STUDIES ON PLANT HYPERTENSINASE*

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From the kidneys and other organs of normal animals, an enzyme, hypertensinase, has been extracted (1). This enzyme has the ability to destroy the pressor substance, hypertensin (2), probably a polypeptide, which results from the action of the enzyme on the substrate hypertensinogen, a globulin fraction in the blood plasma. Intestinal mucosa and kidney are the richest animal sources of this enzyme which is also present in practically all other animal tissues (3). An enzyme, or group of enzymes, capable of inactivating hypertensin, has also been extracted from plants, for example mushrooms (4) and yeast (5).

The present communication deals with an attempt to obtain a potent, nontoxic preparation of hypertensinase, the study of which might help to clarify the part played by this enzyme in the humoral mechanism of experimental renal hypertension.

Methods

During the search for a rich source of hypertensinase, intestinal mucosa, kidney, and plants of various kinds were extracted and compared and it was found that many plants are a much richer source than animal tissues of enzyme activity like that of hypertensinase. Aqueous extracts of jackbean meal, brewer's yeast, wheat, corn, barley, oatmeal, garlic, onion, parsley, navy and lima beans, potatoes, lentils, soya beans, and peas contained variable amounts of hypertensinase but were toxic when injected intravenously into animals. Jackbean meal, for instance, proved to be a highly potent source of hypertensinase, but it was not possible to separate the hypertensinase from the urease, either by precipitation with neutral salts, acetone, or alcohol, by changes of pH, or by inactivation of the urease by oxidation. Crystalline urease did not show any hypertensinase activity.

During the course of these experiments it was found that the bran portion of the cereal grains contained the major part of the hypertensinase activity and that extract of wheat bran yielded a potent enzyme solution which was relatively less toxic than the other plant extracts studied. Purification and con-

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centration of the wheat bran extract were therefore undertaken and the results of this study are given below.

Method of Extraction and Purification of Hypertensinase Obtained from Wheat Bran

Extraction.—25 pounds of finely ground wheat bran was thoroughly mixed with 25 pounds of washed fine sand. The mixture was moistened with distilled water and thoroughly kneaded. Care was taken to break up all lumps and still maintain the material in a moist state, so that it could be poured. It was allowed to stand for 24 hours at 3–4°C.; then the mixture was poured into a large metal percolator (6) filled with cold distilled water. The next day the mixture was allowed to percolate and the fluid was filtered through sand and cotton at the bottom of the percolator at a rate of 10 drops (about 0.05 cc.) per minute. The daily yield was kept in frozen state at −35°C. Of this dark brown extract, 1 cc. usually contained about 100 units of hypertensinase, 1 unit being the amount capable of completely inactivating one unit of hypertensin.

Purification.—During the entire process of purification the wheat bran proteins were maintained at a high concentration and the reagents were added slowly by capillary pipette. All procedures were carried out continuously at 1°C. and the conditions of pH and ionic strength indicated below were strictly controlled.

