THE PURIFICATION OF HYPERTENSIN II

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Hypertensin, a pressor substance, has been found in the arterial blood of many human beings (1) with essential hypertension as well as in dogs (2) with experimental renal hypertension. The purification of this material led to the discovery that it exists in two forms (3). The first, hypertensin I, is the initial product of the reaction of the renal enzyme, renin, upon its plasma substrate. Hypertensin I is converted to the second form, hypertensin II, by the action of a chloride-activated enzyme in the plasma. Hypertensin II is a powerful vasoconstrictor substance and is responsible for elevations in blood pressure produced by the renin-hypertensin system (4).

Hypertensin I has been purified by countercurrent distribution (5) and its quantitative amino acid composition determined (6). The chloride-activated-converting enzyme has been obtained in a concentrated and partially purified form (4). It is the purpose of this paper to describe an improved method of obtaining renin, improvements in the purification of hypertensin I, the enzymatic conversion of this substance to hypertensin II, and the final purification of the latter material.

EXPERIMENTAL

Assays.—All assays were conducted on intact, anesthetized rats (7) and results are expressed in Goldblatt units (8). Fractions to be assayed were diluted in alkaline saline (5). When assays were performed upon fractions containing large amounts of ammonium sulfate it was necessary to eliminate the ammonium ion. This was accomplished by diluting an aliquot estimated to contain 1 unit with 5 ml. of 0.75 M $NaHCO_3$ and evaporating the resulting solution to dryness under vacuum. Upon addition of 25 ml. of distilled water to the residue, a solution was obtained sufficiently free of ammonium ion to permit assay.

Preparation of Renin:

The following method, which is derived from the work of Katz and Goldblatt (9), Dexter, Haynes, and Bridges (10), and Haas, Lamfrom, and Goldblatt (11), is well adapted to large scale batches and yields a highly active product.

One hundred pounds of fresh frozen hog kidneys were thawed and ground into 50 liters of cold distilled water. The mixture was stirred for 1 hour and strained through gauze. The

pulp was reextracted by stirring for a similar period with a second portion of 50 liters of water. After straining, the pulp was discarded and the two filtrates combined. A 20 per cent solution of trichloroacetic acid was then added slowly with stirring until the pH was 2.8. The temperature was maintained at 10°C. or less during this addition. The mixture was then filtered through paper on large gravity funnels in the refrigerator. After thorough draining the bulky precipitates were discarded. The pH of the clear yellow filtrate was adjusted to 5.0 with 2.5 N NaOH and 500 gm. of solid ammonium sulfate were added per liter of filtrate. One pound of celite filter aid was added and after thorough stirring the active precipitate was gathered on paper on large Büchner funnels. The filtrate was discarded. The precipitate was suspended in 5 liters of cold distilled water and the temperature lowered to 0–1°C. The suspension was then carefully adjusted to pH 1.6 by addition of 4 N H_2SO_4 with constant stirring. At the end of 15 minutes the pH was made to 4.3 with a strong NaOH solution. The volume was then adjusted to 10 liters with cold distilled water and solid ammonium sulfate was added to a final concentration of 2.5 M. The suspension was then filtered on Büchner funnels and the filtrate discarded. The active precipitate was washed with two 10 liter portions of cold 2.5 M ammonium sulfate solution; the solid material being thorougly suspended by stirring and filtered both times on a Büchner funnel.

The washed precipitate was suspended in 5 liters of cold distilled water in order to dissolve the renin. The pH of the suspension was adjusted to 7.5 and after thorough stirring was filtered on a Büchner funnel. The precipitate was washed once more in a similar fashion with an additional 5 liter portion of water. After filtering, the Celite-precipitate cake was discarded. The combined filtrate, with a volume of about 10 liters, was stored in the frozen state.

Renin prepared by this procedure contained little or no hypertensinase. The chloride ion was also absent—a necessary condition for preparing hypertensin I. Two liters of this preparation acting for a half-hour, converted into hypertensin I all of the substrate extracted from 100 liters of horse plasma.

