THE PURIFICATION OF HYPERTENSIN I

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The pressor substance, hypertensin, has been shown to be present in the arterial blood of many patients with hypertensive cardiovascular disease (1) as well as in the blood of animals with experimental renal hypertension (2–4) and may, therefore, be concerned in causing the elevation of blood pressure in both of these conditions. In the course of attempts to purify hypertensin, it was found that under certain conditions two pressor compounds may actually be formed (5). The first, designated hypertensin I, is the initial product of the action of the renal enzyme renin upon its plasma substrate. The second, hypertensin II, is produced by the action on hypertensin I of a chloride-activated enzyme in the plasma. The relative importance of these two compounds in hypertension is not known. It, therefore, seems necessary that both materials be purified and chemically characterized. Accordingly, this paper describes the purification of hypertensin I, which appears to be the most powerful pressor material known.

Methods

Assays.—All assays were conducted on intact anesthetized rats (6). Blood pressures were recorded directly from the carotid artery using an ink-writing capillary mercury manometer. Injections were made into the jugular vein. All assay samples were compared in potency to a hypertensin solution standardized in the dog and the results accordingly were expressed in Goldblatt units (7). Highly purified preparations, when used for assay, were diluted in an alkaline saline solution prepared by dissolving 8 gm. of NaCl, 0.22 gm. of $NaCO_3$, and 0.84 gm. of $NaHCO_3$ in water and diluting to 1 liter. Use of this solution, with a pH of approximately 10, is necessary to prevent adsorption of hypertensin onto the walls of the glassware. This pH has no apparent deleterious effect on the activity of diluted hypertensin. The assay values reported are accurate to ± 10 per cent.

Determination of Nitrogen.—Samples containing 5 to 50 μg. of nitrogen were digested using 2 m. eq. of H_2SO_4 and minimal amounts of saturated $K_2S_2O_8$. These were diluted and nesslerized to give a final volume of 5 ml., and the NH_3 present was measured colorimetrically at 460 mμ. This method is accurate to ± 5 per cent.

Preparation of Renin.—Fresh hog kidneys were processed in lots of 100 pounds according to the method of Katz and Goldblatt (8), modified by Dexter (9). One additional precipitation of the active material at pH 2.0 with saturated NaCl was used (10, p. 101) in order to eliminate renal hypertensinase. This procedure has been previously described in detail (5).

Preparation of Substrate.—Fresh citrated horse plasma in lots of 100 liters was fractionated with $(NH_4)_2SO_4$, dissolved in water, and treated to destroy residual hypertensinase by treatment with acid at a pH of 3.8 for 30 minutes. Details of this preparation have been previously given (5).

The purification of hypertensin, unless otherwise stated, was carried out at room temperature. All hypertensin fractions when not being processed were stored in the deep freeze at −20°C. The adjustments of the pH of solutions were carried out using NaOH or HCl. The normality used does not appear critical. Evaporations were conducted under reduced pressure so that the temperature of the evaporating material rarely reached 40°C., and usually was 20–25°C.

EXPERIMENTAL

Preparation of Crude Hypertensin I.—

The substrate derived from 25 liters of horse plasma was dialyzed in volumes of 5 to 8 liters at pH 7.0 using an artificial kidney (11). The dialyses were conducted against cold tap water for 18 hours during which time NH_3 was completely removed. Dialysis was then continued against running distilled water for an additional 6 hours. At this time neither ammonium nor chloride ion was detectable in the dialysate which was brought into equilibrium with the substrate. The organic nitrogen content of evaporated samples of this dialysate was also negligible. At the completion of the dialysis the incubation with renin was carried out without delay. The temperature of the substrate was raised to 37.5°C. and the amount of renin derived from 25 pounds of kidneys was added. The pH of the mixture was always between the limits of 7.0–7.6 and, therefore, was not adjusted.

