THE EXISTENCE OF TWO FORMS OF HYPERTENSIN

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The pressor substance hypertensin, which is the product of the action of the renal enzyme renin upon its plasma substrate, has been shown to be present in the arterial blood of many patients with hypertensive cardiovascular disease (1) as well as in the arterial blood of animals with experimental renal hypertension (2–4). Hypertensin may, therefore, be concerned in causing the elevation of blood pressure in both of these conditions. In the course of attempts to purify this material it was found that certain preparations, when examined by means of the countercurrent distribution method, revealed not one but two pressor substances. Further study of this completely unexpected finding showed that the initial pressor substance, hypertensin I, formed as a result of the action of renin on its substrate, is quickly converted to a second substance with approximately equal pressor activity, hypertensin II, apparently by an enzyme in plasma, which is activated by chloride ion. It is the purpose of this paper to describe the preparation of hypertensin I from suitable preparations of renin and substrate, to demonstrate its conversion to hypertensin II by the action of the plasma enzyme, and to determine which ions are effective in activating this enzyme.

Methods

Preparation of Renin.—The renin was prepared from fresh hog kidneys according to the method described by Katz and Goldblatt (5) and modified by Dexter (6). This preparation contained a considerable amount of hypertensinase activity which was eliminated (reference 7, page 101) in the following fashion. Renin, prepared from 100 pounds of hog kidneys by ammonium sulfate precipitation, was dissolved in 1.5 liters of water. The solution was chilled to 0–5°C. and sufficient solid sodium chloride was added to insure complete saturation. 0.1 N HCl, saturated with NaCl, was then dripped into the solution slowly and with constant stirring until the pH dropped to 2.0. The solution was centrifuged at high speed at 0–5°C. The supernatant containing the hypertensinase was decanted and discarded. The precipitate was dissolved in 1 liter of cold 0.1 M Na_2HPO_4. The light brown solution was adjusted to pH 7.5, put into small diameter cellophane bags, and dialyzed exhaustively against cold distilled water. The resulting product, with a volume of about 1.25 liters, was found to be uniformly free of hypertensinase. Usually 1 volume of this enzyme preparation was adequate to catalyze the formation of hypertensin in 25 volumes of the substrate preparation described below in a period of 30 to 45 minutes when this mixture was incubated at 37°C. and pH 7.5.

Preparation of Substrate.—Horse blood was collected in $\frac{1}{10}$ volume of 4 per cent sodium

citrate and was allowed to stand for 48 hours at 0–5°C. This and subsequent steps were all carried out at these temperatures. The clear supernatant plasma was drawn off and its pH adjusted to 6.0 with 1.0 N HCl. A 4.0 M solution of ammonium sulfate was added slowly with stirring until the concentration of this salt reached 1.3 M. The solution was filtered by gravity and the precipitate discarded. Additional 4.0 M ammonium sulfate solution was added to the filtrate over a period of several hours with continuous stirring until the salt concentration rose to 2.2 M. The precipitate was gathered on large Buchner funnels, resuspended, and thoroughly washed in a 2.5 M solution of $(NH_4)_2SO_4$. The precipitate was again collected on Buchner funnels and dissolved in distilled water, the volume of which was $\frac{1}{5}$ that of the original plasma. The temperature was brought to 25°C. and the pH adjusted to 3.8 with 2.5 N HCl. These conditions were maintained for 30 minutes; during this period, residual hypertensinase was destroyed (reference 7, page 133). The pH was then adjusted to 7.5 with 2.5 N NaOH and the solution dialyzed in an artificial kidney (8) in the cold against tap and then distilled water until it was completely free of ammonia and chloride. The volume at this stage was approximately $\frac{1}{4}$ that of the original plasma and usually contained enough substrate to yield 1.0 to 1.5 Goldblatt units (9) of hypertensin per ml. when incubated with renin. Its pressor effect was assayed by intravenous injections into rats and compared to that produced by an injection of a standardized hypertensin solution.