Preparation of Substrate:

Horse blood was collected in $\frac{1}{10}$ volume of a 4 per cent solution of sodium citrate containing phenylmercuric acetate in a concentration of 1–2000. The blood was chilled as rapidly as possible to 1–3°C. by means of a siliconized refrigerated copper coil which was put directly into the blood. After standing for 48 hours the clear supernatant plasma was drawn off and its pH adjusted to 6.0 with 2.5 N H_2SO_4. This and all subsequent operations were performed at refrigerator temperatures and the volumes of reagents described are for a batch of 100 liters of plasma. A 4.0 M solution of ammonium sulfate was added slowly with stirring until the concentration of this salt reached 1.3 M. One kg. of hyflo super-cel was added and the mixture filtered on cloth on a large vacuum filter. The precipitate was discarded. The ammonium sulfate concentration in the filtrate was raised to 2.3 M by a second slow addition of the 4.0 M ammonium sulfate solution. One kg. of hyflo super-cel was again added and the mixture filtered for the second time. The filtrate was rejected. The precipitate was collected and washed twice with 25 liters of a cold 2.5 M ammonium sulfate solution. These washings were performed by thoroughly suspending the precipitate in the solution and then filtering the mixture by vacuum. During the second filtration the filter was covered by a rubber membrane and the precipitate squeezed into a hard dry cake. The protein was then dissolved by treating the cake with two separate portions of 8 and 4 liters of cold distilled water. Each time the cake was thoroughly stirred with the water and the mixture filtered by vacuum. The filtrates were combined and the volume adjusted to 20 liters. The concentration of protein in this solution was between 9 and 10 per cent.

Preparation of Crude Hypertensin I:

A 20 liter volume of substrate preparation was warmed to 25°C. and the pH adjusted to 3.8 with 2.5 N H_2SO_4. These conditions were maintained for 30 minutes in order to destroy hypertensinase. At the end of this time the pH was adjusted to 7.5 and the temperature raised to 37.5°C. 2 liters of renin was added, the mixture stirred, and incubation continued until the maximum yield of hypertensin was obtained. This time period, determined in advance by small scale experiments, was usually of 30 minute duration. The reaction was stopped by precipitation of the proteins with 4 volumes of 95 per cent alcohol. The mixture was stirred, adjusted to pH 5.5, and filtered on large gravity funnels. After draining, the proteins were washed with 4 volumes of 80 per cent alcohol and refiltered. The precipitate was discarded and the filtrates evaporated under vacuum to 5 liters. The pH of this solution was adjusted to 5.0. The material was then extracted twice using 1/2 volumes of diethyl ether. The ether layers were discarded and the aqueous layers vacuum evaporated to volume of 700 ml. At this concentration solid ammonium sulfate separated from solution.

Purification of Hypertensin I:

Four individual crude preparations of hypertensin I representing a total of 80 liters of the substrate or 400 liters of horse plasma and containing approximately 120,000 units were pooled. Distilled water was added, with constant stirring, until the excess solid ammonium sulfate was just dissolved and the solution was saturated with this salt. The volume of the solution was about 4 liters. The pH was adjusted to 7.5, 20 gm. of Celite were added, and, after thorough stirring, the precipitate collected by vacuum filtration. The inactive filtrate was discarded. The precipitate was extracted three times with 500, 300, and finally 200 ml. of 0.01 N HCl. On each extraction the pH of the suspension was adjusted to 2.0, stirred for 10 minutes and filtered by vacuum. The Celite plus insoluble materials were discarded and the combined acid washes with a volume of about 1 liter were adjusted to pH 7.0.

From this point the purification was accomplished by methods not differing greatly from those previously described (5). The material was successively extracted into neutral *n*-butanol, reextracted into acid, extracted into *n*-butanol from an acid salt-saturated solution, chromatographed on alumina, treated to remove $Al(OH)_3$, and salted-out at pH 2.0. One additional salting-out was performed at pH 7.0.

