spectrophotometric assay of angiotensin-converting enzyme. Incubations for the spectrophotometric assay of HHL hydrolysis by angiotensin-converting enzyme are carried out at 37° in disposable 13 × 100 mm tubes. Each 0·25-ml assay mixture contains the following components at the indicated final concentrations: potassium phosphate buffer,* 100 mM; sodium chloride, 300 mM; HHL, 5 mM; and enzyme, 0–10 mU per 0·25 ml of assay volume. The enzyme, in a volume of 0·15 ml or less, is added last to initiate the reaction and tubes are incubated, usually for 30 min, in a New Brunswick Gyrotory shaker.

The enzymic reactions are terminated by addition of 0·25 ml of 1 N HCl; the HCl is added before the enzyme in zero-time control assays. The hippuric acid formed by action of the angiotensin-converting enzyme on HHL is extracted from the acidified solution into 1·5 ml of ethyl acetate by vortex mixing for 15 sec. The unreacted HHL is less than 1 per cent extracted, while 91 per cent of the hippuric acid extracts into the ethyl acetate layer. After a brief centrifugation, a 1·0-ml aliquot of each ethyl acetate layer is transferred to a clean tube by means of an Eppendorf automatic pipette modified to allow the tips to extend the required distance into the tubes. The ethyl acetate aliquots are evaporated by heating at 120° for 30 min in a Temp-Blok module heater. The hippuric acid is redissolved in 1·0 ml water and the amount formed is determined from its absorbance at 228 mμ (Fig. 2).

Definition and calculation of units. For the spectrophotometric assay, 1 unit of angiotensin-converting enzyme activity is defined as the amount catalyzing the formation of 1 μmole hippuric acid from HHL in 1 min at 37° under standard assay conditions.

* The buffer is prepared to yield a pH of 8·3 in the presence of 300 mM NaCl; the substrate solution is also adjusted to pH 8·3.

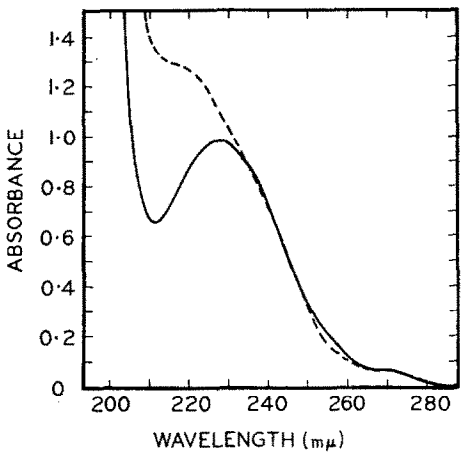

FIG. 2. Ultra-violet absorbance spectra of hippuric acid (——) and hippuryl-L-histidyl-L-leucine (– –). The concentration of each peptide was 0·1 mM in H_2O.

For a 30-min assay, the number of units of angiotensin-converting enzyme activity is equal to: $[A_{228}-A_{228}$ (control)$] \times 5{\cdot}6 \times 10^{-3}$. The conversion factor ($5{\cdot}6 \times 10^{-3}$) is derived from the following: ϵ_{228} (hippuric acid) $= 9{\cdot}8\ mM^{-1}\ cm^{-1}$; the fraction of hippuric acid extracted = 0·91; the fraction of the ethyl acetate extract transferred = 0·67; the incubation time = 30 min.

Other enzyme assays. Pancreatic chymotrypsin and trypsin or similar activities in tissue extracts were assayed spectrophotometrically by hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and *p*-toluene sulfonyl-L-arginine methyl ester (TAME) respectively.[16] Leucine aminopeptidase or similar activities were assayed by following the hydrolysis of L-leucinamide[17] and carboxypeptidase A by hydrolysis of hippuryl-L-phenylalanine.[18]

RESULTS

Spectrophotometric assay of angiotensin-converting enzyme activity

The fixed-time spectrophotometric assay of hippuric acid production from HHL is more specific for angiotensin-converting enzyme than is the previously described ninhydrin assay,[13] and can be employed over a much wider range of substrate and enzyme concentrations. Chymotrypsin and aminopeptidases have no activity on HHL; carboxypeptidases that might cleave this substrate are not present in significant concentration in lung extracts, as judged by the lack of activity on hippuryl derivatives of histidine, leucine, glycine, phenylalanine and arginine. The production of hippuric acid from HHL is linear for at least 30 min with amounts of angiotensin-converting enzyme up to 12 mU per assay (Fig. 3).

