

## THE PREPARATION, PURIFICATION, AND AMINO ACID SEQUENCE OF A POLYPEPTIDE RENIN SUBSTRATE

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The enzyme renin, which is found in extracts of kidney cortex, acts upon a protein substrate contained in the alpha-2 globulin fraction of the plasma (1) to produce the decapeptide hypertensin I. This peptide is further degraded by a plasma enzyme (2) to the powerfully vasoconstrictor octapeptide hypertensin II which appears to be the effector substance of the renin-hypertensin pressor system (3, 4).

Since hypertensin has been found in the blood of many human beings with hypertensive cardiovascular disease (5) as well as in animals with experimental renal hypertension (6, 7) it is of great interest to discover a method of preventing its action *in vivo*. Owing to the recent purification and structural delineation of both hypertensin I and hypertensin II (8-12) this problem can now be rationally approached. Three separate methods appear feasible. First, it might be possible to prepare structural analogs of hypertensin II capable of interfering with the vasoconstrictor reaction. Second, the conversion of hypertensin I to hypertensin II might be prevented by compounds inhibiting the hypertensin-converting enzyme. Finally, the production of hypertensin I from renin substrate might be prevented by the inhibition of renin. Since renin is the initial and rate-limiting substance in the renin-hypertensin system it would seem that this last approach would be the most likely to succeed. This view is re-enforced by the observation that immunization with heterologous renin has been used successfully in the treatment of dogs with experimental renal hypertension (13).

In order to prepare compounds capable of inhibiting renin it is necessary that the structure of its substrate be known. Inasmuch as renin substrate is a protein which has been only partially purified (14) the full determination of its structure is difficult. It appeared unlikely that the entire renin substrate molecule would be required for the action of renin since the specificity of the common proteolytic enzymes is based on one or at most two amino acid residues. An attempt was therefore made to partially degrade the renin substrate by the action of the enzyme trypsin. This effort has proven successful and has yielded a polypeptide renin substrate amenable to purification and structural determination.

## EXPERIMENTAL

*Preparation of Protein Renin Substrate.*—The fraction of horse plasma precipitating between 1.3 and 2.3 M ammonium sulfate was prepared according to a method previously described (9). The protein content of this product was determined and the solution diluted to a concentration of 5 per cent. Hypertensinase was destroyed by a 30 minute incubation at pH 3.8 and 25°C. The solution was then cooled to ice box temperatures, adjusted to pH 6.0, and a 4.0 M solution of ammonium sulfate added slowly with constant stirring until a concentration of 1.5 M had been obtained. Two per cent hyflo super-cel was added and the mixture filtered on cloth on a large vacuum funnel. The precipitate was discarded and the concentration of ammonium sulfate in the filtrate increased to 2.2 M by a second slow addition of the 4.0 M solution. After addition of 1 per cent hyflo super-cel the precipitate was gathered by vacuum filtration. The filter was covered with a rubber membrane and the precipitate was squeezed to a hard dry cake. The filtrate was discarded. The cake was suspended in a minimum volume of distilled water and, after thorough stirring, the hyflo super-cel was removed by filtration. The resulting protein solution was dialyzed in an artificial kidney (15) against iced tap water until free from the ammonium ion and finally against cold distilled water for a period of 6 hours. The protein content was determined and adjusted to 5 per cent with distilled water.

This procedure yielded approximately 280 gm. of protein per 100 liters of horse plasma. Upon incubation of small samples of the product with an excess of renin, hypertensin activity was produced which amounted to about 70 Goldblatt units (16) per gm. of protein.

*Preparation of Polypeptide Substrate.*—The protein substrate was brought to a temperature of 19°C. and was adjusted to pH 7.75 with 2.5 N NaOH. Anhydrous alcohol which contained 0.04 per cent hydrocinnamic acid was added to a final concentration of 20 per cent. At this point the pH was readjusted to 7.75 if necessary. The temperature was 26.5°C. Two times crystallized trypsin (50 per cent  $MgSO_4$ <sup>1</sup>), in the amount of 1.00 gm. per liter of protein solution, was dissolved in a volume of ice cold distilled water equal to one-tenth the volume of the protein solution. After the trypsin had completely dissolved the enzyme was added to the alcoholic protein solution. About 5 minutes elapsed between the additions of alcohol and trypsin. The temperature of the mixture fell to 25°C. and the pH to 7.4 or 7.5. These conditions were maintained for a length of time previously determined in small scale experiments to yield the highest concentration of polypeptide substrate. At the end of the incubation period the mixture was poured with stirring into sufficient 95 per cent alcohol to raise the concentration to 75 per cent. The pH was adjusted to 5.5 and the mixture filtered through paper on gravity funnels. In small scale preliminary experiments aliquots of the alcoholic filtrate were simply evaporated to dryness and the residue dissolved in a similar volume of saline. In large scale experiments the filtrate was evaporated to one-half the volume of the original protein solution. The concentrate, now aqueous, was adjusted to pH 3.0 with 2.5 N HCl and was extracted twice with one-half volumes of diethyl ether. The ether extracts were discarded. The aqueous phase was further evaporated to a volume one-twentieth that of the original protein solution.