The solution at this stage, with a volume of 35 ml. contained 75,000 units with a purity of 4350 units per mg. of N. It was adjusted to pH 10.5 and placed in the first 4 tubes of a 10 ml. phase, 200 tube Craig-Post countercurrent machine. The machine was operated for 700 transfers using the method of single withdrawal as described by Craig (12). The solvents consisted of redistilled secondary butanol and a 0.01 M $NaHCO_3$-Na_2CO_3 buffer having a pH of 10.5. At the conclusion of the distribution, assays and nitrogen determinations revealed the pattern illustrated in Fig. 1.

The active fractions were pooled, their pH adjusted to 4.0, and evaporated to a volume of 50 ml. The solution was adjusted to pH 2.0, saturated with NaCl, and filtered. The salt filtrate containing about 2,000 units was discarded. The precipitate was dissolved in 30 ml. of 0.01 HCl and filtered. The filtrate representing the finally purified hypertensin I was made to pH 6.0 and stored in the

frozen state. Upon assay and determination of nitrogen the preparation was found to contain 70,000 units with a specific activity of 7100 units per mg. of N.

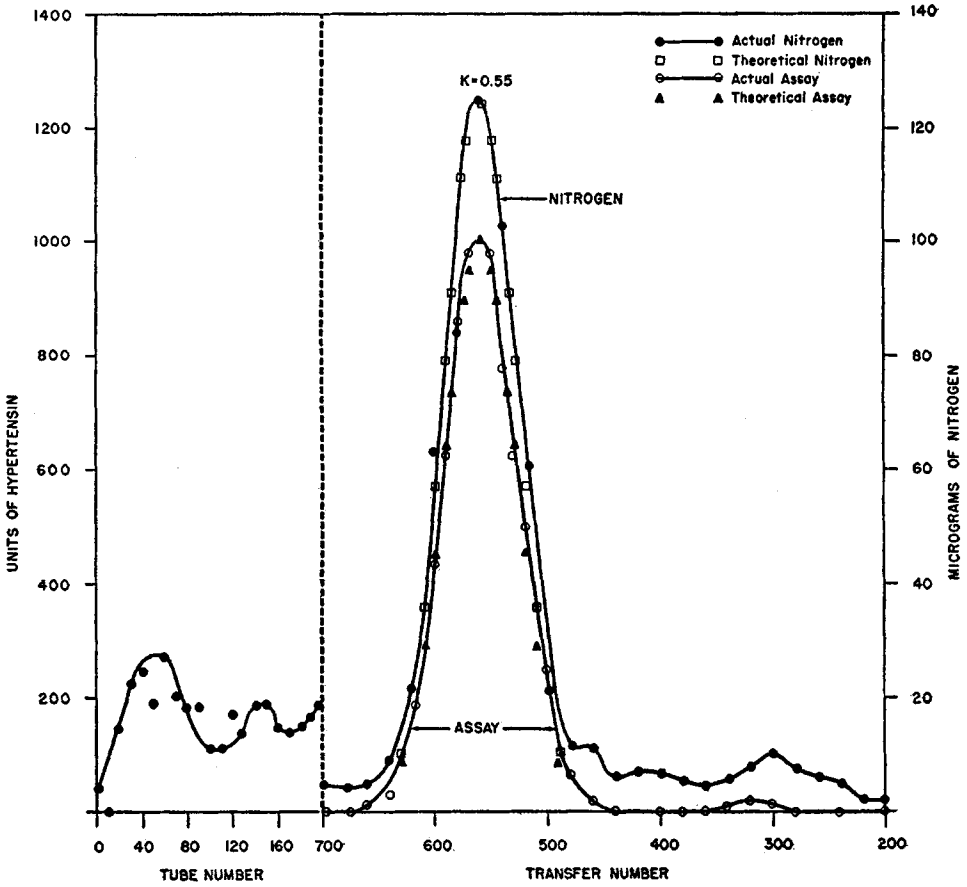

FIG. 1. Countercurrent distribution of hypertensin I.

