THE PREPARATION AND FUNCTION OF THE HYPERTENSIN-CONVERTING ENZYME

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It has recently been found that hypertensin may exist in two forms (1). The first, hypertensin I, is the initial product of the action of the renal enzyme renin upon its plasma substrate. The second, hypertensin II, is produced from hypertensin I by the action of an enzyme contained in the plasma which requires chloride ion for its activation.

Both forms of hypertensin are pressor upon intravenous injection into intact animals and give blood pressure responses which do not differ significantly in duration or general contour. The recent work of Helmer (2), using isolated aortic strips, suggests that hypertensin II is a true vasoconstrictor material while hypertensin I is not. Experiments have been performed, using isolated perfused rat kidneys which show that this is true. Hypertensin I therefore becomes pressor upon intravenous injection only as a result of its immediate change to hypertensin II by the converting enzyme in the plasma.

Hypertensin I has been purified by means of countercurrent distribution (3) and its quantitative amino acid composition determined (4). It is desirable that this material be converted to hypertensin II by means of the converting enzyme and that the chemical reaction involved be elucidated. A method has therefore been devised by which the converting enzyme can be prepared in a highly concentrated and partially purified form.

EXPERIMENTAL

Perfusion of the Isolated Rat Kidney.—Large male rats were anesthetized with sodium amytal (9.0 mg./100 gm.) and the peritoneal cavity opened with a long midline incision. Heparin (1 mg./100 gm.) was given by direct injection into the exposed vena cava. The left kidney was freed from its bed, the renal artery exposed, and cannulated *in situ* with 22 gauge needle stock inserted into a short piece of polyethylene tubing. The kidney was then removed from the animal and the tubing connected to a positive displacement, variable speed, rubber tubing pump which was arranged to deliver a physiological salt solution (5) from a reservoir at any desired rate between 0.8 and 3.2 ml. per minute. A "T" fitting was inserted in the line leading from the pump to the kidney to permit the connection of a small bore mercury manometer equipped for inkwriting on the kymograph (6). This arrangement allowed the recording of the pressure required to force a constant stream of perfusion fluid through the

kidney. No attempt was made to control the temperature of the perfusion. Immediately after the connection of the kidney to the pump perfusion was instituted at pressures of 150 to 250 mm. of Hg for a period of 15 to 30 minutes in order to wash out all traces of blood. At the end of this time the speed of the pump was reduced until the perfusion-pressure was in the range of 50 to 100 mm. of Hg. Injections of solutions to be tested could then be made into a rubber tube connecting the pump with the reservoir of fresh perfusate.

The solutions used for testing were prepared from purified hypertensin I, with a purity of 7150 Goldblatt units (7) per mg. of N and from purified hypertensin II which had a purity of 13,700 units per mg. of N. Both solutions were prepared in alkaline saline (3) and were assayed in the intact rat (6) and found to have equal pressor activity.

As shown in the recording of Fig. 1 the injection of 0.05 units of hypertensin II resulted in large and sustained elevations in the perfusion pressure while similar doses of hypertensin I were completely without effect. In other experiments the dose of hypertensin I was increased twelve times without any evidence of vasoconstrictor activity.

Preparation of the Converting Enzyme.—Horse blood was collected in one-tenth volume of 4 per cent sodium citrate, chilled to 0–3°C. and centrifuged as soon as possible. The plasma was removed and adjusted to a pH of 6 with 2.5 N H_2SO_4 and mixed immediately with one

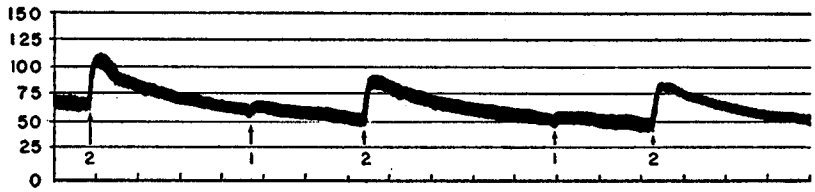

FIG. 1. Perfusion of isolated rat kidney: *1*, injection of 0.05 units of hypertensin I; *2*, injection of 0.05 units of hypertensin II. Perfusion pressure in mm. of Hg, time in minutes.

third volume of 4.0 M ammonium sulfate solution. This portion of the procedure was performed within a period of 3 hours after the collection of the horse blood.

Additional 3.5 M ammonium sulfate solution was added slowly with constant stirring until the molarity reached 1.6. A precipitate formed which was removed by filtration on large gravity funnels and discarded. The molarity of the ammonium sulfate in the filtrate was then raised slowly to 2.2 and the resulting precipitate gathered on Buchner funnels.

