

THE ISOLATION AND ASSAY OF HYPERTENSIN FROM BLOOD*

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The identification of the vasopressor substance responsible for the elevation of the blood pressure in renal hypertension would be a contribution to the knowledge of the mechanism causing this phenomenon. One of the pressor substances suspected of being the chemical mediator of hypertension is the polypeptide hypertensin which is produced by the enzymatic action of renin upon its substrate, α -2-globulin.

Hypertensin has not yet been isolated in pure form although it has been obtained in a state of purity which had a pressor potency equal to that of epinephrine (1). The molecular weight of hypertensin is approximately 2700. To judge from paper chromatography it contains the following amino acids: lysine, histidine, glycine, alanine, serine, proline, valine, tyrosine, leucine (or isoleucine), aspartic acid, and glutamic acid. It has a characteristic pressor effect when injected intravenously, the maximum rise in blood pressure occurring in 1 minute or less and returning to normal in 3 minutes or less. Continuous injection produces an increase in blood pressure which is maintained during the entire period of injection. The assay of hypertensin must be accomplished by biological methods, usually by its effect upon blood pressure following intravenous injection into an intact anesthetized animal.

The identification of hypertensin in an unknown solution, although never absolute, can be made reasonably certain by using methods for the preparation of extracts which are as chemically specific as possible. Further evidence can be obtained that the pressor material is hypertensin by use of simple inactivation tests with proteolytic enzymes as well as by the demonstration of its pharmacological properties.

Although hypertensin has been isolated from the circulating arterial blood of dogs with benign experimental renal hypertension by dialysis in an artificial kidney, it was not possible to conclude from these experiments that it was the vasoconstrictor substance responsible for the elevation of blood pressure (2, 3). As a result of these studies, it was concluded that a direct method of isolating hypertensin from blood must be developed.

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This study describes a chemical method for the isolation of hypertensin directly from blood and its biological assay. Blood samples were drawn directly from the femoral artery of dogs into 95 per cent ethanol, which stopped the action of the enzymes renin and hypertensinase, and precipitated the proteins of the blood. The hypertensin in the filtrate was then concentrated and sufficiently purified for final assay on anesthetized rats.

Methods

Preparation of Extracts.—265 gm. (250 ml.) of whole blood was drawn directly from the femoral artery of unanesthetized dogs into one liter of approximately 95 per cent ethanol. The ethanol was contained in a 2 liter heavy duty Erlenmeyer flask in which there was maintained a partial vacuum. A 17-gauge needle, which was used for puncturing the femoral artery, was connected to the Erlenmeyer flask by a polyethylene catheter with an internal diameter of 0.047 inches. The flask was placed on a torsion balance, tared, and measurements of the sample accomplished by weighing. The flask was weighed and shaken intermittently during the period of collection in order to obtain a rapid and thorough mixing of the blood and ethanol. 1½ to 4 minutes was required to collect the samples of blood. (It took less than 5 seconds for the blood to pass from the femoral artery through the polyethylene tubing into the ethanol.)

After the sample was collected, the blood and ethanol mixture was shaken for 5 minutes and then filtered through No. 42 Whatman filter paper on an 18 cm. Buchner funnel. The mat of the precipitate, while still unbroken and undisturbed, was washed by drawing through it 1 liter of approximately 80 per cent ethanol (800 ml. of 95 per cent ethanol diluted to 1000 ml. with water). The precipitate was then discarded and 10 ml. of a solution containing 7 m. eq. of HCl and 2 gm. of NaCl was added to the filtrate which was then stored in the deepfreeze from 1 to 14 days.

A grayish white precipitate formed in the filtrate during its storage at -15° in the deepfreeze. The clear supernatant filtrate was decanted and the remainder was centrifuged at high speed for 10 minutes at -15° . The supernatant obtained by centrifuging was combined with that which was decanted and the precipitate discarded. The filtrate was vacuum-concentrated to 100 ml. at temperatures below 20° with the aid of 1 ml. of antifoam solution (toluene saturated with antifoam A, Dow Corning Corp.). The pH of the solution, about 2.5, was adjusted to 5.5 with 2.5 N NaOH in order to precipitate any of the proteins that remained in the filtrate. This procedure resulted in a solution which had a negative Biuret test for protein. The mixture was then centrifuged at high speed at 0 to 3° for 15 minutes and the supernatant decanted. The precipitate was washed by shaking with 50 ml. of 2 per cent NaCl and recentrifuged in a similar manner. The clear wash was then combined with the first supernatant and the pH was readjusted to 2.5 by the addition of HCl. The solution was then extracted twice in the ice box at 5 to 10° with 200 ml. of diethyl ether. 15 to 30 minutes was allowed for the separation of the phases and then the ether extracts were discarded. Extraction with ether was necessary to remove the materials which interfered with the flow of the filtrate through the alumina column.

The aqueous layer was vacuum-concentrated to 10 ml. with the aid of 0.2 ml. of antifoam solution. This acid solution, saturated with NaCl, was then extracted twice with 25 ml. of *n*-butanol. Under these conditions, hypertensin passes quantitatively into the butanol.