Properties of the angiotensin-converting enzyme of rabbit lung

pH optimum. The rate of hydrolysis of HHL by the angiotensin-converting enzyme of rabbit lung acetone powder extract was maximal at pH values from 8·1 to 8·3 in the presence of 300 mM NaCl (Fig. 4). The maximal activity obtained in 100 mM tris–acetate buffer was only 60 per cent of that obtained in 100 mM potassium phosphate or sodium borate buffers.

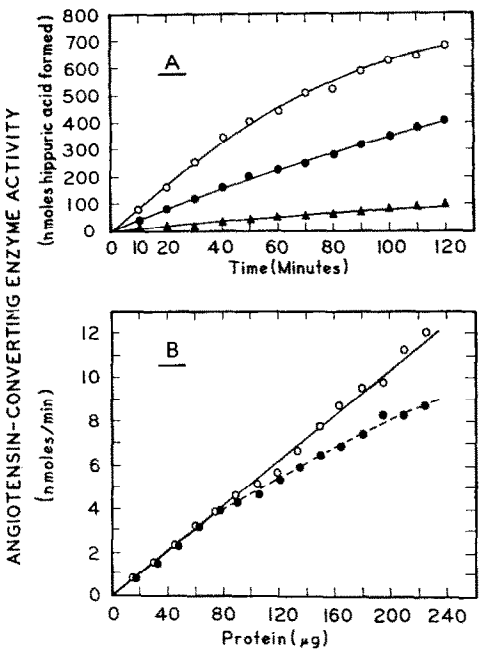

FIG. 3. Effect of incubation time (A) and enzyme concentration (B) on the activity of angiotensin-converting enzyme of lung, measured as the rate of hydrolysis of hippuryl-L-histidyl-L-leucine. Assay conditions were as described in the text. (a) Activity vs. time of incubation. Three different concentrations of rabbit lung acetone powder extract were employed: 15 μg protein per assay (▲—▲); 75 μg protein per assay (●—●); and 150 μg protein per assay (○—○). (b) Activity vs. enzyme concentration. Fixed time assays were incubated for 20 min (○—○) or 60 min (●- -●).

Chloride-ion dependence. Activity was optimal in the presence of 300 mM NaCl (Fig. 5); this rate is 7·5 times greater than the rate in assays lacking chloride ion. The extract used in this experiment was thoroughly dialyzed against chloride ion-free buffer, and the pH of the assay mixture at each chloride ion concentration was adjusted to 8·3.

Effect of substrate concentration. The optimal concentration of HHL for assay of the rabbit lung angiotensin-converting enzyme was 5–10 mM (Fig. 6); inhibition by substrate was observed at higher concentrations. The apparent Michaelis constant for HHL, calculated from these data by the method of Lineweaver and Burk,[19] was 2·6 mM.

Metal-ion effects. The complete inhibition of the rabbit lung angiotensin-converting enzyme in the presence of 0·1 mM EDTA (see below) can be reversed by dialysis, suggesting a tightly bound metal ion, not easily removed from the enzyme. Enzyme subjected to prolonged dialysis against 1 mM EDTA was not reactivated by subsequent dialysis against buffer, but activity could be restored to varying degrees by addition of divalent cobalt, zinc or manganese ions at a concentration of 0·2 mM (Fig. 7). Manganese ion at optimal concentration restored only 40 per cent of the original activity; zinc ion restored exactly 100 and cobalt ion 160 per cent of the activity found before the EDTA treatment. No activity was restored by divalent calcium, magnesium, barium, copper or ferrous ions. Cobalt ion also stimulated the