EXPERIMENTAL

Preparation of Hypertensin I.—

2.5 liters of the substrate solution, free of ammonia and chloride and with a pH of 7.5, was warmed to 37°C. 500 ml. of renin with a similar pH and also free of ammonia and chloride was added to the substrate with mixing. This amount of renin is greatly in excess of the amount required for complete conversion of substrate and was used in this experiment only in an effort to demonstrate the initial product of the action of renin. After 6 minutes' incubation, the mixture was poured into 12 liters of 95 per cent alcohol and stirred thoroughly while the pH was adjusted to 5.5 with 2.5 N HCl, the samples for determination of pH being diluted 1 to 4 with distilled water. The precipitated proteins were removed by filtering on large gravity funnels and thoroughly washed with 12 liters of 80 per cent alcohol. After removal of the precipitate, the filtrates were combined and concentrated to a volume of 500 ml. by evaporation under reduced pressure at a temperature less than 40°C. The solution was then acidified with sufficient 2.5 N HCl to bring the pH to 3.0 and extracted three times with 500 ml. portions of diethyl ether. The ether extracts were discarded and the fat-free aqueous layer, after adjustment to a pH of 6.0 with 2.5 N NaOH, was further concentrated to a volume of 150 ml. by evaporation under reduced pressure. At this point, the solution was adjusted to a pH of 7.0 by the addition of 2.5 N NaOH and the active material was extracted into three portions of *n*-butanol totalling 450 ml. in volume. The active material was recovered from the butanol by extraction into one 100 ml. and two 50 ml. portions of 0.05 N H_3PO_4. This preliminary passage through butanol effected a considerable purification with little loss of active material. The combined acid extracts, containing 3300 Goldblatt units of hypertensin, were brought to pH 7.0 with 2.5 N NaOH and concentrated to 25 ml. by evaporation *in vacuo*. This material was subjected to countercurrent distribution with 25 ml. of 0.05 N sodium phosphate buffer (pH 7.0) in the lower phase, and the same volume of *n*-butanol in the upper. Both of the solutions were saturated with each other. The distribution apparatus used was a modification of the simple machine described by Craig (10) for preliminary work. After 49 transfers, all the tubes were acidified by the addition of 1.0 ml. of 3 N H_3PO_4; time was allowed for re-equilibration, and the inactive butanol layers were discarded. Samples of the lower aqueous layer, after removal of residual butanol by evaporation and suitable dilution, were assayed (11) for their pressor activity in the rat.

The results of these assays, expressed in Goldblatt units, are shown in curve A, Fig. 1. The curve reveals one pressor substance contained in tubes 16 to 34, which has been designated hypertensin I. The solutions from tubes numbered 17 through 31 were pooled. After the pH was brought to 7.5 and the solution was concentrated to 250 ml. it was stored at −20°C. for subsequent experiments. Upon assay, this preparation was found to contain a total of 2000 Goldblatt units.

Demonstration of Hypertensin II.—

Two 50 ml. aliquots of the solution of hypertensin I described above, containing 400 units each, were warmed to 37°C. and mixed at the same temperature with 400 ml. portions of substrate, one of which had been made 0.1 molar with sodium chloride, while the other remained salt-free. After incubation for 1 hour, each solution was mixed with 4 volumes of 95 per cent ethyl alcohol, brought to a pH of 5.5 with 3.0 N H_3PO_4, and filtered by gravity to remove the precipitated protein. The precipitate was washed with 4 volumes of 80 per cent alcohol, again filtered by gravity, and discarded. The combined alcoholic filtrates were concentrated by evaporation to about 100 ml., adjusted to pH 3.0 by the addition of 3 N H_3PO_4, and extracted three times with equal volumes of ether. The ether extracts were discarded; the fat-free aqueous layers were adjusted to pH 7.0 and then concentrated *in vacuo* to a volume of 25 ml. Each of these two preparations was subjected to a 50 tube countercurrent distribution exactly as described for the preparation of hypertensin I.

The results in Fig. 1 show that hypertensin I, incubated with substrate in the absence of sodium chloride (curve B), appeared in tubes 16 to 30 with a maximum in tube 20 and was, therefore, virtually unchanged from the starting material (curve A). However, the activity of the sample incubated with added sodium chloride appeared (curve C) in tubes 4 to 12, with a maximum in tube 6. This fraction, designated hypertensin II, apparently was produced from hypertensin I by the action of a plasma enzyme which was activated by chloride ion. Chloride ion alone will not produce this conversion, nor will boiled plasma with chloride present.