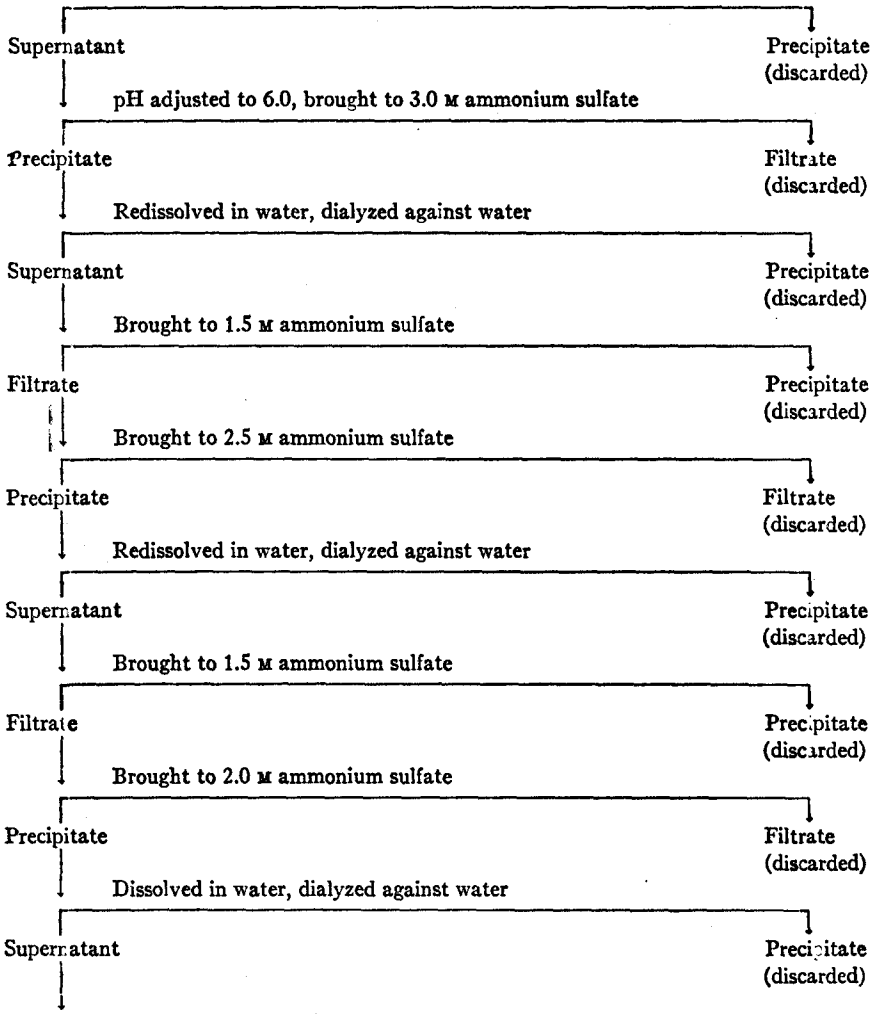
Five liters of the original wheat bran extract, at pH 6.2, was diluted with an equal volume of distilled water. The pH was adjusted to 4.1 by dropwise addition of 1 N HCl solution through a capillary pipette, while the mixture was vigorously stirred. The resulting suspension was allowed to settle at 0°C., overnight, after which the major portion of the clear supernatant fluid was syphoned off. The remaining portion of the mixture was centrifugalized at 0°C., the precipitate discarded, and the supernatant fluid combined with the first portion. This fluid was then adjusted to pH 6.0 by the addition of 1 N NaOH, again during vigorous stirring. To this solution was then added sufficient solid ammonium sulfate to produce a molarity of 3.0. The resulting precipitate was filtered in the cold, on large Büchner funnels, and the filtrate discarded. The precipitate was pressed, dissolved in 500 ml. of distilled water, and the resulting solution was enclosed in cellophane tubing and dialyzed in the cold against repeated changes of distilled water until free of sulfate ion. The precipitate which formed was separated by centrifugalization and discarded, and the supernatant fluid was further fractionated as follows: 4 M solution of ammonium sulfate was added gradually through a capillary pipette to bring the fluid to 1.5 M. The precipitate which formed was collected by filtration on a Büchner funnel, and discarded. The clear filtrate was then brought to 2.5 M by the addition of sufficient 4 M ammonium sulfate. The resulting precipitate was also collected, by filtration, on a Büchner funnel and the filtrate discarded. The precipitate, first pressed, then dissolved in 100 ml. of distilled water, was dialyzed against distilled water until free of sulfate. The dialysis was continued for 2 days. The euglobulin precipitate which formed was removed by centrifugalization and discarded. The clear supernatant fluid was fractionated a second time, as follows:—