Incubation was continued until a maximum yield of hypertensin was obtained. This time period was determined by small scale pre-incubation experiments and maximum yields generally occurred at the end of 1 hour. At this time, the reaction was halted by the precipitation of the proteins with 4 volumes of 95 per cent alcohol. After thorough stirring, the mixture was made to a pH of 5.5, and filtered on large gravity funnels. After thorough draining, the precipitated proteins were washed with 4 volumes of 80 per cent alcohol and refiltered by gravity. The proteins were then discarded and the combined clear yellow filtrates evaporated to a volume of 1 liter. The pH was adjusted to 3.0 and the solution extracted exhaustively with diethyl ether. The fat-free material was then adjusted to pH 6.0 and further evaporated to 500 ml. and stored pending further treatment.

The material at this point was a turbid yellow solution usually containing 7500 units with a purity of about 40 units per mg. of N; a good share of the nitrogenous material is probably protein not removed in the alcoholic precipitation.

It is very important that the dialysis be most exhaustive since the chloride ion must be completely removed in order that type I instead of type II hypertensin be obtained (5). In addition, the removal of as much as possible of all dialyzable impurities from the substrate preparation makes the subsequent purification of the hypertensin considerably easier. The artificial kidney with a dialyzing area of 18,000 sq. cm. of cellophane is an efficient apparatus for the removal of these impurities.

Preliminary Purification.—At this point, the active material from 16 batches prepared as described above, derived from 400 liters of plasma and 400 pounds of kidneys, was combined. This large pool contained approximately 120,000 Goldblatt units of hypertensin activity and 3.0 gm. of N.

The pooled material with a volume of 3.5 liters was adjusted to pH 2.0 and centrifuged. The dark green precipitate containing only 3750 units with a very low purity was discarded. The active supernatant solution was adjusted to pH 7.0 and saturated with NaCl which precipitated the major portion of the active material. This was then gathered on paper by gravity filtration. The filtrate contained only 3200 units and was discarded. The active precipitate was dissolved in 1 liter of 0.01 N HCl. Insoluble material containing 2600 units at a purity of 5 units per mg. of N was centrifuged out and discarded.

Approximately 100,000 units was present at this point, with a purity of 450 units per mg. of N. The large gain in purity in these steps is probably due to the removal of proteinaceous material as well as the removal of small molecular weight materials which were not precipitated in the saturated salt solution.

Extraction into Neutral Butanol.—

The active acid supernatant solution with a volume of 1 liter was made to pH 7.0 and extracted three times with equal volumes of *n*-butanol. The majority of the hypertensin passes into butanol under these conditions. The residual aqueous layer, which contained 11,000 units at a purity of 162 units per mg. of N, was set aside for possible further processing. The bulk of the active material was then extracted from the *n*-butanol into four 750 ml. portions of .015 N HCl. The residual butanol which contained only a few units of hypertensin was then discarded.

The total number of units at this stage was 85,000 with a purity of 1300 units per mg. of N which represents a threefold increase from the previous stage. A large amount of active material failed to be extracted by neutral butanol and remained in the residual aqueous layer. Part of this may have been hypertensin II, which would not extract into butanol under these conditions.

Extraction into Acid Butanol and Chromatography on Alumina.—

The clear acid extract was evaporated to a volume of 1 liter. The pH was adjusted to 1.0 and the solution was extracted with 2 volumes of *n*-butanol over a period of 1.5 hours. During this time, NaCl was added slowly with continuous agitation until the mixture was completely saturated. At the conclusion of this period the butanol was separated and the aqueous phase re-extracted with another 2 volumes of butanol. The two butanol phases were then combined and the aqueous phase containing negligible amounts of hypertensin was discarded.

The butanol extract was then run over a chromatographic column prepared from 300 gm. of acid-treated Al_2O_3 (3) which quantitatively adsorbed the hypertensin. The column was washed with 2 liters of 85 per cent alcohol which eluted only 3500 units, which were discarded. The hypertensin was then eluted from the column by washing it with 1250 ml. of boiling water.