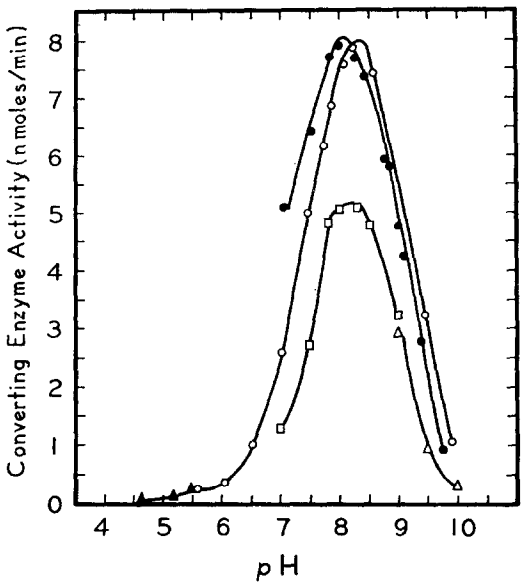

FIG. 4. Effect of pH on the activity of angiotensin-converting enzyme of lung, measured as the rate of hydrolysis of hippuryl-L-histidyl-L-leucine. Rabbit lung acetone powder extract was dialyzed overnight against 1 mM potassium phosphate buffer, pH 7·8; for each assay, 150 μg of extract protein was incubated for 40 min under standard assay conditions, as described in the text. Each 100 mM buffer was adjusted to yield the indicated pH in the presence of 300 mM NaCl: sodium acetate (▲—▲); potassium phosphate (○—○); sodium borate (●—●); Tris acetate (□—□); and sodium bicarbonate (△—△).

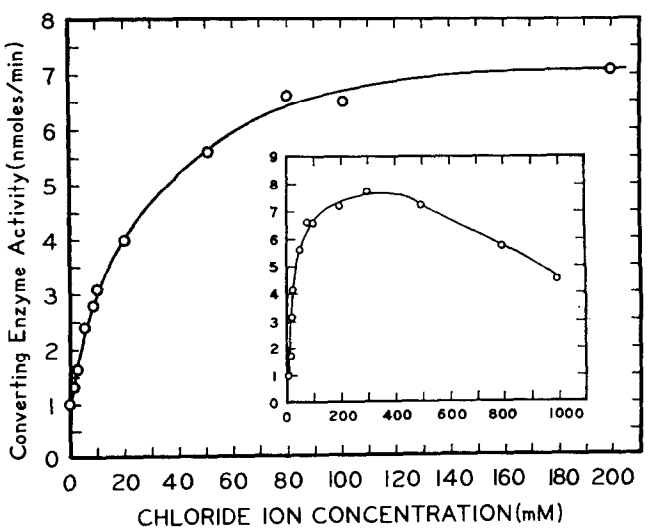

FIG. 5. Effect of chloride ion on the activity of angiotensin-converting enzyme of lung, measured as the rate of hydrolysis of hippuryl-L-histidyl-L-leucine. Rabbit lung acetone powder extract was dialyzed overnight against 1 mM potassium phosphate buffer, pH 7·8; for each assay 150 μg of extract protein was incubated 40 min under standard assay conditions, as described in the text. Separate buffers were employed for each chloride ion concentration, each adjusted to give a final pH of 8·3.

