The Isolation of Hypertensin from the Circulating Blood of Dogs by Dialysis in an Artificial Kidney

By Joseph R. Kahn, M.D., Leonard T. Skeggs, Ph.D., and Norman P. Shumway, M.D.

A vasopressor substance has been dialyzed out of the circulating blood of dogs by means of an artificial kidney. This substance is similar to hypertensin. It has been recovered from normotensive and hypertensive dogs and from animals with malignant hypertension. The method for concentration of this substance in the dialyzate is described and concentration data which, due to the loss in recovery, is only roughly quantitative, is given.

The present view of the mode of development of experimental renal hypertension is that a chemical mechanism causes the elevation of the blood pressure. The components of this mechanism are the renal enzyme, renin, its plasma substrate, hypertensinogen, and the vasopressor substance, hypertensin, resulting from the interaction of renin and hypertensinogen. It is of paramount importance for the acceptance of the renin-hypertensin theory that the vasopressor substance, hypertensin, be present in the circulating blood and be recoverable from it in sufficient amounts to account for the elevation of blood pressure in animals with experimental renal hypertension. The presence of renin in the circulating blood has been demonstrated by indirect means but the presence of hypertensin in the circulating renal venous or systemic blood of man and experimental animals has not been demonstrated beyond question. It has been presumed that the failure to recover hypertensin was due to its destruction by the enzyme, hypertensinase, or the immediate removal of hypertensin from the blood by its combination with smooth muscle in the walls of the arterioles. The other possibility is that hypertensin is an artefact, formed only

in vitro by the action of renin on hypertensinogen. Hypertensin is a polypeptide of low molecular weight, about 2700, and slowly dialyzable through a cellophane membrane. We found that it took one to two hours, in aqueous solution, to reach equilibrium across the membrane in the artificial kidney. It occurred to us that, if hypertensin be present in the circulating blood of normal or hypertensive animals, it might be separated from it by subjecting the blood to dialysis in a suitable artificial kidney. For this purpose, we used the artificial kidney described by one of us.

Experiments

Dogs were used in all of the experiments. They received morphine sulfate, 7.5 to 15 mg., and atropine sulfate, 0.5 mg., before the beginning of the experiment. The artificial kidney was connected to the femoral artery and femoral vein by means of rubber tubing.

The pressure in the femoral artery produces a rapid flow of blood through the kidney making it unnecessary to introduce a mechanical pump into the blood side of the system.

The circulating blood of the animals was dialyzed for three or four ninety-minute periods against 500 ml. of circulating dialyzate. The composition of the dialyzing solution was Na⁺ 148, Ca⁺⁺ 5, K⁺ 3, Mg⁺⁺ 3, Cl⁻ 126, HCO₃⁻ 24, HPO₄⁻² 2, and lactate⁻ 7 mEq./liter plus 100 mg. of glucose/100 ml. The pH of the solution was 7.35 to 7.45. At the end of each period the dialyzate was collected, measured and placed in round bottom flasks in which it was frozen by rotation of the flasks in a

From the Department of Medicine and Surgery, Veterans Administration Hospital, and the Institute of Pathology and Department of Medicine, Western Reserve University, Cleveland, Ohio.

Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Circulation, Volume II, September, 1950

363
mixture of dry ice and acetone. All of the dogs were heparinized. At the beginning of the experiment, they were given 4 mg./Kg. of heparin, intravenously, and throughout the experiment, they were given 0.5 mg./Kg./hour. The blood flow through the kidney was regulated by a screw clamp on the rubber tubing which returned the blood from the kidney to the femoral vein. Only in the hypertensive dogs was the blood flow of such magnitude that it had to be reduced by constriction of the lumen of the rubber tubing. The blood flow through the kidney ranged between 100 to 200 ml./minute. Four units of the kidney were used. The dialyzing surface was 6,800 sq. cm., and the capacity was 200 ml. of blood and 200 ml. of dialyzate. Number 300 mm. moisture proof DuPont cellophane was used as the dialyzing membrane.