*Method of Assay.*—Renin, prepared as previously described (9), was thoroughly dialyzed, made 0.5 M with respect to  $NaH_2PO_4$  and adjusted with NaOH to yield pH 7.5 upon 10-fold dilution.

Unknown solutions to be assayed were diluted in saline to an estimated strength of 0.1 unit per ml. Nine ml. of the diluted sample was then mixed with 1 ml. of renin and incubated for 15 minutes at 37°C. A similar control sample was also prepared in which heat-coagulated rather than native renin was used. At the end of the incubation period, the pH was adjusted

<sup>1</sup> Nutritional Biochemical Co., Cleveland.

to 5.5 and the mixture heated on a boiling water bath for 10 minutes. After cooling, the proteins were removed by centrifugation and the supernatant solution assayed for its pressor activity in the rat (17). When assaying highly purified preparations it was necessary to use siliconized glassware in the preparation of the samples (18). The results of assays are expressed in Goldblatt units (16).

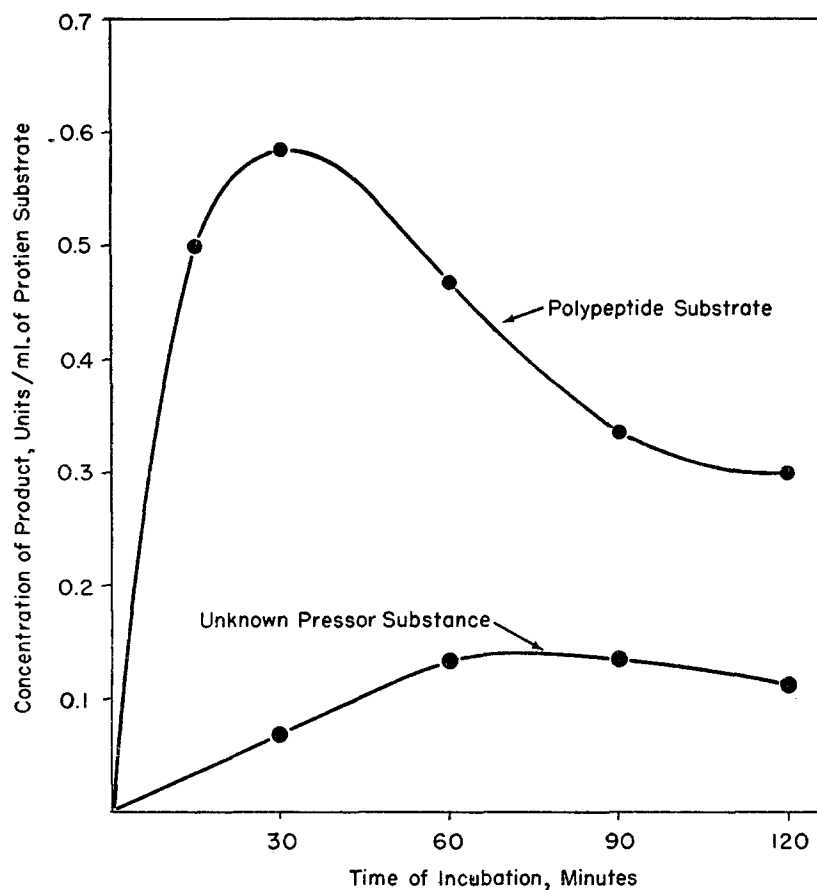


FIG. 1. Incubation of protein renin substrate with trypsin.

*The Effect of Various Conditions of Incubation.*—The results of a typical preliminary experiment can be seen in Fig. 1. Samples were withdrawn from the main incubation mixture at various times and were processed as described above. The lower curve in the figure, which was obtained by assay of those samples incubated with heat-coagulated renin, represent the appearance in the incubation mixture of an unknown, directly pressor substance (UPS). A similar curve would have been obtained had the solutions been assayed without treat-

ment with coagulated renin. The upper curve represents the difference between the samples incubated with native renin and those treated with heat-coagulated renin and therefore illustrate the concentration of polypeptide substrate in the mixture.