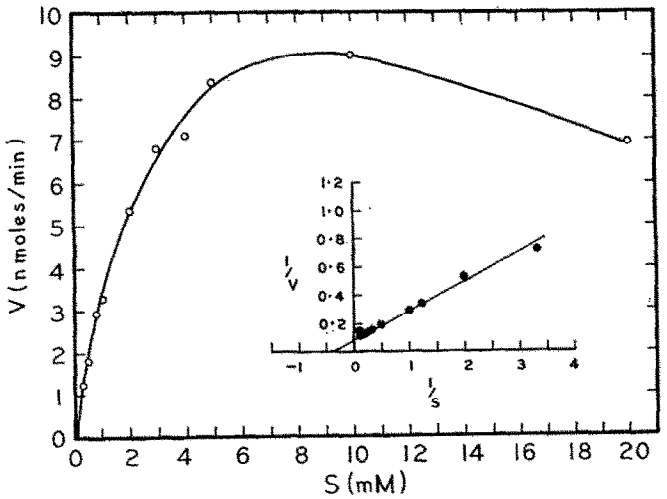

FIG. 6. Effect of substrate concentration on the activity of angiotensin-converting enzyme of lung, measured as the rate of hydrolysis of hippuryl-L-histidyl-L-leucine. Rabbit lung acetone powder extract was dialyzed overnight against 1 mM potassium phosphate buffer, pH 7·8. To determine the initial rate of hydrolysis at each HHL concentration, 150 μg of extract protein was incubated for 5, 10, 15 and 20 min, using the standard assay system described in the text. The insert shows a Lineweaver–Burk plot of the same data.

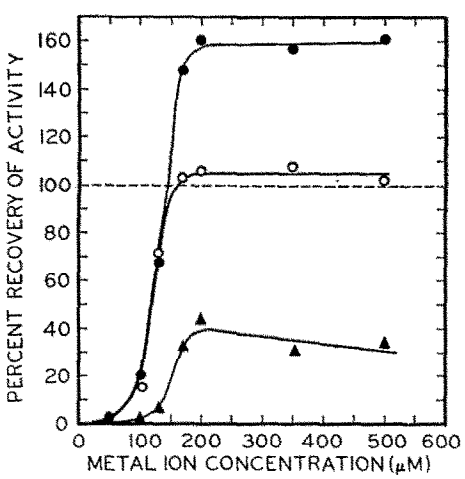

FIG. 7. Reactivation of EDTA-treated, dialyzed angiotensin-converting enzyme by addition of divalent metal ions. The angiotensin-converting enzyme activity of rabbit lung acetone powder extract, measured as hydrolysis of hippuryl-L-histidyl-L-leucine, was inhibited 100 per cent by three dialyses, each against 200 vol. of 1 mM EDTA. Free EDTA was completely removed by five dialyses, each against 200 vol. of 1 mM potassium phosphate buffer, pH 7·8; after this treatment the enzyme remained completely inactive. Activity could be restored to varying extents by addition of $MnCl_2$ (▲—▲), $ZnCl_2$ (○—○) or $Co(NO_3)_2$ (●—●). Incubations were carried out for 30 min, as indicated in the text, using 150 μg of extract protein per assay and the indicated final concentration of metal ion.

activity of uninhibited angiotensin-converting enzyme by 60 per cent, presumably by exchanging with the naturally occurring metal ion to form a more active metalloenzyme.

Inhibitors. A great number of standard enzyme inhibitors, having various mechanisms of action, were tested for their effect on the angiotensin-converting enzyme from lung, without preincubation, at concentrations ranging from 0·1 to 1000 μM. Those compounds found to be inhibitory fall into four classes (Table 1). Agents in the first three classes probably inhibit the angiotensin-converting enzyme by affecting the tightly bound metal ion required for activity, the first two classes of compounds by chelation, and the heavy metal ions by exchange reactions to yield inactive metalloproteins. The most effective inhibitors in these classes were EDTA and $CdBr_2$, although other chelating agents may inhibit at lower concentrations if preincubated with the enzyme.