There was a marked fall in the blood pressure of two of the animals after they had been attached to the artificial kidney for ten minutes. The blood pressure gradually rose again until it reached normal in one or two hours. It has been found that the fall in blood pressure was due to a toxic material which was present in the cellophane and which could be removed by boiling the cellophane in distilled water for twenty-four hours. Subsequent to this procedure no further hypotensive periods were observed in the dogs. Hypertensin was recovered from the dialyzates of these two animals which sustained a fall of blood pressure. Of what importance the recovery of hypertensin is in these two animals cannot be stated, since hypertensin was also present in the dialyzates of three normal animals in which there was no appreciable fall of blood pressure.

After the kidney was assembled, it was sterilized by passing steam through both the blood and dialyzate sides of the apparatus for thirty minutes and then was washed with five liters of sterile pyrogen-free saline.

The dialyzate, that is the solution against which the blood was dialyzed, was pumped through the kidney by means of an electrically driven rotary pump at a rate of 200 cc./minute and at a negative pressure of 10 mm./Hg. The dialyzate flowed through a copper coil placed in a water bath which was maintained at 39 C. This maintained the temperature of the blood circulating through the kidney at 38.5 C. A thermometer in a T tube was inserted into the rubber tubing connecting the kidney with the femoral vein. In this manner, changes in the temperature of the blood could be regulated and noted throughout the experiment. A needle attached to a mercury manometer was inserted into the rubber tubing connecting the femoral artery with the kidney, so that the approximate mean blood pressure of the animal could be observed throughout the experiment. (See figs. 1 and 2.)

Before the experiment was started, 400 ml. of blood was collected by arterial puncture from a donor dog into a flask containing 40 mg. of heparin and 20 cc. of normal saline. This blood was used to fill the artificial kidney and the rubber tubing connecting it to the animal. The remaining 200 ml. of blood was given by a slow intravenous drip throughout the early period of the dialysis. The pressure of the blood in the kidney was then raised by addition of blood to it with a syringe until
it was equal to the arterial pressure of the dog whose blood was being dialyzed. By this procedure, there was no loss of blood or fall of blood pressure when the dialysis was started.

Normal donor dogs were used in three experiments. This procedure had to be abandoned because of the possible presence of renin or hypertensin in the blood of these animals. As a matter of fact, hypertensin was demonstrated in the dialyzate of all three of the animals which received blood from normal donor dogs. In order to be certain that the hypertensin, which was recovered in the dialyzate, came from the animals subjected to dialysis, bilaterally nephrectomized donor dogs were used. The animals were nephrectomized from 18–36 hours before they were bled.

All of the animals lost water during dialysis. The average loss was 100 ml. during the first ninety-minute period and 40–60 ml. during each subsequent one. Animals with hypertension may lose as much as 200 ml. of water during each period of dialysis. There is a filtration pressure across the cellophane membrane of 180 mm./Hg in an animal with a mean blood pressure of 170 mm./Hg; the artificial kidney, therefore, acts by ultrafiltration as well as by dialysis. In order to avoid dehydration, the animals must be given a constant intravenous infusion of dialyzate equal to the volume of water lost during each period of attachment to the artificial kidney.

At the conclusion of each experiment 150 mg. of protamine in 100 ml. of saline was administered intravenously to the animals to bring the clotting time back to normal.

**Preparation of the Dialyzate**

The dialyzates from the individual dialyzing periods, each approximately 600 ml., are shell frozen immediately in three-liter round bottom flasks in a dry ice-acetone bath. These are lyophilized, tightly stoppered and stored in the cold until further processing is possible. The dialyzate from each period is worked up as an individual preparation.