The aqueous eluate was adjusted to pH 7.0 and heated to 80°C. The heavy flocculation of aluminum hydroxide was removed by centrifugation, washed with hot water, and discarded. The supernatant fluids were combined and evaporated to a volume of 48 ml. During this concentration, two additional small precipitates containing aluminum hydroxide were formed. These contained about 2000 units and were removed by centrifugation and discarded.

The concentrated eluate contained 71,000 units with a purity of approximately 3300 units per mg. of N. It is important during the extraction into acid

butanol that the salting out process be accomplished gradually in order to avoid the formation of large masses of precipitate which are difficult to extract.

Salting Out in Acid Medium.—

The concentrated eluate, containing approximately 1500 units per ml., was adjusted to a pH of 2.0 and saturated with NaCl. The highly active precipitate which formed was separated on a small medium porosity sintered glass filter. The precipitate was then washed two times with 2 ml. of saturated NaCl. The filtrate, containing 1500 units and some aluminum was discarded. The active material on the filter was then dissolved and washed through the funnel with 40 ml. of distilled water.

The filtrate contained 69,600 units with a purity of 4850 units per mg. of N. In addition to increasing the purity this step removes most of the residual aluminum hydroxide.

Countercurrent Distribution.—

A 106 tube, 10 ml. phase modified Craig machine (12) was used for countercurrent distribution. The hypertensin solution was divided into two batches of 34,800 units each and two separate distributions were made. The initial solution in each case was adjusted to a pH of 10.0 and was loaded into the first three tubes. The solvents consisted of *sec*-butanol and 0.01 N $NaHCO_3$–Na_2CO_3 buffer having a pH of 10.0. The method of single withdrawal as described by Craig was used for a total of 374 transfers. This large number of transfers insured that all of the hypertensin had passed from the machine and that the maximum resolving power had been obtained. After assays and nitrogen determinations had located the active band, the appropriate fractions from the two distributions were pooled. The pooled material was adjusted to pH 3.0 and the solution evaporated to a volume of 20 ml.

The assay and nitrogen data from both distributions were practically identical. The data obtained from one of the two distributions are given in Fig. 1. Not shown in the figure are the results for the solution remaining in the machine which, though inactive, contained 1.69 mg. of N. The effluents between transfer numbers 246 and 326 were considered to contain a single homogeneous component since both the assay and the N values paralleled each other, and both very closely fit the theoretical curves for a single component. This fraction, when combined with the corresponding fraction from the second distribution and evaporated as described above, was found to contain 55,000 Goldblatt units at 7000 units per mg. of N. The activity recovered amounted to only 79 per cent of the previous figure. Had no loss of activity occurred the purity would have been over 8500 units per mg. of N. Inasmuch as the active fraction collected appears as a single component, the data suggest that active and inactive hypertensin I migrate under these conditions as a single component. Should this be true, the change from the active to the inactive compound under these conditions must entail only a small alteration in a relatively large molecule.

The nature of the inactivation in the above type of experiment is not clear.

Countercurrent distribution, in every way similar to the one described, has been carried out at 3–5°C. without significant increase of purity or yield. The