The aqueous layer obtained from the butanol extractions was highly depressor and had an absorption spectrum similar to that of adenylic acid.

The hypertensin was removed from the butanol by adsorption onto a column of alumina. The alumina (activated 80 to 200 mesh, Fischer Scientific Co.) was prepared in the following fashion: 600 gm. of alumina was shaken intermittently for 1 hour with 3 liters of concentrated

hydrochloric acid diluted 1:5 with distilled water. The hydrochloric acid was filtered off, the alumina was washed thoroughly with distilled water (about 30 gallons) until the wash water, when treated with silver nitrate, gave only a faint opalescence. The alumina was then air-dried for 48 hours. The column was prepared by pouring a slurry of 12 gm. of alumina and butanol into a glass tube 14 mm. in diameter. The alumina column was supported on a small wisp of cotton and a glass bead placed in a constricted portion of the tube. The tube was tapped to settle the adsorbent. After the butanol extract had passed through the alumina, the column was washed with 200 ml. of 85 per cent ethanol which removed additional depressor materials. This wash was discarded.

The hypertensin was eluted from the alumina by washing the column with 200 ml. of 50 per cent ethanol. This solution was vacuum-concentrated and then transferred to a centrifuge tube with the aid of small washes of water until the final volume was 10 ml. The pH was adjusted to 7.0-7.4 with 0.1 N NaOH using two drops of 0.01 per cent phenol red indicator. The precipitate which formed was removed by centrifuging at high speed at 1 to 3° for 15 minutes. The supernatant was transferred to a 50 ml. conical centrifuge tube and then one drop of 0.1 N HCl and caprylic alcohol was added. The solution was then evaporated to dryness in this tube under vacuum at 40°. The residue was redissolved in 1 ml. of distilled water and the color of the indicator was again made to a faint pink by using minute amounts of 0.1 N NaOH. The tube was capped and centrifuged at a low speed for 5 minutes. The supernatant fluid was transferred to a small test tube which was stoppered and placed in the deepfreeze until assayed.

Recovery Experiment.—The chemical method was tested for its ability to recover hypertensin from blood by processing and assaying samples prepared in the following manner: 250 ml. of blood was collected from each of 8 normal dogs by direct puncture of the femoral artery. The blood was heparinized, pooled, and then redistributed in samples of 250 ml. 0.2 unit of hypertensin was added to 6 of the samples. No hypertensin was added to the other 2 samples in order to determine the amount of pressor material present in 250 ml. of arterial blood collected in this fashion. The hypertensin was usually added to the liter of ethanol just prior to the addition of the 250 ml. sample of blood. It was found, however, that the addition of hypertensin directly to the blood 30 seconds prior to its mixture with the ethanol did not substantially alter the amount of hypertensin recovered.

To determine the per cent of recovery in all of the experiments, two control samples to which hypertensin had been added were processed in parallel with groups of 6 experimental samples drawn from normal or hypertensive dogs.

Inactivation Experiments.—The effect of trypsin on the pressor activity of the extracts was determined by incubating approximately 1 mg. of the dialyzed enzyme (Armours crystallized) with 1.0 ml. of the extract for 2 hours at 39°. Each extract was made 0.1 M with respect to sodium phosphate buffer pH 7.4. Merthiolate at a concentration of 1 to 25,000 was used as a preservative. The enzyme was inactivated and removed from the solution after incubation and before assay, by heating to 100° for 10 minutes followed by centrifugation. Each test was accompanied by its own control which consisted of the incubation of the extract in similar fashion with previously heat-inactivated trypsin.

Assay Methods.—The pressor effect of the preparations was assayed by intravenous injection into rats and compared to that produced by an injection of a standardized hypertensin solution. All assays are expressed in terms of Goldblatt units. A detailed description of the assay methods has been previously published (3).

EXPERIMENTAL

A pool of extracts representing 23 different preparations purified as above was found on analysis to contain 0.22 m. eq. of Na, 0.0011 m. eq. of K, 2.71 mg. of total N₂, and 28.5 mg. of total solids as computed for a single extract of 250

ml. of blood. The extraneous material present did not have a depressor effect upon the blood pressure of the test animal.

An experiment designed to test the ability of the chemical method to recover hypertensin added to individual samples of blood drawn from a single pool of normal dog blood is shown in Table I. Five such experiments have been conducted. The recovery of added hypertensin in all five experiments, which consisted of 39 individual samples, was between 40 and 65 per cent with an over-all average of 50 per cent.