The most potent inhibitors of the action of the angiotensin-converting enzyme on HHL were peptides: [Ile^5]-angiotensin I, bradykinin and [Asn^1, Val^5]-angiotensin II all showed significant inhibitory potency at 10 μM. The first two peptides are substrates for the enzyme and the third is the amide derivative of a product of its action. The

TABLE 1. INHIBITION OF THE ANGIOTENSIN-CONVERTING ENZYME OF LUNG*

Compound	Per cent inhibition at (μM): 0·1	1	10	100	1000
Chelating agents					
EDTA	0	4	84	93	99
o-Phenanthroline	0	0	0	51	97
Diphenylthiocarbazone	0	0	0	74	98
Diethyldithiocarbamate	0	0	0	7	17
α, α-Dipyridyl	0	0	0	1	21
Sulfhydryl compounds					
Dithiothreitol	0	1	10	60	91
Dimercaptopropanol	0	0	0	30	81
Cysteine	0	0	0	12	87
Thioglycolic acid	0	0	4	22	78
2-Mercaptoethanol	0	0	2	9	49
Heavy-metal ions					
$HgCl_2$	0	0	11	84	98
$CdBr_2$	0	8	51	86	95
$Ni(NO_3)_2$	0	0	0	72	93
$CuSO_4$	0	0	13	42	95
$Fe(NH_4)_2(SO_4)_2$	0	0	0	13	76
Peptides					
[Ile^5]-angiotensin I	0	0	50	94	—
[Asn^1, Val^5]-angiotensin II	0	0	35	85	—
Bradykinin	2	13	68	96	98
SQ 20,475†	59	87	95	99	—

* Each compound was added to the assay system at the indicated final concentration without preincubation with the enzyme. Incubations were carried out for 60 min with 150 μg of rabbit lung acetone powder extract protein in the standard assay system described in the text.

† SQ 20,475 is a pentapeptide: L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline.

pentapeptide, L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline (SQ 20,475), originally isolated from the venom of *Bothrops jararaca* as a bradykinin-potentiating factor,[20–22] and later shown to be an inhibitor of the angiotensin-converting enzyme,[22,23] was approximately 100 times more potent than any other inhibitor tested; it did not significantly inhibit trypsin, chymotrypsin, leucine aminopeptidase or carboxypeptidase A at a concentration of 1 mM. Two other bradykinin-potentiating peptides, human fibrinopeptides A and B,[24–26] did not inhibit the angiotensin-converting enzyme from rabbit lung at a concentration of 1 mM.

The groups of inhibitors that had no effect on the angiotensin-converting enzyme at 1 mM included: the serine esterase inhibitor, diisopropylfluorophosphate (2-hr preincubation); histidine alkylating agents, 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) and L-1-tosylamido-2-phenylethylchloromethylketone (TPCK)* (each preincubated for 2 hr); thiol-alkylating and mercaptide-forming reagents, iodoacetic acid, iodoacetamide, *N*-ethylmaleimide and *p*-hydroxymercuri-benzoate; heavy metal-complexing agents, cyanide, azide and sulfide; and carbonyl-complexing agents, hydroxylamine and aminooxyacetic acid.

Stability. The angiotensin-converting enzyme activity in crude rabbit lung extracts was stable for at least 6 months when stored at 5° at pH values ranging from 5 to 9; sodium chloride was not required for stability, although it was a near absolute requirement for activity; divalent metal ions and sulfhydryl compounds had no effect on stability. At higher temperatures, the enzyme was readily denatured; 60 per cent of the activity was lost after 15 min of preincubation at 60°.

DISCUSSION

The difficulty of attempting to follow purification of the angiotensin-converting enzyme from lung extracts by means of existing biological assay techniques prompted us to try a simpler peptide substrate resistant to degradation by other tissue peptidases and the product of which could be quantitatively determined by simple chemical methods. McDonald and others[27–35] have successfully employed dipeptidyl-β-naphthylamides for assay of enzymes that hydrolyze dipeptide residues from the *N*-terminal end of polypeptides; these substrates are dipeptide analogs of chromogenic substrates (amino acyl-β-naphthylamides) commonly employed to assay aminopeptidases.[36–39] It thus seemed possible that a dipeptide analog of the simple carboxypeptidase substrate, hippuryl-L-phenylalanine,[18] could be used to assay the angiotensin-converting enzyme. The peptide that we synthesized was hippuryl-L-histidyl-L-leucine (HHL). Because of insufficient spectral difference between HHL and the product, hippuric acid, a direct kinetic spectrophotometric assay was not feasible. However, hippuric acid formed by action of the angiotensin-converting enzyme could be quantitatively determined in a fixed-time assay after its separation from unreacted HHL by extraction into ethyl acetate.