The 1.6 to 2.2 M ammonium sulfate precipitate was dissolved in water and the protein concentration of the resulting solution determined and adjusted to 4 per cent. The protein solution was reprecipitated twice between the concentration limits of 1.6 and 2.2 M ammonium sulfate. The final precipitated protein was dissolved in water to yield a blue-green solution which was dialyzed in a sterilized cellophane bag against cold distilled water until it was free of the ammonium ions. Fractions prepared in this fashion contained a considerable amount of the converting enzyme but also contained hypertensinase. It was then fractionated by means of isoelectric precipitation.

The protein content of the dialyzed solution was adjusted to 2 per cent. The solution was chilled to 0–1°C. and the pH adjusted to 5.2 using a concentrated sodium acetate buffer pH 4.0 which resulted in a light green precipitate that was gathered by centrifugation. The supernatant fluid was discarded. The precipitate was dissolved in 0.05 M sodium phosphate buffer at a pH of 7.8. The pale green, opalescent solution was placed in ampoules, shell-frozen, and lyophilized.

This procedure yielded 5.5 gm. of enzyme for every 10 gallons of horse blood. It was free of hypertensinase. All the procedures were carried out at low temperatures and fractions

were frozen when not being processed. The converting enzyme was stable at pH 4, although it was entirely destroyed in 20 minutes at pH 3 and a temperature of 25°C.

Estimation of Enzyme Activity.—The enzyme was suitably diluted so that the protein content was between 0.025 and 0.25 per cent. It was prepared in a 0.05 M sodium phosphate buffer with a pH of 6.5 and a sodium chloride content of 1 per cent and was sterilized by Seitz filtration. 0.2 ml. of a sterile solution of hypertensin I containing 100 units was added to 9.8 ml. of the buffered enzyme and this mixture incubated for 1 hour at 37.5°C. At the end of this period the solution was placed in a boiling water bath for 10 minutes and after

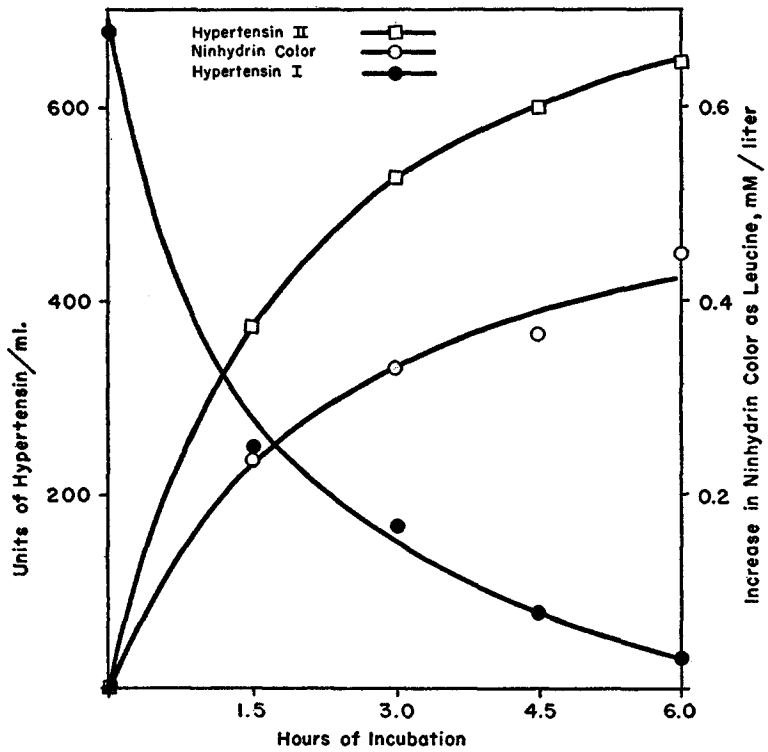

FIG. 2. The enzymatic conversion of hypertensin I to hypertensin II.

cooling the pH adjusted to 7. The precipitate which formed was removed by centrifuging and was discarded. A 5 ml. aliquot of the supernatant solution was then subjected to an 8 tube countercurrent distribution (8) in which the lower phase was composed of 0.1 M sodium phosphate with a pH of 7 containing 15 per cent sodium chloride while the upper consisted of redistilled secondary butanol. Tubes 0 and 1 containing hypertensin II were pooled and aliquots of both phases removed, suitably diluted, and assayed in the rat. Hypertensin I was present in tubes 3, 4, 5, 6, 7 and was treated in a similar fashion. Tube 2 contained approximately 20 per cent of the total pressor activity, but since previous experiments had shown this to be a mixture of hypertensin I and II, it was discarded. This experimental method allowed the estimation of the amount of hypertensin II formed as well as the amount

of hypertensin I remaining after the action of the enzyme. Hypertensin and the enzyme are easily destroyed by bacterial contamination so that it is necessary to conduct these procedures under sterile conditions. The converting activity cannot be determined accurately in crude plasma fractions because of the presence of hypertensinase.