A preliminary experiment for the purpose of testing the method for its ability to isolate hypertensin from the circulating blood of dogs is shown in Table II. The samples that were drawn from the dogs of this group prior to constriction of their renal arteries had pressor activity equivalent to 0.02 to 0.05 units of hypertensin per liter of blood. After removal of the right kidney and marked

TABLE I
Recovery of Hypertensin Added to Blood

Sample No.	Hypertensin added	Hypertensin found	Recovery of hypertensin	
	<i>units</i>	<i>units</i>	<i>units</i>	<i>per cent</i>
17 A	0	0.02	—	—
17 B	0.2	0.13	0.11	55
17 C	0.2	0.13	0.11	55
17 D	0.2	0.13	0.11	55
17 E	0.2	0.12	0.10	50
17 F	0.2	0.12	0.10	50
17 G	0.2	0.14	0.12	60
17 H	0	0.01	—	—

constriction of the left renal artery by a Goldblatt clamp (4), hypertension and nitrogen retention developed in all animals. Samples of blood were drawn 48 and 72 hours after application of the clamps. The concentrations of hypertensin found in 10 dogs ranged between 0.10 and 1.6 units per liter of blood. All animals surviving 72 hours were sacrificed. The presence of the malignant phase of experimental renal hypertension was confirmed either by the occurrence of multiple hemorrhages in the gastrointestinal tract and other organs or by the microscopic observation of necrotizing arteriolitis.

The pressor activity of 8 final extracts from dogs with acute hypertension was tested for its susceptibility to destruction by trypsin. In all cases the pressor activity of the extracts was completely destroyed. The control tubes which contained the inactivated trypsin were strongly pressor.

15 samples of blood from 9 dogs which had been bilaterally nephrectomized for 48 to 72 hours were processed and tested for the presence of pressor activity. 10 samples contained no pressor material, while 6 contained barely detectable

amounts which were insufficient to permit identification. The maximum concentration of pressor activity was equivalent to 0.016 units of hypertensin per liter of blood. The contour of the pressor response obtained in the rat was indefinite and not comparable to that obtained by the injection of similar amounts of hypertensin.

An additional experiment designed to estimate the degree of specificity of the method for hypertensin was performed. Representative pressor materials were added to separate duplicate samples drawn from a pool of normal dog blood. The amounts added were in each case sufficient to yield a minimum of 20 easily significant pressor doses in the rat. The following substances were

TABLE II
Assay of Hypertensin from the Blood of Dogs before and after Constriction of the Renal Artery with Development of Malignant Hypertension

Dog No.	Before constriction	48 hrs. after constriction			72 hrs. after constriction		
	Hypertensin found	Blood urea nitrogen	Mean femoral blood pressure	Hypertensin found	Blood urea nitrogen	Mean femoral blood pressure	Hypertensin found
	<i>units per liter</i>	<i>mg. per 100 ml.</i>	<i>mm. Hg</i>	<i>units per liter</i>	<i>mg. per 100 ml.</i>	<i>mm. Hg</i>	<i>units per liter</i>
43	0.03	75	185	0.10	104	190	0.10
67	0.04	190	190	1.2	Dead	Dead	Dead
41	0.02	96	165	0.20	207	155	0.25
55	0.05	150	160	0.17	146	175	1.0
62	0.02	146	175	0.12	Dead	Dead	Dead
76	No sample	No sample	185	0.20	218	165	1.6
80	0.05	104	155	0.28	159	175	0.36
82	0.02	93	185	0.24	153	190	0.28
83	0.04	86	165	0.29	141	155	1.0
89	0.02	119	160	0.10	150	.70	0.21

used: 0.1 pressor unit of pitressin, 2.5 mg. of ACTH (Armour and Co.), 1.0 μ g of arterenol, 0.1 mg. tyramine hydrochloride, and 1.0 μ g. of epinephrine. In no case did these substances add additional significant pressor activity to the final extracts.

DISCUSSION

The blood samples are drawn as rapidly as possible from the dog into ethanol in order to avoid the possible error that could occur due to the production of hypertension by the extravascular action of renin on its substrate or by the destruction of the hypertensin by hypertensinase.

The chemical method described represents a compromise between an effort to obtain both a high recovery and an adequate purity of hypertensin. A re-

covery rate as high as 80 per cent can be obtained by eluting the adsorbed hypertensin from the alumina column by boiling water; however, in addition to the hypertensin, a depressor substance is also recovered which interferes with the final assay in the rat.

The pressor material obtained from dogs with malignant hypertension was identified as hypertensin by the following means. The material was completely destroyed by trypsin, eliminating all known pressor materials with the exception of pitressin and ACTH. These two possibilities were excluded by examination of the contour of the pressor response obtained as well as by failure to recover these substances by the method used when reasonable amounts were added to blood samples.

There was not sufficient pressor activity in the extracts of blood of normal dogs to permit inactivation experiments with trypsin. For this reason, samples of blood from bilaterally nephrectomized dogs were prepared for assay. The lack of pressor activity in these preparations would indicate that the material obtained from normal dogs was of renal origin.

It must be emphasized that the pressor activity of the blood extracts of both normal dogs and those with malignant hypertension may have been increased by the production of hypertensin by circulating renin released from the animals' kidneys as a result of withdrawing 250 ml. of blood in 1½ to 4 minutes. Experiments are now in progress in which attempts are being made to obtain blood samples from dogs under more physiological conditions.

SUMMARY

A method has been described for isolation and assay of hypertensin from the blood of dogs. An ethanol filtrate of blood drawn from the femoral artery is prepared and concentrated by evaporation. The hypertensin is extracted into *n*-butanol from which it is adsorbed into alumina, and subsequently eluted with dilute ethanol. The eluate is then evaporated to dryness, dissolved in water, and assayed by intravenous injection into rats.

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