It is apparent that the polypeptide substrate appears in the incubation mixture only as an intermediate product. Extended incubation leads to disappear-

TABLE I  
*The Effect of Various Conditions of Incubation upon the Yield of Polypeptide Substrate*

Experiment No.	Hydrogen ion concentration	Concentration of ethanol	Concentration of hydrocinamic acid	Time for maximum yield	Concentration of polypeptide substrate	Concentration of UPS
	pH	per cent	mg. per ml. of protein substrate	min.	u. per ml. of protein substrate	u. per ml. of protein substrate
176	7.5	0	0	45	0.20	0.15
182	7.5	10	0	45	0.40	0.10
181	7.5	20	0	75	0.50	0.10
177	7.5	30	0	60	0.30	0
178	7.5	40	0	75	0.25	0
*	7.5	20	0.1	30	0.58	0.07
214	7.5	20	0.5	60	0.55	0.05
190	7.5	20	1.0	60	0.45	0.05
222	6.5	20	0.1	60	0.45	0.05
223	8.5	20	0.1	30	0.30	0
215	7.5	20	0.1	60	0.35	0.10
208B	7.5	20	0.1	30	0.50	0.10

Aliquots of the same preparation of protein substrate (5 per cent protein) and of the same 2 x crystallized trypsin (50 per cent  $MgSO_4$ ) were used in all experiments. The concentration of alcohol was calculated prior to the addition of the trypsin solution. Trypsin was added in a 1 per cent solution in a volume of cold distilled water equal to one-tenth the volume of protein substrate. The temperature of the incubations was 25°C.

\* Results are the average of three experiments and are presented more fully in Fig. 1.

Experiment 215: Sodium chloride was added to a concentration of 0.115 M.

Experiment 208B: Protein substrate and alcohol were mixed and maintained at pH 7.75 and at 25°C. for 30 minutes before addition of the trypsin solution.

ance of the substrate activity probably due to cleavage of the arginyl-valine bond which is susceptible to the action of trypsin both in hypertensin I and hypertensin II molecules.

The conditions described are the best thus far found for the preparation of polypeptide substrate. As shown in Table I the omission of alcohol from the incubation mixture decreased greatly the amount of substrate which is obtained. The use of more than 20 per cent alcohol is not of value although it is interesting to note that in these cases no UPS is found. Alcohol is said to destroy the protein renin substrate (18). It is clear from the results here reported

that alcohol does no damage to the site of action of renin upon the protein substrate molecule, nor upon that part which may be released as hypertensin I. Possibly alcohol denatures or distorts the protein substrate molecule thus preventing adequate contact with the enzymatically active sites of renin.

Also of great value is the addition of hydrocinnamic acid; a known, potent inhibitor of both carboxypeptidase and chymotrypsin (19). Although  $2 \times$  crystalline trypsin was used it seems probable that this material contained at least small amounts of the aforementioned enzymes as impurities. It is not known whether the hydrocinnamic acid would be of value with more rigorously purified trypsin.

The identity of the UPS has not been established nor is its manner of appearance comprehensible at present. In short, 8-tube, countercurrent distributions the material demonstrated a similarity to hypertensin II. However, trypsin is known to act upon the carboxylamide linkage of arginine or lysine residues and therefore would not be expected to sever the phenylalanyl-histidine bond to yield hypertensin II from the substrate molecule. Both hypertensin II and the UPS are in fact destroyed by this enzyme. It is theoretically possible that chymotrypsin, present as an impurity, might sever the phenylalanyl-histidine bond yielding hypertensin II. In this event the product would be expected to have a very short life due to vulnerability of the tyrosylisoleucine bond (12) contained in the hypertensin II molecule. It is also possible that the UPS is hypertensin resulting from the action of carboxypeptidase upon the newly formed polypeptide substrate molecule. Finally, UPS may arise through the action of trypsin upon an unknown protein and may have no connection with the renin-hypertensin system.

*Preparation of Crude Polypeptide Substrate.*—A total of 1020 liters of citrated horse plasma was fractionated in 6 batches with ammonium sulfate and dialyzed, yielding 2840 gm. of semipurified protein renin substrate. This material was incubated with trypsin as previously described. The reaction was stopped and the proteins precipitated with alcohol. After removal of the proteins by filtration, the solution was evaporated, defatted with diethyl ether and then further evaporated to a volume of 3 liters.

Assay and determination of the nitrogen concentration of the crude concentrate showed the presence of 23,500 units of polypeptide substrate having a purity of 2.2 units per mg. of N. Only a negligible amount of an unidentified pressor substance was present. Had the 2840 gm. of protein renin substrate been incubated directly with renin approximately 200,000 units of hypertensin I could have been expected with a purity of 40 units per mg. of N.

*Salting Out a Neutrality.*—The crude concentrate, with a volume of 3 liters, was adjusted to pH 7.0. Sodium chloride was added to full saturation. After addition of a small amount of celite the mixture was filtered on a Buchner funnel. The filtrate, which contained little active material, was discarded. The filter cake was suspended in 1 liter of distilled water. After adjustment to pH 2.0 the mixture was stirred vigorously for 10 minutes and then filtered as

before. The filter cake was extracted twice more in a similar manner using 0.01 N HCl. The final filter cake was discarded and the filtrates combined yielding a light yellow solution with a volume of 3 liters.