It was found that the enzyme exhibited its maximum activity at a pH of 6.5 in the presence of 0.05 M phosphate. Experiments conducted using 0.1 M citrate and 0.1 M tris(hydroxymethyl)aminomethane indicated that no advantage resulted from the use of either of these buffers. In the case of citrate optimal activity was found to be at a pH of 7.5 or higher, while the maximum activity was obtained with the tris buffer at a pH of 8.

The Use of the Converting Enzyme.—A small scale experiment was conducted prior to the conversion of a large amount of purified hypertensin I to hypertensin II. A solution of hypertensin I containing 710 units per ml. was prepared in a 0.05 M sodium phosphate buffer at a pH of 6.5. Sodium chloride was added to a concentration of 1 per cent. The solution was sterilized by immersion in boiling water for 15 minutes. The lyophilized enzyme was dissolved in a volume of the same buffer so that its concentration was 3 per cent, and was passed through a Seitz filter. One volume of the enzyme was added to 6 volumes of the hypertensin, and the mixture incubated for 6 hours at 37°C. At suitable intervals during the period of incubation small samples were withdrawn from the main incubation mixture. The samples were placed on a boiling water bath for 10 minutes and aliquots were taken for eight tube countercurrent distribution and assay. The ninhydrin reaction was also applied to similar aliquots according to the method of Moore and Stein (9). The results of this experiment are illustrated in Fig. 2. As the concentration of hypertensin I decreased there was a corresponding increase in the hypertensin II concentration until at the end of 6 hours 96 per cent had been converted. This conversion was accompanied by a corresponding increase in the ninhydrin color value.

DISCUSSION

The finding that hypertensin I is neither a vasoconstrictor nor a directly pressor substance for the isolated kidney is very surprising because the blood pressure responses obtained from the intravenous injection of the two forms of hypertensin into intact animals are identical. It must therefore be concluded that the converting enzyme is present in the plasma of test animals in very large excess in order that the conversion might proceed at a rate at least equal to that of the rate of destruction or utilization of hypertensin II. This example is in marked contrast to the relatively slow production of hypertensin I by the action of renin on its substrate which in all probability is still the rate-limiting reaction in the renin-hypertensin system.

Inasmuch as enzymes having peptides as their substrates are frequently metalloproteins (10) it was of interest to test the effect of cyanide and of versene[1] upon the activity of this enzyme. It was found that the addition of either of these substances to incubation mixtures in concentrations of 0.01 M caused a marked reduction in the amount of hypertensin converted. Attempts were therefore made to reduce the activity of the enzyme by electrodialysis and by dialysis against 0.005 M cyanide. In neither case was the activity of the enzyme preparation decreased by the treatment. The addition of 8 different metallic ions to concentrations of 0.0001 M was also of no value in increasing the activity

[1] Disodium ethylenediaminetetraacetate.

of the enzyme preparations. Therefore it seems possible that the enzyme is a metalloprotein.

The enzyme has been used to convert a preparation of hypertensin I containing 250,000 units with a specific activity of approximately 7700 units per mg. of nitrogen. After termination of the reaction and suitable removal of the enzyme a preparation of hypertensin II was obtained which contained 228,000 units with a purity of 9900 units per mg. of nitrogen. Since the specific activity was actually increased by the process it seems probable that only very small amounts of impurities are introduced by the enzyme preparation.

SUMMARY

It has been shown by use of isolated, perfused rat kidneys that hypertensin II is a potent vasoconstrictor substance while hypertensin I is not. Hence it would appear that in intact animals the pressor activity of hypertensin I results from its rapid conversion to hypertensin II.

An enzyme which effects this conversion has been procured from horse plasma in a semipurified form by means of ammonium sulfate fractionation and isoelectric precipitation. A method is described for estimating the activity of the enzyme. An example of the use of the preparation in converting purified hypertensin I to hypertensin II has been described.

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