A number of experimental observations leave little doubt that the rabbit lung enzyme assayed by conversion of HHL to hippuric acid is identical with the angiotensin-converting enzyme. The HHL-hydrolyzing enzyme purified 70-fold from dog lung[13] rapidly and quantitatively cleaved angiotensin I to angiotensin II plus

* This inhibitor was preincubated with the enzyme in 3% methanol; the final concentration in the assay was only 0·04 mM, due to solubility limitations.

histidylleucine, as determined by biological and thin-layer chromatographic assays. The enzyme from rabbit lung acetone powder extract is inhibited markedly by the more tightly bound substrate, angiotensin I. It shares two other very specific properties with the angiotensin-converting enzyme: chloride ion activation and inhibition by low concentrations of peptides isolated from the venom of *Bothrops jararaca;*[23] one of these, SQ 20,475 (BFP V-3-A of Ferreira *et al.*[23]), has been synthesized and found to be a reasonably specific inhibitor of the angiotensin-converting enzyme, whether assayed biologically with angiotensin I as substrate[23] or spectrophotometrically with HHL, using rabbit lung extract. Inhibition of the HHL-hydrolyzing activity of the lung enzyme by such agents as EDTA, dimercaptopropanol and *o*-phenanthroline, and the lack of inhibition by diisopropylfluorophosphate, agree with results obtained by various workers studying the angiotensin-converting enzyme from plasma.[11,12,40–42] The pH optimum, the chloride ion-activation curve, and the observed reactivation of EDTA-inhibited enzyme by cobalt, zinc and manganese ions are very similar to the results obtained by Dorer *et al.*[11] using a purified angiotensin-converting enzyme isolated from plasma. Since the lung enzyme is located in close proximity to the blood in the pulmonary circulation,[3,4,43] membrane-bound lung enzyme escaping into the circulation may be the source of the plasma enzyme.

We have chosen to assay the angiotensin-converting enzyme from lung tissue rather than from plasma because of its greater concentration in this tissue and because numerous experimental results demonstrate the central physiological role of the lung as the site of the conversion *in vivo* of circulating angiotensin I to angiotensin II.[3–8]

The angiotensin-converting enzyme which we have purified 70-fold from dog lung,[13] in addition to catalyzing the hydrolysis of HHL and angiotensin I, also removes the C-terminal phenylalanylarginine residue from bradykinin. The enzyme from rabbit lung acetone powder extract described in this paper is inhibited by two classes of "bradykinin-potentiating" compounds: sulfhydryl compounds and peptides isolated from the venom of *Bothrops jararaca.* Although the results of Ryan *et al.*[44] imply that the angiotensin-converting enzyme is not the rate-limiting kininase activity of lung, an enzyme similar or identical to the angiotensin-converting enzyme of lung might serve as an important kininase activity in other tissues. Kininase II isolated from kidney and plasma by Erdös and Yang[45,46] cleaves the C-terminal dipeptide residue from bradykinin and has recently been shown to cleave histidylleucine from both angiotensin I and HHL.[47] Kininase II is stimulated by cobalt ion, inhibited by EDTA, dimercaptopropanol, *o*-phenanthroline and heavy metal ions, but not by diisopropylfluorophosphate or sulfhydryl-reactive reagents. One apparent difference between the angiotensin-converting enzyme and kininase II is that the latter is not strongly inhibited by cadmium ion.