The material at this stage contained 21,400 units with a purity of 19.3 units per mg. of N. An 8.8-fold purification is obtained by this step.

*Butanol Extraction.*—The preparation was adjusted to pH 7.0 and extracted twice with 1500 ml. of *N*-butanol. The aqueous layer, containing 1320 units of low purity was discarded. The combined butanol extracts were extracted once with 750 ml. of 0.03 N HCl and then 3 times with similar volumes of 0.01 N HCl. The inactive butanol layer was discarded.

The acid extract was found to contain 18,000 units with a purity of 52 units per mg. of N. Nearly 3-fold purification is obtained by this step.

*Purification with Alumina.*—The acid extract, with a volume of 3 liters and a pH of 2.0 was made 5 per cent with respect to NaCl and extracted twice with *N*-butanol. The aqueous layer, which contained 1440 units with a purity of only 14.5 units per mg. of N, was discarded. The butanol extracts were combined and stirred with 150 gm. of acid-treated alumina for a period of 1 hour. The alumina was separated by decantation and the butanol again treated with a second portion of alumina in an exactly similar fashion. The butanol was separated from the alumina and being inactive was discarded. The two portions of alumina were combined and washed twice with 1500 ml. portions of 75 per cent alcohol. The active material was then eluted from the alumina with two 1500 ml. portions of boiling water. In each extraction the slurry of alumina in water was stirred vigorously for 10 minutes and then filtered by vacuum. The alumina was discarded and the two eluates combined, heated to 80°C., and adjusted to pH 7.0. The flocculant precipitate of aluminum hydroxide which formed was removed by centrifugation and was discarded. The clear colorless supernatant solution was adjusted to pH 4.0 and evaporated to a volume of 60 ml.

Upon assay of the preparation at this stage 15,750 units were found having a purity of 183 units per mg. of N. Thus extraction into butanol followed by absorption and elution using alumina provided a 3.5-fold increase in purity. More importantly however, this step eliminates emulsifying agents and makes it possible to perform a countercurrent distribution.

*First Countercurrent Distribution.*—The material was adjusted to pH 10.5, saturated with secondary butanol, and loaded into the first 7 tubes of a 200 tube, 10 ml. phase Craig-Post countercurrent distribution apparatus (20). The solvents were 0.01 N NaHCO<sub>3</sub>-0.01 N Na<sub>2</sub>CO<sub>3</sub> and redistilled secondary butanol. The machine was operated for 590 transfers using the method of single withdrawal. A single active band ( $K = 1.02$ ) was found upon assay of the effluent fractions. The material remaining in the tubes of the machine was inactive. The active fractions were combined, equilibrated with a small amount of water, and adjusted to pH 4.0. The material was then evaporated to a volume of 23 ml.

A total of 15,000 units were found at this stage with a purity of 720 units per mg. of N. A nearly 4-fold purification is achieved by this step.

*Isoelectric Precipitation.*—The preparation, with a volume of 23 ml. was adjusted to pH 7.4, resulting in the appearance of a light flocculant precipitate which was separated by

centrifugation. The supernatant solution containing 800 units with a purity of only 50 units per mg. of N was discarded. The precipitate was suspended in a small amount of water and was adjusted to pH 2.0. After thorough stirring a very light precipitate persisted which was removed by filtration through a sintered glass funnel of medium porosity. The filtrate with a volume of 25 ml. was clear and light yellow in color.

It was found upon assay that a 3-fold purification has been effected. 14,200 units were present with a purity of 2200 units per mg. of N.

*Second Countercurrent Distribution.*—The solution was adjusted to pH 2, saturated with redistilled secondary butanol, and loaded into the first 3 tubes of the countercurrent apparatus. The solvents used in this case were 0.01 N HCl containing 2.0 per cent NaCl and secondary butanol. The machine was operated for 200 transfers at which time the distribution was stopped. Sufficient assays were performed to locate the active band which was found between tubes 135 and 165. All inactive tubes were then emptied and refilled with fresh solvents. The apparatus was then arranged for recycling and was operated for 468 additional transfers. Assays and nitrogen determinations were performed on every tenth tube.

The results are illustrated in Fig. 2. The curves obtained are similar to those predicted by theory for one component (20).

The solvents from the active tubes 460 through 505 were combined, and evaporated to 125 ml. This solution was extracted four times with one-half volume of secondary butanol. The aqueous layer which was then inactive and contained a large amount of NaCl was discarded. The butanol extracts were combined and after the addition of a small amount of water were evaporated to 17 ml. This solution, now aqueous, was adjusted to pH 7.0. The light flocculant precipitate which formed was separated by centrifugation and washed 3 times with 4 ml. portions of distilled water. The combined filtrate and washings contained 1600 units with a purity of 2100 units per mg. of N. The precipitate was dissolved in 25 ml. of water with the aid of a few drops of dilute HCl and the resulting solution clarified by filtration through sintered glass.