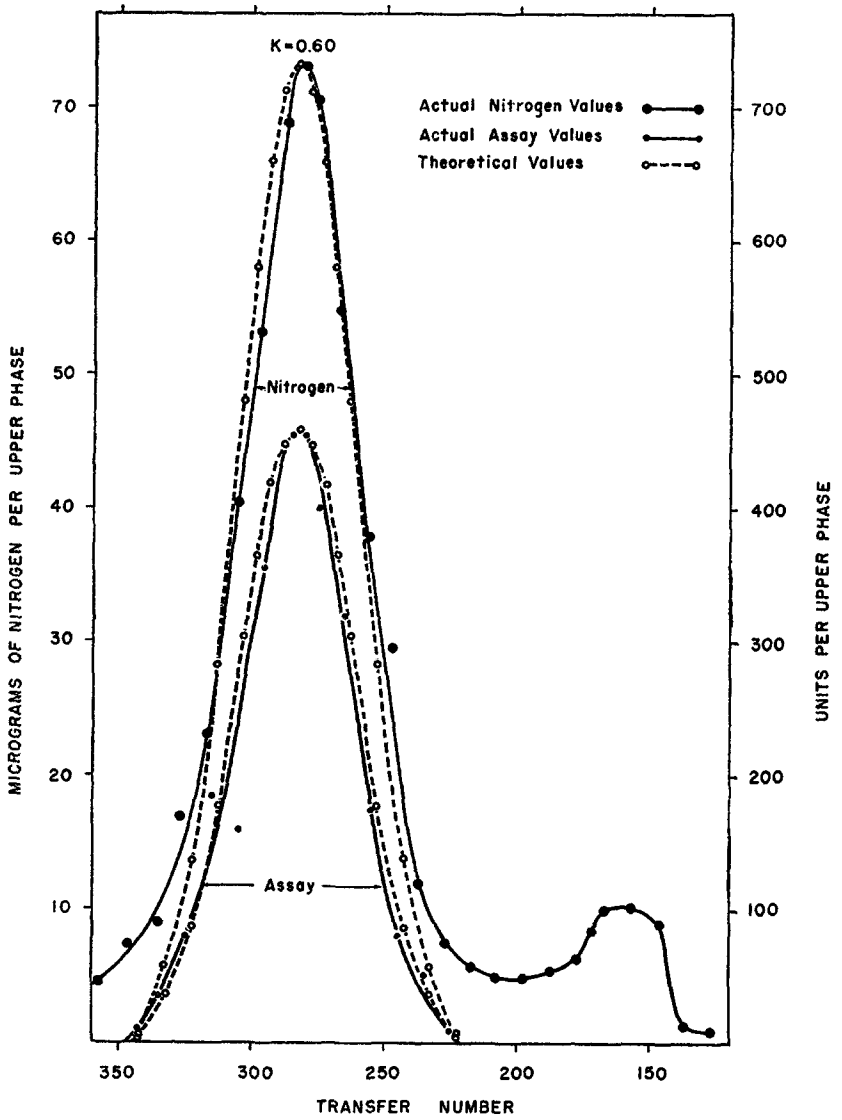

FIG. 1. Countercurrent distribution of hypertensin I.

alkalinity used could not have caused the inactivation since hypertensin I is quite stable even in 0.1 N NaOH for several hours at room temperature. Atmospheric oxygen could not have caused the inactivation since dilute solu-

tions of hypertensin are not harmed by exposure to 100 per cent oxygen for periods of many hours.

It was found necessary with high purity material to use a buffer of pH 10.0 in order to avoid adsorption onto the glass walls of the machine. Losses due to adsorption as high as 50% were encountered at pH 7.0 or less. Profound alterations in the distribution coefficient were also found when pure fractions were run at pH 7.0. It is unfortunate that at a pH of 10.0 the solubility of hypertensin is so low that the largest amount of material usable in this size machine is in the order of 25 to 50 mg.

Elimination of Buffer Salts.—

The pooled active single components from the countercurrent distributions containing 55,000 units in a volume of 20 ml. were adjusted to pH 7.0 and saturated with NaCl. The active precipitate was then separated on a previously weighed fine porosity sintered glass funnel. The precipitate was washed twice with 0.5 ml. portions of distilled water, twice with 1.0 ml. portions of absolute alcohol, and finally twice with anhydrous ether. The filtrate and washings, containing 15,600 units of good purity, were set aside for recovery of additional material. The funnel containing the hypertensin in the form of a white powder was then dried to constant weight in high vacuum over P_2O_5. After a constant weight was attained, the funnel was returned to the filter flask and the precipitate was dissolved from the plate with 100 ml. of distilled water added in small portions, giving a clear colorless filtrate. After the precipitate was redissolved, the funnel was redried to constant weight as described above. The content of sodium in the solution was determined by the flame photometer in order that a correction for the weight of the NaCl might be applied.