The dialyzate is reconstituted in concentrated form in the original flask by the addition to the dried cake of 5 ml. of 0.5 normal hydrochloric acid for each 100 ml. of original dialyzate. The flask is rotated until solution is complete, and the carbon dioxide gas is eliminated. The pH is adjusted to approximately 6.0, using small amounts of 2.5 normal sodium hydroxide and bromthymol blue as an external indicator. A thick yellow syrup is obtained at this stage, and it usually contains 80–85 mEq. of sodium. Nine volumes of anhydrous ethyl alcohol,
on its axis at 120 rpm in a large horizontal glass cylinder partially stoppered and containing 10 ml. of distilled water. In this manner, these relatively small volumes of dialyzate are exposed to a constantly moving dialyzing membrane with an area of 65 sq. cm. Dialysis is continued for ten to twelve successive five-minute periods, the dialyzing water being removed and replaced with 10 cc. of distilled water at the end of each period until the total sodium within the cellophane bag is decreased to less than 0.15 mEq. The material is then removed from the bag and stored as fraction I. The dialyzing water is combined and evaporated under vacuum (at less than 30 C.) to a volume of 5-6 ml., placed in the same cellophane bag and subjected to the same dialyzing procedure. The dialyzing water in this case is discarded, and the material remaining in the bag, fraction II, is combined with fraction I. (The loss of hypertensin during these dialyses is 50-60 per cent.) One-half volume of ethyl ether, C.P., is then added to the solution which is shaken thoroughly and centrifuged. The ether layer is discarded together with the insoluble material formed at the ether-water interface. The clear water layer is de-etherized and evaporated to a small volume under vacuum. The pH is adjusted to 7.3±0.2 using sodium hydroxide, and the final volume is adjusted, so that each ml. represents 250 ml. of the original dialyzate. The preparation is frozen until it is assayed. The final product is clear, yellow and has a sodium concentration between 100 and 200 mEq. per liter, a potassium concentration of 5 to 15 mEq. per liter. The overall recovery of hypertensin, as estimated by adding it to one of two identical aliquots of the original dialyzate, is between 10 and 30 per cent. Performing all of the procedures in the cold does not improve the yield of hypertensin nor do small changes in the pH values appreciably change it.

Assays of the dialyzates are performed on mature rats anesthetized with Nembutal, 5 mg. per 100 Gm. of body weight. Both vagus nerves are cut and the trachea is cannulated. A needle is inserted and tied into the left jugular vein; this is used for intravenous injections of the material to be assayed. The right carotid artery is cannulated and connected with a calibrated double rubber membrane manometer (Phipps and Bird) equipped with a pen for ink writing on a kymograph.

Rats are used in this experiment since 0.01 of a unit of hypertensin (Goldblatt) produces an appreciable rise in blood pressure, the average rise in the arterial pressure from this dose is 25 mm./Hg. The average normal blood pressure of the anesthetized rat with the vagi sectioned is between 100 and 175 mm./Hg.

All of the tests on the dialyzates are done with small volumes ranging between 0.25 and 0.50 ml. The rises in the rats' blood pressure are compared with those obtained by the injection of 0.01 unit of hypertensin prepared from standardized lyophilized powder. One unit of this standard gave an average rise in blood pressure of 32 mm./Hg in 12 nembutalized rabbits and an average 30 mm. rise in seven unanesthetized dogs. This unit is approximately equal to the Goldblatt unit.

Eleven of the dialyzates which had pressor effect were tested for their susceptibility to destruction by one or several of the following substances: hypertensinase, trypsin, acid (pH 1.0 at 100 C. for ten minutes) and alkali (pH 12.0 at 100 C. for ten minutes).

The hypertensinase used in these experiments is prepared by hemolyzing one volume of freshly drawn rat blood by adding it to eight volumes of water. One volume of 10 per cent sodium chloride and one volume of 1:1000 solution of Merthiolate are then added. The pH of this solution is adjusted to 7.4±0.1, centrifuged and 0.5 volume of the clear supernatant fluid is added to a suitable aliquot of the active preparation. The mixture is incubated for two hours at 37.5 C. Following incubation, the solution is kept frozen until it is assayed.

A solution of trypsin is prepared by dissolving a small amount of Armour's crystalline trypsin in water. It is then dialyzed to eliminate MgSO₄ and a sufficient amount of 1:1000 solution of Merthiolate is added to give a final concentration of 1:5000. The pH of the solution is adjusted to 7.4. This solution of the enzyme is prepared so that it contains 1.03 to 1.50 mg. of nitrogen per ml. as determined by the
micro Kjeldahl method. One-fifth volume of this enzyme preparation is added to a suitable aliquot of the active dialyzate, and the mixture is incubated for two hours at 37.5 °C. At the end of the period of incubation, the mixture is placed in a boiling water bath for ten minutes. After cooling and centrifuging, the clear supernatant fluid is withdrawn and frozen until it is ready to be assayed.