The clear colorless filtrate represented the final product. Upon assay and determination of nitrogen, a total of 10,800 units were found with a purity of 5900 units per mg. of N. It was assumed from the known specific activity of hypertensin I (1800 units per micromole or 7700  $\mu$ ./mg. of N (9)) that 6.0  $\mu$ M were present. Approximately 45 per cent of the polypeptide substrate product in the crude concentrate was found in the final product. The over-all purification was about 2700-fold.

Descending paper chromatograms using both butanol-acetic acid-water (4, 1, 5) and phenol-water (100, 39.5) solvents gave only single ninhydrin and Durrum-positive spots (21) with  $R_f$  values of 0.57 and 0.87, respectively.

*Amino Acid Analysis.*—A sample of polypeptide substrate estimated to contain 0.5  $\mu$ M and 900 units was hydrolyzed in 1 ml. of 6 N HCl in a sealed tube at 105°C. for 22 hours. After removal of the excess acid by evaporation a portion of the hydrolysate estimated to be 0.1  $\mu$ M was chromatographed on Whatman No. 1 filter paper in two dimensions, using as solvents water saturated tertiary amyl alcohol and phenol-water (100, 39.5). After spraying with ninhydrin (saturated solution in *N*-butanol) and heating, a pattern of amino acids was

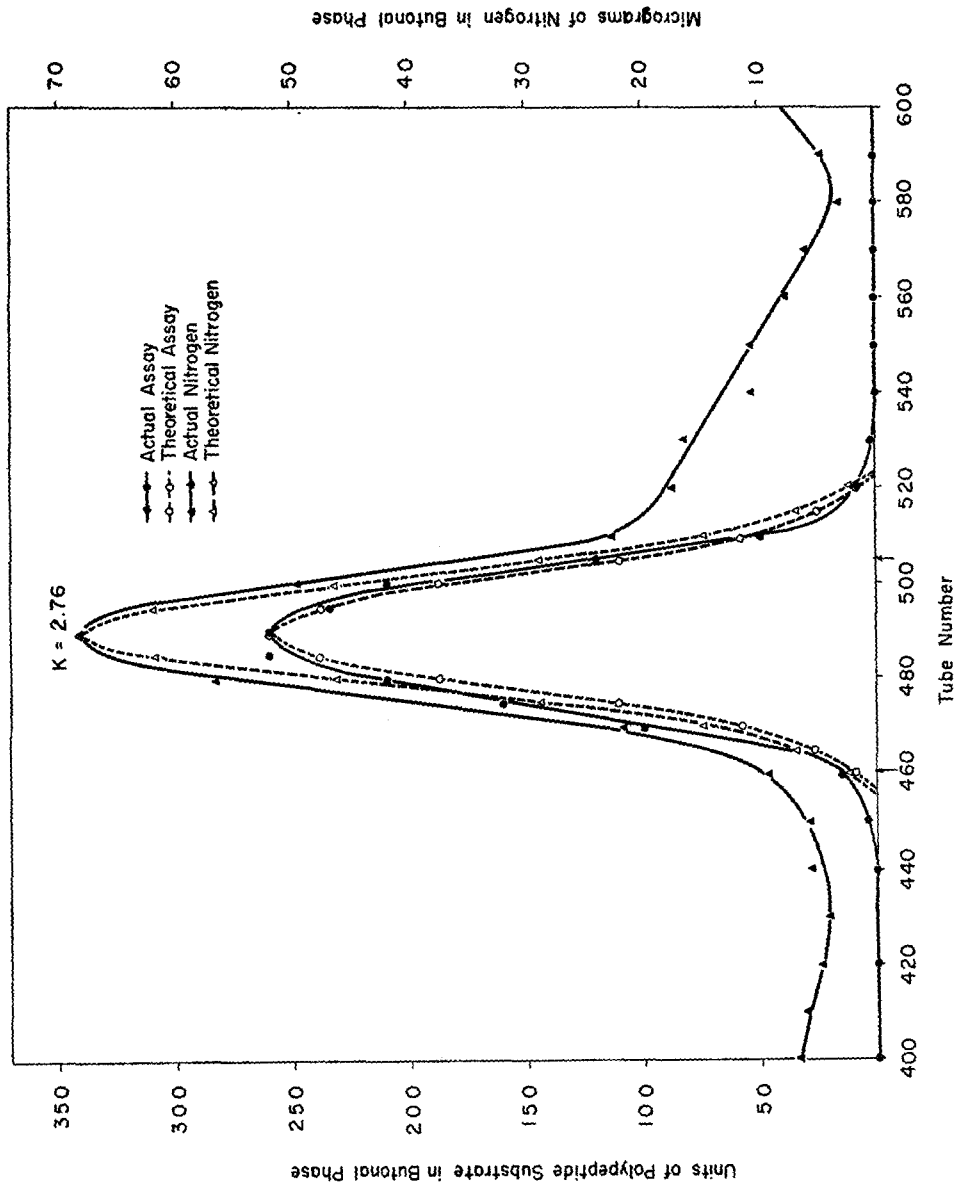


Fig. 2. Second countercurrent distribution of polypeptide renin substrate. The material in tubes 460 to 505 was used in preparation of the final purified product.