The volume of 4 M ammonium sulfate required to bring the fluid to 1.5 M with respect to ammonium sulfate was calculated. The 4 M ammonium sulfate solution was then enclosed in a cellophane membrane and permitted to dialyze, with rotation, against the fluid, until equilibrium was reached. This took 48 hours. The resulting precipitate was collected on a Büchner funnel and discarded. The clear filtrate was then brought up to 2.0 M by equilibrium with the requisite amount of 4 M ammonium sulfate enclosed in a cellophane tube. Again a precipitate formed, and this was collected on a Büchner funnel, and the filtrate discarded. The precipitate was pressed, washed once with 2.0 M ammonium sulfate, dissolved

TABLE I

Flow Diagram Representing the Purification of Wheat Bran Hypertensinase

Wheat bran extract, + equal volume distilled water, mixture brought to pH 4.1 by addition of 1 N HCl.



in 100 ml. of distilled water, and dialyzed free of sulfate ion against repeated changes of distilled water, in the cold. The small amount of precipitate formed in the dialysis was removed by centrifugalization and discarded. The supernatant fluid had only 24 per cent of the hypertensinase activity of the original wheat bran extract, but, on the basis of nitrogen content, was 15 times purer than the original extract.

All fractions were neutralized and rendered isotonic before they were tested for hypertensinase activity, as described below.

Table I presents a flow diagram of the steps used in the purification of the hypertensinase.

Hypertensinase Assay

Dilutions of the original preparation and of all fractions during the process of purification to be tested for hypertensinase activity were mixed with 1 cc. of physiological phosphate buffer at pH 7.8. To these mixtures 1 dog unit of hypertensin was added and the test tubes were incubated for 1 hour at 40°C. in a water bath. The reaction was stopped by placing the test tubes in an ice bath at 0°C. A dog unit of hypertensin is the amount necessary to raise the direct mean blood pressure of an unanesthetized trained dog 30 mm. Hg. The maximum rise from a dose of this size is reached in about 1 minute and the return to normal occurs in another minute or two. The samples were warmed to body temperature before injection into unanesthetized trained dogs. In these dogs, blood pressure determinations were made by the direct method, a 20 gauge needle, attached to a mercury manometer, inserted into a femoral artery. The quantity of undiluted fluid with hypertensinase activity which completely destroyed the vasoconstrictor effect of 1 dog unit of hypertensin at 40°C. for 1 hour was considered to contain 1 unit of hypertensinase. Rabbits under the influence of nembutal, injected intravenously, also proved to be reliable test animals when 1 dog unit of hypertensin was used as the control test. In these animals the blood pressure was determined by means of a cannula, attached to a mercury manometer, inserted into a carotid artery.

Properties of Plant Hypertensinase

This enzyme is a non-dialyzable pseudoglobulin. It is destroyed at 56°C., or over, and remains active in a pH range of 3.9 to 9.5, at body temperature. Its optimum activity is at 40°C., and it is not appreciably active at 0°C. It is precipitable by neutral salts, but it is denatured by organic solvents, at room temperature, and at 0°C.

Plant hypertensinase destroys the activity of hypertensin and pepsitensin (7) if incubated with them for 1 hour at 40°C., but it does not affect the activity of renin, adrenalin, or hydroxytyramine.

Experiments with Purified Plant Hypertensinase

Intramuscular Injection.—Into a dog (No. 10-46), with experimental renal hypertension and average direct mean femoral blood pressure of 220 mm. Hg, 10 cc. of extract containing 6000 units of hypertensinase was injected intramuscularly daily for 6 days. During this period there was no reduction of the blood pressure. It was thought that the hypertensinase, injected intramuscularly, might not have been absorbed. If hypertensinase is to act on hypertensin in the plasma, it must first be absorbed into the blood stream. Therefore it was considered advisable to study the absorption of hypertensinase before further therapeutic experiments with hypertensinase were attempted.