Since the foregoing experiments were done with the use of horse blood and hog renin as starting materials, it was important to determine whether similar results could be obtained with human materials. This was done using a dialyzed non-fractionated human plasma as the substrate preparation and human renin prepared according to the method of Dexter (6). The human hypertensin prepared by the salt-free incubation of these materials gave a distribution pattern similar to that of horse hypertensin I. Furthermore, the incubation of this hypertensin with human plasma containing chloride produced a pressor material which yielded a distribution pattern similar to that of horse hypertensin II. It was shown, moreover, that a highly purified mixture of horse hypertensins I and II (5000 units per mg. of N) had a pressor action when injected into humans. A dose of 1.5 to 2.5 units was injected intravenously and the blood pressures were determined by auscultation. The results shown in Table I

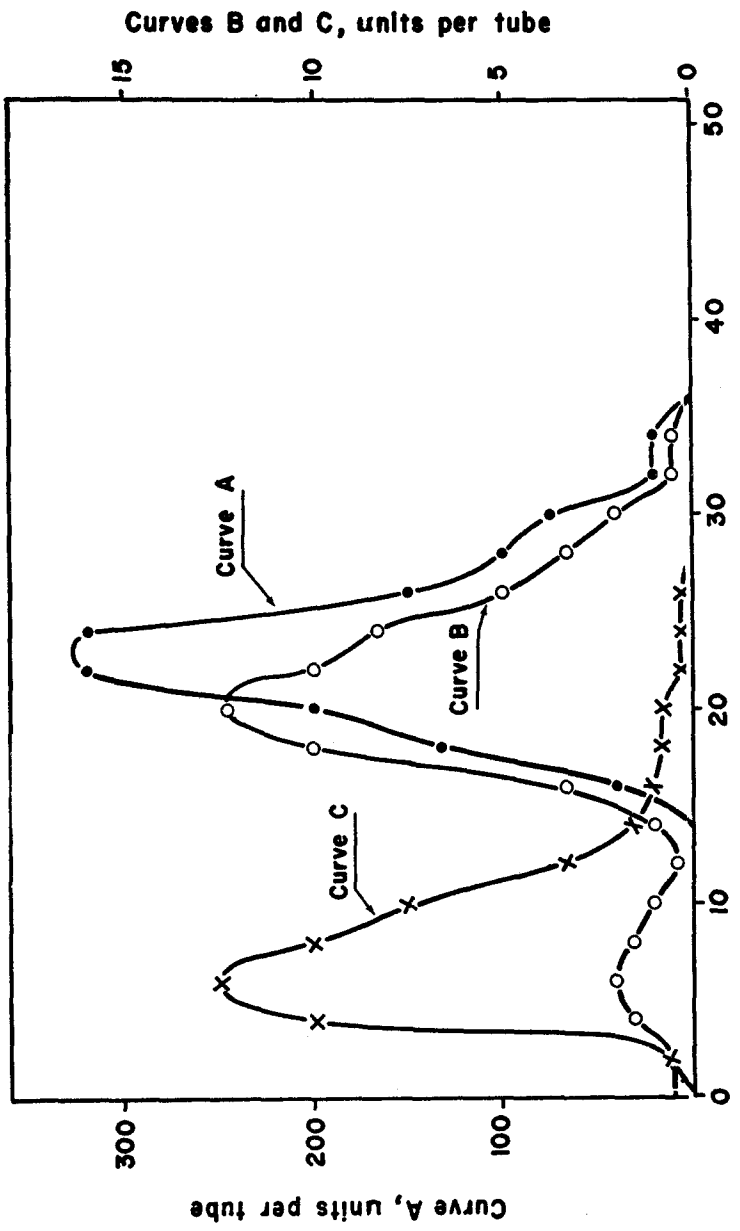

FIG. 1. 50 tube countercurrent distribution of preparations of hypertensin I, curves A and B, and hypertensin II, curve C.

indicate that the human being is quite sensitive to the pressor activity of horse hypertensins and that the sensitivity is not changed by repeated injection.

Effects of Different Ions on the Activation of the Substrate Enzyme.—The methods used for this phase of the investigation were simpler than those described in the previous experiments.