The angiotensin-converting enzyme from lung appears to be a metalloprotein with a unique substrate specificity. The nature of the metal ion is not known, but results with specific chelating agents and reactivation studies with EDTA-inhibited enzyme suggest zinc as a likely candidate. The similar peptidase, carboxypeptidase A, is a zinc-containing metalloprotein.[48] Specificity studies with a 70-fold purified enzyme from dog lung[13] indicate that the enzyme is a carboxypeptidase which cleaves dipeptide residues rather than amino acid residues from the C-terminus of polypeptides. Two or three dipeptides appeared to be sequentially released from bradykinin; the lack of further activity of the angiotensin-converting enzyme on angiotensin II is

probably due to the presence of a penultimate proline residue in the structure of this peptide. The enzyme from lung did not yield biologically active angiotensin II from endo-His^{8a}-angiotensin I, an analog of the natural substrate that contains an extra histidine residue between the eighth and ninth amino acids.[49] There is no way, however, that a dipeptide-cleaving enzyme could produce angiotensin II from this undecapeptide substrate; a dodecapeptide of similar structure should be converted to angiotensin II by cleavage of two dipeptide residues. Further elucidation of the nature of the angiotensin-converting enzyme will require studies with an enzyme preparation of greater purity; from the data presently available, however, the enzyme appears to be a metalloprotein which would officially be designated an α-carboxypeptide dipeptidohydrolase; Yang and Erdös[47] have suggested the trivial name dipeptidyl carboxypeptidase.

REFERENCES

1. L. T. Skeggs, W. H. Marsh, J. R. Kahn and N. P. Shumway, *J. exp. Med.* **99**, 275 (1954).
2. L. T. Skeggs, J. R. Kahn and N. P. Shumway, *J. exp. Med.* **103**, 295 (1956).
3. K. K. F. Ng and J. R. Vane, *Nature, Lond.* **216**, 762 (1967).
4. K. K. F. Ng and J. R. Vane, *Nature, Lond.* **218**, 144 (1968).
5. P. Biron and C. G. Huggins, *Life Sci.* **7**, part I, 965 (1968).
6. S. Oparil, C. A. Sanders and E. Haber, *Fedn Proc.* **28**, 1789 (1969).
7. Y. S. Bakhle, A. M. Reynard and J. R. Vane, *Nature, Lond.* **222**, 956 (1969).
8. P. Biron, L. Campeau and P. David, *Am. J. Cardiol.* **24**, 544 (1969).
9. C. G. Huggins and N. S. Thampi, *Life Sci.* **7**, part II, 633 (1968).
10. N. S. Thampi, G. Schoellmann, M. W. Hurst and C. G. Huggins, *Life Sci.* **7**, part II, 641 (1968).
11. F. E. Dorer, L. T. Skeggs, J. R. Kahn, K. E. Lentz and M. Levine, *Analyt. Biochem.* **33**, 102 (1970).
12. H. J. G. Hollemans, J. Van Der Meer and W. Kloosterziel, *Clin. chim. Acta* **23**, 7 (1969).
13. D. W. Cushman and H. S. Cheung, *Fedn Proc.* **28**, 3019 (1969).
14. M. Roth, A. F. Weitzman and Y. Piquilloud, *Experientia* **25**, 1247 (1969).
15. M. Bodanszky and V. duVigneaud, *Biochem. Prep.* **9**, 110 (1962).
16. B. C. W. Hummel, *Can. J. Biochem. Physiol.* **37**, 1393 (1959).
17. M. A. Mitz and R. J. Schlueter, *Biochim. biophys. Acta* **27**, 168 (1958).
18. J. E. Folk and E. W. Schirmer, *J. biol. Chem.* **238**, 3884 (1963).
19. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
20. S. H. Ferreira, *Br. J. Pharmac. Chemother.* **24**, 163 (1965).
21. S. H. Ferreira, in *Hypotensive Peptides* (Eds. E. G. Erdös, N. Back, F. Sicuteri and A. F. Wilde), pp. 356–367. Springer, New York (1966).
22. L. J. Greene, J. M. Stewart and S. H. Ferreira, in *Int. Symp. on Cardiovascular and Neuroactions of Bradykinin*, Fiesole, Italy (July 21–25, 1969), to be published by Plenum Press. Abstr. in *Pharm. Res. Comm.* **1**, 159 (1969).
23. S. H. Ferreira, L. J. Greene, V. A. Alabaster, Y. S. Bakhle and J. R. Vane, *Nature, Lond.* **225**, 379 (1970).
24. J. A. Gladner, P. A. Murtaugh, J. E. Folk and K. Laki, *Ann. N.Y. Acad. Sci.* **104**, 47 (1963).
25. A. J. Osbahr, J. A. Gladner and K. Laki, *Biochim. biophys. Acta* **86**, 535 (1964).
26. J. A. Gladner, in *Hypotensive Peptides* (Eds. E. G. Erdös, N. Back, F. Sicuteri and A. F. Wilde), pp. 344–55. Springer, New York (1966).
27. J. K. McDonald, S. Ellis and T. J. Reilly, *J. biol. Chem.* **241**, 1494 (1966).
28. J. K. McDonald, T. J. Reilly, B. B. Zeitman and S. Ellis, *Biochem. biophys. Res. Commun.* **24**, 771 (1966).
29. S. Ellis and J. M. Nuenke, *J. biol. Chem.* **242**, 4623 (1967).
30. J. K. McDonald, T. J. Reilly, B. B. Zeitman and S. Ellis, *J. biol. Chem.* **243**, 2028 (1968).
31. J. K. McDonald, F. H. Leibach, R. E. Grindeland and S. Ellis, *J. biol. Chem.* **243**, 4143 (1968).
32. J. K. McDonald, B. B. Zeitman, T. J. Reilly and S. Ellis, *J. biol. Chem.* **244**, 2693 (1969).
33. V. K. Hopsu-Havu and S. R. Sarimo, *Hoppe-Seyler's Z. physiol. Chem.* **348**, 1540 (1967).
34. V. K. Hopsu-Havu, P. Rintola and G. G. Glenner, *Acta chem. scand.* **22**, 299 (1968).
35. V. K. Hopsu-Havu and T. O. Ekfors, *Histochemie* **17**, 30 (1969).