This solution represented the final product. The dry weight of solid material going into solution, after correcting for the presence of 0.7 mg. of NaCl, was 32.3 mg. The nitrogen content was 5.16 mg., or 15.97 per cent N. Careful assays of this hypertensin showed it to have a specific activity of 7050 units per mg. of N, or 1125 units per mg. of solid.

A total of 36,300 units was recovered in the final fraction. An additional 12,000 units was later obtained from the filtrate and washings of this fraction. The over-all recovery from 120,000 crude units was, therefore, 48,300 units or 40 per cent.

DISCUSSION

The pressor potency of hypertensin I is most remarkable. When tested in the rat (6) it was found that our preparation of hypertensin I had four times the pressor power of *l*-arterenol (Winthrop-Stearns, Inc., New York) on a weight for weight basis. Similar comparisons made in the atropinized cat anesthetized with chloralose showed it to be eight times as powerful as *l*-arterenol. When one considers that *l*-arterenol has a small molecular weight while the weight of hypertensin I is undoubtedly comparatively large, it would appear that the ratios of activity on a molar basis must be still many times greater.

An effort to purify hypertensin was made by Edman in 1945. In order to facilitate comparison of the products we have tested our material in the cat according to his technique (13). It was found that 1 mg. of hypertensin I had the pressor activity of 1400 mg. of tyramine phosphate. Edman states that 1 mg. of his material had activity equivalent to 39 mg. of tyramine phosphate. Additional evidence that the present material is considerably improved may be gained from the fact that although Edman's material gave a positive ninhydrin reaction, our material reacts with difficulty if at all with this reagent.

The purification of hypertensin has been the subject of two recent publications. One mg. of the hypertensin produced by Clark (14) was equivalent to 5.8 mg. of tyramine hydrochloride. The purity of the hypertensin (angiotonin) prepared by Bumpus (15) is not stated. In neither case do the authors state which type of hypertensin was prepared.

It has not been possible as yet to obtain further evidence as to the degree of purity of the present product other than by countercurrent distribution. Filter paper chromatography and filter paper electrophoresis were not successful due to excessive trailing, irreversible adsorption, and inactivation. Bulk column chromatography appears to fail for similar reasons. Some success has been met with in conventional electrophoresis, although the degree of spreading is such as to cast doubt on the value of the method. It seems possible that solubility studies may yield a solution to this problem.

Preliminary results with two dimensional paper chromatography of acid hydrolysates indicated the presence of nine different amino acids. These have been tentatively identified as aspartic acid, tyrosine, histidine, arginine, proline, valine, isoleucine, leucine, and phenylalanine. Three other barely detectable ninhydrin spots were found on the chromatograms which were thought to be due to the presence of very small amounts of impurities. No information has yet been obtained on the possible presence of tryptophane since it would presumably have been destroyed under the conditions of the hydrolysis. These data can only be regarded as approximate owing to the limitations of paper chromatography. It would appear probable, however, that our preparation contains less than the 13 amino acids which were reported by Bumpus to be present in his preparation.

A more critical study of the purity, molecular weight, isoelectric point, and amino acid composition of the purified hypertensin I will be the subject of another communication.

SUMMARY

The purification of hypertensin I has been described. The final product which is four times as powerful a pressor agent as *l*-arterenol, is obtained with an over-all recovery of 40 per cent. The product consists of a single component in countercurrent distribution, having a nitrogen content of 15.97 per cent and

a specific activity of 7050 Goldblatt units per mg. of N or 1125 units per mg. of solid. Acid hydrolysis and paper chromatography indicate in a preliminary fashion that there are about nine amino acids present in the intact polypeptide.

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