Several control tests are performed using heat inactivated trypsin and hypertensinase.

The susceptibility of the pressor substance in the dialyzate to destruction by alkali or acid is tested by changing the pH of suitable aliquots to 1.0 or 12.0. These solutions are heated in boiling water for ten minutes, cooled, and the pH readjusted to 7.3±0.2.

**Results**

*Group I:* In 9 normal dogs the circulating blood was subjected to dialysis for 3 or 4 ninety-minute periods. Blood from bilaterally nephrectomized donor dogs was used to fill the artificial kidney. Hypertensin was recovered in the dialyzates of only two animals. The amount of hypertensin recovered was 0.04 unit per liter of dialyzate from each animal. No hypertensin was detected in the dialyzate of the remaining 7 animals.

*Group II:* Two normal dogs were subjected to dialysis for three ninety-minute periods. Blood from normal donor dogs was used to fill the artificial kidney. Hypertensin was recovered in the dialyzate from both of these animals. The amount of hypertensin recovered was 0.05 and 0.12 unit per liter of dialyzate.

*Group III:* Two normal dogs in which there was a marked fall in blood pressure during the experiment were subjected to dialysis for three ninety-minute periods. Blood from bilaterally nephrectomized donor dogs was used to fill the kidney. Hypertensin was recovered from the dialyzates of both of the animals. The amounts of hypertensin were 0.05 and 0.4 unit per liter of dialyzate.

*Group IV:* Nine normal dogs were given multiple intravenous injections of widely varying amounts of renin and subjected to dialysis for ninety minutes. Hypertensin was recovered in the dialyzates of only three of these animals. The amount of hypertensin recovered was 0.1, 0.15, and 0.7 unit per liter of dialyzate.
Group V: Two bilaterally nephrectomized dogs were subjected to dialysis for three ninety-minute periods. Blood from bilaterally nephrectomized donor dogs was used to fill the artificial kidney. No hypertensin was recovered in the dialyzates from either of the animals.

Group VI: Four dogs with malignant hypertension, produced by the Goldblatt method, were subjected to a dialysis for three ninety-minute periods. Blood from bilaterally nephrectomized donor dogs was used to fill the artificial kidney. Hypertensin was recovered in the dialyzate of all four animals. The amount of hypertensin recovered was 0.4, 0.07, 0.5, and 0.04 unit per liter of dialyzate.

Discussion

By the use of an artificial kidney, a pressor substance has been dialyzed out of the circulating blood of intact animals which conforms to the usual tests for hypertensin: intravenous injections of 0.25 to 0.50 ml. produces an immediate steep rise in blood pressure, the maximum rise occurs in one minute or less, and the return to normal in three minutes or less. Repeated injections of the same magnitude do not produce tachyphylaxis. The pressor effect of intravenous injections of the dialyzate was unaffected by previous injections of cocaine, atropine or 933F but was potentiated by a previous injection of tetraethylammonium chloride. By the methods that were used in the preparation of the dialyze, the pressor material has been found to be water and alcohol soluble, ether insoluble, and slowly dialyzable.

In the dialyzates in which there was a sufficient amount of the pressor material, this substance was found to be destroyed by trypsin, hypertensinase, boiling at pH 12 but not at pH 1 (fig. 3). Eleven such preparations were tested by one or more of the above methods. In all cases the pressor effect was destroyed.

Summary

A pressor substance has been dialyzed out of the blood of intact dogs by means of an artificial kidney. This substance conforms to all the usual tests described by Goldblatt and Edman for the identification of hypertensin.

References


The Isolation of Hypertensin from the Circulating Blood of Dogs by Dialysis in an Artificial Kidney

JOSEPH R. KAHN, LEONARD T. SKEGGS and NORMAN P. SHUMWAY

Circulation. 1950;2:363-368
doi: 10.1161/01.CIR.2.3.363

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/2/3/363

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/