revealed which corresponded exactly to that of hypertensin I except for the presence of one additional amino acid whose  $R_f$  values were those of serine.

The remaining hydrolysate (0.4  $\mu\text{M}$ ) was quantitatively analyzed by the method of Levy, Geschwind, and Li (22). The amino acids were treated with fluorodinitrobenzene (FDNB)<sup>2</sup> and the resulting dinitrophenyl (DNP) derivatives chromatographed two dimensionally in triplicate using "toluene" and 1.6 M phosphate buffer solvents. The separated DNP amino acids thus obtained were excised from the paper and after elution with 1 per cent  $\text{NaHCO}_3$  were measured by their light absorption at 340  $m\mu$  (proline 385). Adjustment of the values thus obtained by means of factors allowed calculation of the molar proportions of the amino acids present.

TABLE II  
*Amino Acid Analysis of Polypeptide Substrate*

	Molar proportions					
	Polypeptide substrate				Hypertensin I*	Hypertensin II†
	1	2	3	Average		
<i>M</i>	<i>M</i>	<i>M</i>	<i>M</i>	<i>M</i>	<i>M</i>	
Leucine . . . . .	—	—	—	—	1.09	—
Isoleucine . . . . .	—	—	—	—	0.95	0.96
Leucine and isoleucine . . . . .	3.10	3.15	3.09	3.11	2.04	0.96
Aspartic acid . . . . .	1.07	1.02	0.98	1.02	1.03	1.02
Proline . . . . .	0.86	0.85	0.84	0.85	1.03	1.07
Valine . . . . .	1.86	1.96	2.06	1.96	1.03	1.08
Tyrosine . . . . .	2.28	2.18	2.31	2.26	0.96	0.96
Phenylalanine . . . . .	1.06	1.02	1.00	1.03	1.04	0.95
Histidine . . . . .	1.82	1.94	1.85	1.87	1.87	0.92
Arginine . . . . .	1.18	1.06	1.15	1.13	0.99	1.06
Serine . . . . .	0.77	0.83	0.74	0.78	—	—

\* Data of Skeggs *et al.* (10).

† Data of Lentz *et al.* (11).

The individual DNP amino acids obtained by this procedure were separated from inorganic salts by extraction with ether from an acidified solution and were rechromatographed with known samples of DNP amino acids in order to confirm their identification.

The results of the quantitative analysis are presented in Table II together with analyses of hypertensin I and hypertensin II which were obtained by the chromatographic method of Moore and Stein (23). It will be noted that the polypeptide substrate contains all of the amino acid residues present in hypertensin I. In addition one residue each of isoleucine or leucine, valine, tyrosine, and serine is present.

*Degradation with Renin.*—A sample of polypeptide substrate containing 1.1  $\mu\text{M}$  or 2000 units was diluted to 50 ml. with water. To this was added 0.5 ml. of hog renin containing

<sup>2</sup> FDNB, fluorodinitrobenzene.

42.5 units having a purity of 123 units per mg. of dry weight. The enzyme solution was 0.01 M with pyrophosphate buffer and 0.03 M with ammonium sulfate.<sup>3</sup> The mixture was adjusted to pH 7.3 with 0.01 N NaOH and was incubated at 39.5°C. In order to prevent losses due to adsorption on glassware it was necessary to siliconize all the equipment used in this experiment. During the incubation period small samples were withdrawn, suitably diluted in saline, and after heating to 100°C. for 10 minutes were assayed in the rat. The results showed that a total of 1680 units of pressor material was obtained after 60 minutes of incubation. Since this was not increased at the end of 90 minutes the solution was evaporated to a small volume without inactivation of the enzyme. The entire concentrated solution was then chromatographed using as a developer *N*-butanol-acetic acid-water (4, 1, 5) on Whatman No. 1 filter paper 6 inches in width which had been prewashed with the solvent. After development the chromatogram was air-dried. Very narrow sample strips were cut from the paper and stained with both ninhydrin and Durrum's reagent (21). Only two bands were revealed. The first ( $R_f$  0.32), fraction A, was both ninhydrin and Durrum-positive. The second ( $R_f$  0.63), fraction B, was ninhydrin-positive but Durrum-negative. The paper strips containing the fractions were cut from the chromatogram and the materials eluted with the aid of several washes of 0.1 N acetic acid. The two solutions were evaporated to remove acetic acid, and after dissolving in a small volume of water, were examined in the ultraviolet spectrophotometer.