10 ml. portions of the salt solution to be tested were added to 100 ml. of a well dialyzed substrate. Care was taken that the pH of this mixture would be within the range 7.0–7.5. Sodium hydroxide or phosphoric acid was used for pH adjustments when it was not possible to use

TABLE I

The Effect of Intravenous Injections of Horse Hypertensin upon the Blood Pressure of Several Human Subjects

Patient	Age	Sex	Dose	Control blood pressure	Maximum blood pressure
	yrs.		*units*	*mm. Hg*	*mm. Hg*
LD	41	M.	2.5	120/82	190/120
				112/80	180/130
SR	44	M.	2.0	120/90	174/140
				120/90	172/130
				128/90	180/140
DS	38	M.	1.5	120/70	160/120
				110/92	165/120
MJ	35	M.	2.0	108/60	130/80
				110/50	130/80
				110/54	125/75
JS	46	M.	2.5	110/72	160/100
				108/70	150/100
JY	33	M.	2.5	126/80	168/108

the acid or base of the salt to be tested. After adjustment of pH, the substrate-salt mixture was brought to 37°C., mixed with 5 ml. of well dialyzed renin, and incubated for 45 minutes. At the end of this period, the mixture was poured into 4 volumes of 95 per cent alcohol, stirred, the pH adjusted to 5.5, and centrifuged. The precipitated proteins were discarded and the supernatant fluid reduced by evaporation to 5 to 10 ml. The pH was adjusted to 8.9 with 2.5 N sodium hydroxide, the solution was made to a volume of 25 ml., and sufficient solid $NaHCO_3$ added to give a concentration of 0.1 M. This solution was then subjected to countercurrent distribution in which the lower aqueous phase consisted of 25 ml. portions of 0.1 N $NaHCO_3$ and the upper phase of similar volumes of *n*-butanol. After 9 transfers, both phases of all tubes were sampled. The aliquots were evaporated to dryness to eliminate butanol, and then suitably diluted for assay.

Two contrasting experiments are shown in Fig. 2. Curve A shows the hypertensin activity after distribution when the incubation mixture had been made

0.1 M with respect to KCl. In this case nearly all of the activity was confined to the water phases of tubes 0 and 1, which had distribution coefficients of 0.03 and 0.06. The active material was therefore identified as hypertensin II. Curve B shows the activity after distribution when the incubation mixture had been 0.1 M with respect to sodium phosphate. Here most of the activity

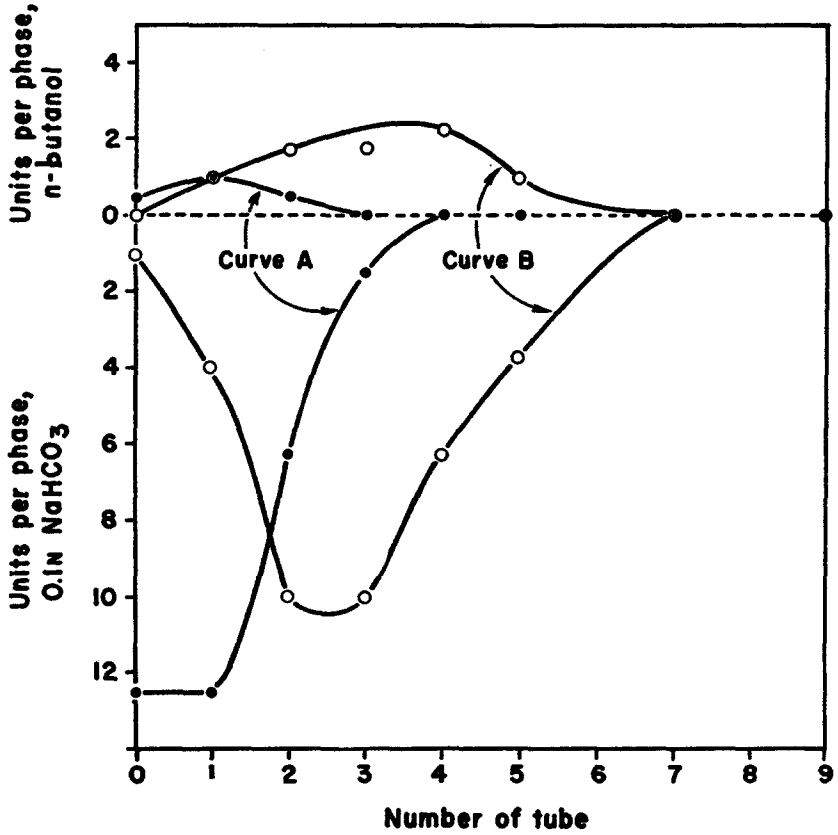

FIG. 2. 10 tube countercurrent distribution. Curve B illustrates hypertensin I produced in a renin-substrate incubation free of inorganic ions. Curve A shows hypertensin II formed in a similar experiment in which the mixture had been made 0.1 M with KCl.