36. M. N. GREEN, K. C. TSOU, R. BRESSLER and A. M. SELIGMAN, *Archs Biochem. Biophys.* **57,** 548 (1955).
37. J. A. GOLDBARG, P. P. ESTEBAN, P. PINEDA and A. M. RUTENBURG, *Am. J. clin. Path.* **32,** 571 (1959).
38. V. K. HOPSU, K. K. MÄKINEN and G. G. GLENNER, *Archs Biochem. Biophys.* **114,** 557 (1966).
39. G. G. GLENNER, P. J. MCMILLAN and J. E. FOLK, *Nature, Lond.* **194,** 867 (1962).
40. G. W. BOYD, A. R. ADAMSON, A. E. FITZ and W. S. PEART, *Lancet* **7588,** 213 (1969).
41. J. W. RYAN, J. K. MCKENZIE and M. R. LEE, *Biochem. J.* **108,** 679 (1968).
42. L. B. PAGE, E. HABER, A. Y. KIMURA and A. PURNODE, *J. clin. Endocr. Metab.* **29,** 200 (1969).
43. Y. S. BAKHLE, *Nature, Lond.* **220,** 919 (1968).
44. J. W. RYAN, J. ROBLERO and J. M. STEWART, *Biochem. J.* **110,** 795 (1968).
45. E. G. ERDÖS and H. Y. T. YANG, *Life Sci.* **6,** Part II, 569 (1967).
46. H. Y. T. YANG and E. G. ERDÖS, *Nature, Lond.* **215,** 1402 (1967).
47. H. Y. T. YANG, E. G. ERDÖS, T. A. JENSSEN and Y. LEVIN, *Fedn Proc.* **29,** 200 (1970).
48. B. L. VALLEE and H. NEURATH, *J. Am. chem. Soc.* **76,** 5006 (1954).
49. K. K. F. NG and J. R. VANE, *Nature, Lond.* **225,** 1142 (1970).