Conversion of Hypertensin I to Hypertensin II:

The final purified products of three hypertensin I preparations, with specific activities ranging from 7100 to 8900 units per mg. of N, derived from a total of 1375 liters of horse plasma, were pooled. The resulting solution, containing 250,000 units with a specific activity of about 7700 units per mg. of N, was diluted to 250 ml. 15 ml. of 0.05 M sodium phosphate was added together with 30 ml. of 10 per cent sodium chloride and the pH carefully adjusted to 6.5. The solution was then sterilized by immersion in a boiling water bath for 15 minutes.

Lyophilized converting enzyme (4) weighing 1.5 gm. was dissolved in 0.05 M sodium phosphate, the pH adjusted to 6.5, and the solution Seitz-filtered. The final volume was 50 ml.

Preliminary incubation of this converting enzyme solution with an aliquot of the hyper-

tensin I was performed in order that the degree of conversion obtained together with the increase in ninhydrin color value might be estimated. These preliminary experiments indicated that an optimal degree of conversion would be obtained at the end of 4.5 hours (4).

Accordingly, the enzyme and hypertensin I solutions were warmed to 37°C., mixed, and incubated for this length of time. The reaction was terminated by the addition of 4 volumes of 95 per cent alcohol. 10 gm. of Celite was added, the pH adjusted to 5.2, and the mixture

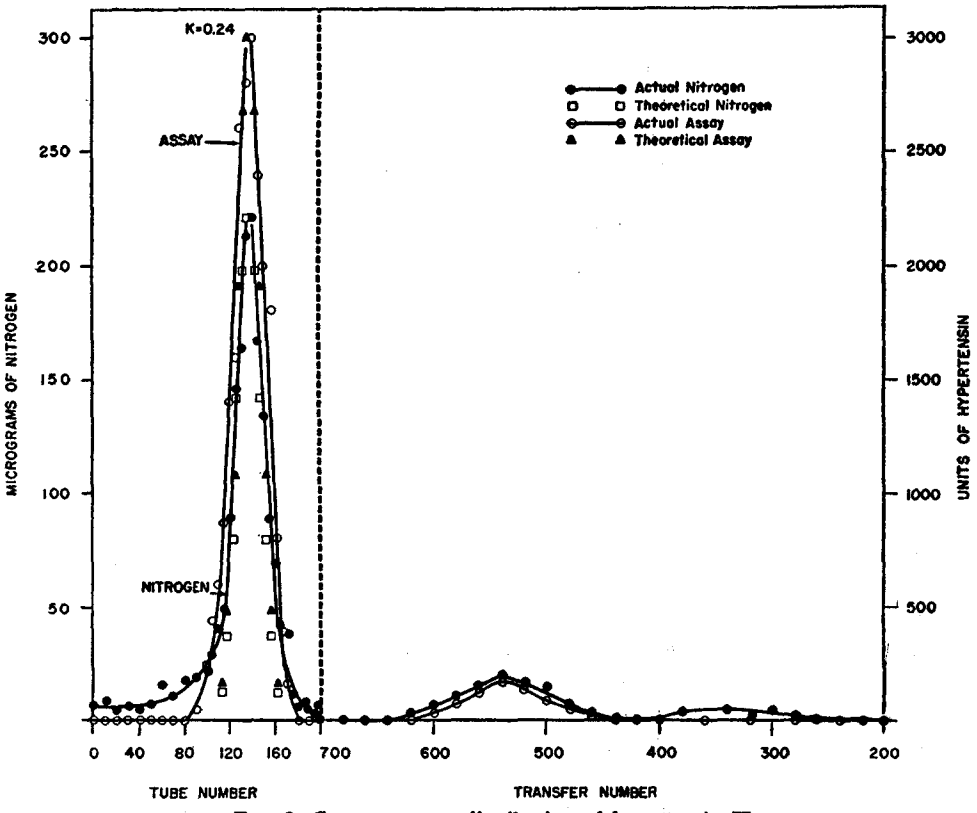

FIG. 2. Countercurrent distribution of hypertensin II.

filtered by vacuum. The filtrate was evaporated under vacuum to a volume of 150 ml., extracted twice with an equal volume of ether, and finally evaporated to 80 ml.