Absorption of Hypertensinase.—Absorption studies were made on dogs and rabbits. Concentrated extract containing 2000 units of hypertensinase was injected intramuscularly into rabbits and samples of the rabbit's plasma were

titrated for hypertensinase activity by the method described above. The hypertensinase activity of the plasma did not show any increase.

On the contrary, even 2 hours after the intravenous injection of 2000 units of hypertensinase into rabbits, a fivefold increase of the hypertensinase activity of the plasma was detected. After 6 hours, the level had returned to normal. In another experiment a total of 7350 units of hypertensinase was injected intravenously into a rabbit. After 90 minutes, the rabbit's plasma still showed an almost fivefold increase of hypertensinase activity.

Similar studies were conducted on dogs. A normal dog of 8.6 kilos was given 1000 units of hypertensinase intravenously. The hypertensinase level of the plasma increased twofold, and it took 6 hours before the plasma returned to the normal level existing before the injection.

Intravenous Injection of Plant Hypertensinase into Cats and Their Reaction to Intravenous Injections of Renin and Hypertensin.—Cats were anesthetized with chloralose, 0.1 gm. per kilo body weight, and the effect on the blood pressure of a third of a dog unit of hypertensin and a third of a dog unit of renin was determined. The dog unit of hypertensin used in this study has already been defined above. The dog unit of renin is the one that has been used in all our studies. The determination of this unit was described in detail in a previous study (6*a*). It is the amount of renin necessary to raise the direct mean femoral blood pressure of at least three unanesthetized dogs 30 mm. Hg. Then the plant hypertensinase was slowly injected into the jugular vein. In a typical experiment, 750 units of hypertensinase were injected into a cat weighing 4.5 kilos. After the hypertensinase level of the plasma had increased twofold, the animal still reacted, but much less, to a unit of hypertensin, but failed to give a rise of blood pressure to 1 dog unit of renin.

Intravenous Injection of Plant Hypertensinase into Normal Dogs, and Their Reaction to Renin and Hypertensin.—The intravenous injection of the concentrated plant hypertensinase caused a sudden and great fall in blood pressure in dogs. Therefore, the material was diluted with equal parts of Ringer's solution and slowly injected by intravenous drip.

In a dog (No. 9–46) weighing 10.9 kilos, extract containing a total of 3090 units of plant hypertensinase was slowly injected during 1 hour. The comparison between the hypertensinase activity of the plasma before and after the injection showed that the hypertensinase level had increased twofold.

In a dog (No. 10–30) weighng 9.5 kilos, extract containing 3000 units of plant hypertensinase was administered intravenously in 1 hour. After the administration of 1500 units, the dog did not react to the intravenous injection of 1 unit of hypertensin and showed a blood pressure rise of only 15 mm. Hg after the intravenous injection of 3 units of renin. At the end of the injection of 3000 units of hypertensinase, 1 unit of renin, injected intravenously, produced no rise in blood pressure, 2 units of renin gave a rise of only 10 mm. Hg and 3 units of hypertensin caused a rise of only 15 mm. Hg. At this time the hypertensinase level of the plasma showed a twofold increase.

In a dog weighing 7.7 kilos (No. 10–47) a total of 2500 units of plant hypertensinase was injected intravenously in 15 minutes. After the completion of the injection, the dog reacted to 1 unit of hypertensin with a 5 mm. Hg rise of blood pressure, to 2 units with 10 mm. Hg and to 4 units with 15 mm. Hg. To 1 unit of renin the dog reacted with a 15 mm. Hg rise in blood pressure. One hour after the completion of the injection the dog reacted to the intravenous injection of 2 units of hypertensin with a rise in blood pressure of 20 mm. Hg and to 1 unit of renin with a rise of 20 mm. Hg. Four hours after the completion of the intravenous injection of the plant hypertensinase, the dog showed a normal response to the intravenous injection of 1 unit of hypertensin, with a rise in blood pressure of 30 mm. Hg, and to 1 unit of renin, with a rise of 35 mm. Hg. The plasma level of hypertensinase, which was increased twofold immediately after the injection of the plant hypertensinase, returned to the normal level 4 hours after the injection was completed.