Both fractions thus obtained had typical tyrosine spectrums. Calculations based upon the extinction of tyrosine at 274.5  $m\mu$  showed the presence of 1.13  $\mu M$  in fraction A, 1.50  $\mu M$  in fraction B. Fraction A contained 960 units of pressor activity as assayed in the rat; fraction B was inactive.

*Analysis of Fraction A.*—An aliquot estimated to contain 0.3  $\mu M$  was hydrolyzed in a sealed tube with 1 ml. of 6 N HCl for 20.6 hours. After removal of the hydrolyzing acid the material was quantitatively analyzed by the FDNB method using duplicate chromatograms. The following amino acids were found with the indicated molar proportions; tyrosine 1.27, phenylalanine 0.99, leucine + isoleucine 2.08, valine 0.95, proline 0.63, aspartic acid 0.95, arginine 1.24, and histidine 1.89. This analysis corresponds to that of hypertensin I.

A second aliquot containing 0.1  $\mu M$  was diluted to 3 ml. and adjusted to pH 8.0. After 0.04 ml. of carboxypeptidase solution (0.04 mg.) had been added (11) the solution was mixed and incubated 1.25 hours at 37.5°C. At this time pressor assays showed that complete destruction of activity had occurred. The enzyme was inactivated by heating the solution to 100°C. for 10 minutes. The solution was evaporated to a very small volume and chromatographed in one dimension using *N*-butanol-acetic acid-water (4, 1, 5) as the developing solvent. After treatment with ninhydrin three amino acids were revealed; namely phenylalanine, histidine, and leucine. In addition one ninhydrin-positive, Durrum-positive spot,  $R_f$  0.32, was observed which was assumed to be that portion of the original molecule unaffected by carboxypeptidase.

The amino acids released by carboxypeptidase from the C terminal of the peptide of fraction A are the same as those obtained after similar treatment of hypertensin I (11).

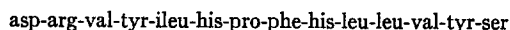
A third aliquot of fraction A, containing 0.3  $\mu M$  was subjected to one cycle of phenylisothiocyanate degradation (24). The phenylthiohydantoin derivative which was obtained

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<sup>3</sup> We are indebted to Drs. Erwin Haas and Harry Goldblatt of Mt. Sinai Hospital, Cleveland, for a supply of purified hog renin.



Inasmuch as the N terminal amino acid of the polypeptide substrate is aspartic acid rather than leucine, the peptide of fraction B must be linked to the leucine carboxyl group of hypertensin I. The structure of the polypeptide substrate is therefore:



*C Terminal Degradation with Carboxypeptidase.*—a sample of polypeptide substrate estimated to contain  $0.45 \mu\text{M}$  was diluted to 30 ml. and adjusted to pH 8.0. After 0.3 ml. of carboxypeptidase solution (0.3 mg.) was added, the solution was mixed and incubated at

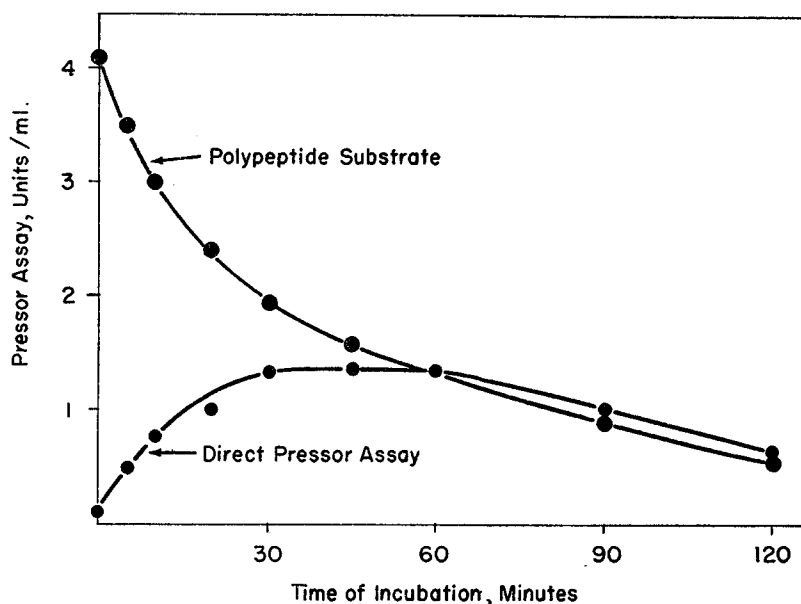


FIG. 3. Incubation of polypeptide renin substrate with carboxypeptidase.