was found in both phases of tubes 2, 3, 4, and 5, which had distribution coefficients of 0.19, 0.19, 0.35, and 0.21, and was, therefore, hypertensin I. Although the degree of resolution obtained in these short distributions did not separate the two types of hypertensin, it provided sufficiently good qualitative identification. This pair of experiments shows that, although hypertensin I was formed in both cases by the action of renin on its substrate, hypertensin I became converted by the plasma enzyme to hypertensin II only when chloride was present. The results of exactly similar experiments using a variety of other ions are

given in Table II. It can be seen that, in addition to Cl^-, the enzyme was activated by NO_3^-, Br^-, and F^-, while HCO_3^-, $SO_4^=$, and $HPO_4^=$ were without effect.

DISCUSSION

The conversion of hypertensin I to hypertensin II occurs readily under the influence of a non-dialyzable protein fraction of plasma in the presence of between 0.0001 and 0.001 M chloride. The chloride ion or the plasma fraction alone has been shown to be ineffective. The nature of the conversion seems to

TABLE II

The Effect of Various Salts upon the Type of Hypertensin Formed

Experiment No.	Salt added	Predominant type of hypertensin
185	No salt	I
238	0.0001 M NaCl	I*
227	0.001 M NaCl	II*
208B	0.01 M NaCl	II
186	0.153 M NaCl	II
194A	0.1 M Na_2HPO_4	I
209B	0.1 M $NaHCO_3$	I
214A	0.1 M NaBr	II
214B	0.1 M NaF	II
204	0.1 M $NaNO_3$	II
194C	0.1 M KCl	II
212	0.1 M K_2SO_4	I
203	0.1 M $CaCl_2$	II
205A	0.1 M $MgCl_2$	II
205B	0.1 M $MgSO_4$	I
199A	0.1 M $(NH_4)_2SO_4$	I
199B	0.1 M NH_4Cl	II
194D	0.1 M Li_2SO_4	I

* These experiments yielded mixtures of the two types of hypertensin.

be enzymatic and the necessary enzyme is present in both horse and human plasma.

The activation of an enzyme by chloride is not unknown. A polypeptidase has been isolated (12) from yeast which requires halide as well as zinc ions. Nitrate in this case will also replace halide to a certain extent.

Hypertensin I has been obtained in this laboratory with a specific activity of 5000 Goldblatt units per mg. of N, indicating a high degree of purity. This product does not give a ninhydrin reaction and yet upon acid hydrolysis yields a large number of ninhydrin-reacting amino acids. The compound is probably, therefore, a polypeptide. It is most natural to assume that hypertensin II results from the removal of a polar group or groups from the hypertensin I molecule leaving that portion of the molecule responsible for its pressor activity unchanged.

In theory, either or both of these compounds might be of importance in human hypertension. It cannot be assumed that owing to the presence of the plasma enzyme and chloride the significant compound would necessarily be hypertensin II, since hypertensin I might exert its effect on the arterioles before being converted to hypertensin II. In any case, the interpretation of previous work done in this laboratory dealing with the assay of hypertensin in the circulating blood of dogs (2–4) and humans (1) with hypertension probably is not affected by the present findings since both types of hypertensin are measured by the methods that were used.

SUMMARY

Two types of hypertensin have been demonstrated by means of countercurrent distribution. The first type is the product of the action of the enzyme, renin, upon its substrate and has been designated hypertensin I. It can be rapidly converted to a second approximately equally pressor compound, hypertensin II, apparently through the action of an enzyme in the plasma which requires halide or nitrate for activation.

A highly purified preparation containing horse hypertensins I and II caused an elevation of blood pressure when injected into human beings.

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