The solution was adjusted to pH 2.0 and saturated with sodium chloride. The precipitate was collected on a small sintered glass funnel. The filtrate together with all subsidiary fractions were saved for the possible isolation of split products. The precipitate after solution in 35 ml. of 0.01 N HCl was filtered and adjusted to a pH of 10.5. At this point it was found to contain 228,000 units with a purity of 9900 units per mg. of N.

The solution was divided into two equal parts. Each was subjected to countercurrent distribution in exactly the same manner as described under the purification of hypertensin I. The results of one of these two distributions are illustrated in Fig. 2. A major peak was found as a single component having a distribution coefficient of 0.24. The small amount of active material between transfer number 460 and 620 represents unconverted hypertensin I.

The desired fractions from both distributions were pooled, the pH adjusted to 4.5 and evaporated to 30 ml. The pH was adjusted to 2.0 and sodium chloride added to saturation. The precipitate was gathered on a sintered glass filter, dissolved in 30 ml. of 0.01 N HCl, and filtered. The filtrate was made to pH 7.5 and stored in the frozen state. This solution was found to contain 165,000 units with a specific activity of 13,700 units per mg. of N.

DISCUSSION

The improvements which have been described in the preparation of renin, of substrate, and in the preparation of crude hypertensin I are of great value in the routine large scale preparation of hypertensin. It has been a constant observation that blood containing actively growing bacteria gives low yields of hypertensin. The use of a preservative and rapid refrigeration of the blood do much to alleviate this problem. The elimination of dialysis of the renin preparation and of its substrate also reduces the opportunity for bacterial action. The technique of washing these two protein products after precipitation with ammonium sulfate is adequate for the removal of small molecular weight impurities prior to the enzymatic reaction to yield hypertensin. The presence of low percentages of ammonium sulfate in the reaction mixture, which is unavoidable in this procedure, does not inhibit the action of renin. It does however, prevent the use of nitrogen determinations in the calculation of purity until the ammonium sulfate has been eliminated.

The enzymatic conversion of hypertensin I to hypertensin II is accompanied by an increase in the ninhydrin color value (4). The reaction is believed to be of a proteolytic nature. The product hypertensin II has a specific activity of 13,700 units per mg. of N. It is therefore the most powerful pressor material known and has nearly twice the specific activity of hypertensin I. Since hypertensin II is the effective vasoconstrictor substance (4) rather than hypertensin I, the gain in specific activity is certainly due in part to a simple change in molecular weight. Its very high specific activity requires that a very large quantity of starting material be used in order to obtain a worthwhile amount of the purified substance. In the present instance 1,375 liters of horse plasma and 300 pounds of kidneys were used in the preparation of approximately 75 mg. of hypertensin II.

Evidence for the purity of the product rests upon the method of preparation in which an already purified material is converted to a new substance by an apparently simple enzymatic reaction. The enzyme preparation contributed very few impurities and was easily removed after the reaction was completed. Countercurrent distribution of the product showed the presence of a single component which was removed from the peak tubes. The evidence for the essential purity of this hypertensin II preparation, as well as of the previously purified hypertensin I, will be greatly strengthened should amino acid analysis of the present product demonstrate a reasonable chemical relationship between the two compounds.

SUMMARY

The enzymatic conversion of hypertensin I to hypertensin II is described together with the subsequent purification of the product by means of countercurrent distribution.

Improved methods are also presented for the preparation of renin and its substrate, as well as in methods for the reaction of these materials and the purification of the resulting hypertensin I.

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