$37.5^{\circ}\text{C}$ . for 150 minutes. Very small samples were withdrawn at frequent intervals, and after destroying the enzyme by heating to  $100^{\circ}\text{C}$ . for 10 minutes were tested for their direct pressor effect as well as for their polypeptide substrate concentration. The assays showed a continuous decrease in the polypeptide substrate concentration. A directly pressor substance, or substances, appeared which also declined upon continued incubation.

Apparently the successive removal of amino acids by the enzyme from the carboxyl terminal produces first hypertensin I and then hypertensin II.

A preliminary experiment illustrating the course of this reaction is shown in Fig. 3. At the end of the incubation period, when the activity was completely destroyed, the enzyme was inactivated by heating to  $100^{\circ}\text{C}$ . for 10 minutes. The solution was evaporated to a small volume. Small aliquots of the material were used in single dimension chromatograms on Whatman No. 1 filter paper using as solvents butanol-acetic acid-water (4, 1, 5), phenol-

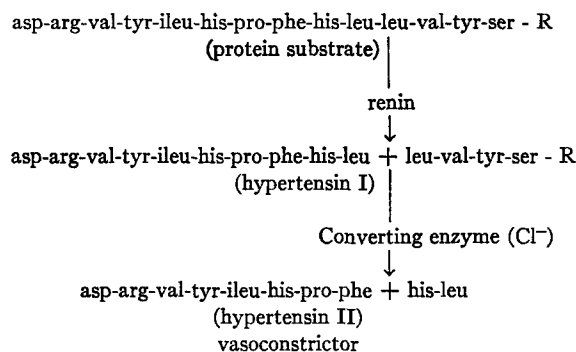
water (100, 39.5), and water-saturated tertiary amyl alcohol. The remaining material was treated with FDNB and the resulting DNP amino acids chromatographed in two dimensions.

The foregoing experiments established that the amino acids released by carboxypeptidase from the C terminal of the polypeptide substrate were serine, tyrosine, valine, leucine, histidine, and phenylalanine. One additional ninhydrin-positive, Durrum-positive spot was obtained in the amino acid chromatograms ( $R_f$  0.27 in butanol-acetic acid-water (4,1,5)) which was assumed to represent that portion of the molecule unaffected by carboxypeptidase.

The finding that the amino acids released from polypeptide substrate by carboxypeptidase are those of fraction B as well as those which are released from hypertensin I by action of this enzyme supports the formula presented in the foregoing section.

#### DISCUSSION

The present understanding of the mechanism of the renin-hypertensin pressor system may be summarized in the following equations.



The enzyme trypsin, with which the original protein renin substrate was degraded to form the polypeptide substrate, is known to hydrolyze only the carboxyl terminal amide or ester linkage of the amino acids arginine or lysine (19). It was expected therefore that one of these two basic amino acids would be found in the carboxyl terminal position in the polypeptide substrate molecule. Since renin breaks the leucyl-leucine bond of the substrate setting free hypertensin I it is clear that the point of attachment of the polypeptide substrate molecule to the parent protein must be at either the serine carboxyl or hydroxyl group. Trypsin would not be expected to sever the peptides serylar-arginine or seryllysine. It is possible however that it might hydrolyze an ester linkage between an arginine or lysine carboxyl and a serine hydroxyl group although no specific prior example of this function is known.

The finding that renin breaks the substrate molecule between two leucine residues is also surprising. Nearly all known proteolytic enzymes having a specific action hydrolyze bonds adjacent to aromatic or basic amino acids. The less specific enzymes will split peptide bonds with little regard to the adjacent amino acid residues. Renin is probably a highly specific proteolytic enzyme whose hydrolytic mechanism very likely differs from that of most of the well known enzymes.

The renin substrate molecule is completely unaffected by a hypertensin-converting enzyme with which it is in intimate contact in the plasma. Neither is the purified polypeptide substrate affected by this enzyme. The leucine carboxyl group of hypertensin I becomes available only after this decapeptide is released from the substrate molecule. It is suggested therefore that this group must be free in order to satisfy the specific requirements of the converting enzyme and allow hydrolysis of the phenylalanyl-histidine bond of hypertensin I.

#### SUMMARY

A purified preparation of a polypeptide renin substrate prepared by tryptic degradation of the protein renin substrate has been analyzed by the fluorodinitrobenzene method and after degradation with renin, carboxypeptidase, and phenylisothiocyanate, has been found to possess the amino acid sequence; asp-arg-val-tyr-ileu-his-pro-phe-his-leu-leu-val-tyr-ser. The first 10 of these amino acids constitutes hypertensin I which is released by cleavage of the leucyl-leucine bond by renin. The remaining 4 amino acids, leu, val, tyr, ser, apparently link hypertensin I to the protein renin substrate.

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