Into a normal dog (No. 10–24) weighing 11.8 kilos an intravenous injection of plant hypertensinase was started, and, after the animal had received 2100 units of hypertensinase, during the first 25 minutes, 2 units of renin were injected intravenously, to which the dog reacted with a rise in blood pressure of 15 mm. Hg. The intravenous injection of hypertensinase was continued at a rate of 25 units per minute and, after 10 minutes, 1 unit of renin was injected intravenously, which gave a rise in blood pressure of 10 mm. Hg. After 5 minutes, 5 units of renin were injected intravenously, to which the dog responded with a 10 mm. Hg rise in blood pressure. Simultaneously with the injection of plant hypertensinase, an intravenous injection of renin was started, at a rate of half a unit of renin per minute. After 14 minutes, the dog still failed to respond to renin. An intravenous injection of 10 units of renin did not produce a rise in blood pressure. Now the rate of intravenous injection of renin was increased to 1 unit per minute. During the next 12 minutes a rise in blood pressure did not occur. Throughout the experiment a total of 4640 units of plant hypertensinase was injected intravenously. After the completion of the plant hypertensinase injection, the hypertensinase level of the plasma had increased twofold.

Into a normal dog (No. 10–41) weighing 10.4 kilos an intravenous injection of plant hypertensinase (37.5 units per cc. in Ringer's solution) was started at the rate of about 100 units of hypertensinase per minute. This rate proved to be too rapid and the injection was stopped after 5 minutes, at which time the blood pressure fell suddenly from 135 mm. Hg to 70 mm. Hg. After another 3 minutes, the blood pressure returned to its previous level and the injection was started again at a slower rate of about 30 units of hypertensinase per minute. During the next half hour enough hypertensinase was injected to add up to a total of 3500 units of hypertensinase. The blood pressure remained at its normal level of 130 to 140 mm. Hg throughout the injection, and the dog had a normal appearance. After the injection was completed, the intravenous injection of 1 unit of hypertensin gave a rise in blood pressure of 15 mm. Hg, 2 units of hypertensin a rise of 15 mm. Hg, 1 unit of renin a rise of 10 mm. Hg, 2 units of renin a rise of 5 mm. Hg, and 5 units of renin gave no rise in blood pressure. Then 200 cc. of arterial blood was withdrawn and treated for the demonstration of hypertensin (8) and hypertensinase. The hypertensinase level in the plasma had increased twofold. There was enough hypertensin present in 200 cc. of plasma to give a rise in blood pressure of 55 mm. Hg when this was injected intravenously into a normal unanesthetized trained dog.

Intravenous Infusion of Plant Hypertensinase into a Hypertensive Dog.—

A dog (No. 10–29) weighing 10 kilos, with experimental renal hypertension and direct mean femoral blood pressure of 180 mm. Hg, received an intravenous injection of plant hypertensinase. The solution contained 50 units of hypertensinase per cc. and, at the slow rate of 1 cc. per minute, a total of 3000 units of hypertensinase was injected. After the injection of

2300 units of hypertensinase, the blood pressure had changed from 180 mm. Hg to 165 mm. Hg. After the injection of 2675 units of hypertensinase the blood pressure had reached the normal level of 120 mm. Hg. At the end of the injection the blood pressure was 130 mm. Hg. After the injection was stopped, the dog reacted to the intravenous injection of 1 unit of hypertensin with a rise in blood pressure of 5 mm. Hg, to the injection of 5 units of hypertensin with a rise of 15 mm. Hg, to the injection of 1 unit of renin with no rise, and to the injection of 5 units of renin with a rise of 25 mm. Hg. At the height of the rise, 200 cc. of arterial blood was withdrawn and treated for the demonstration of hypertensin and hypertensinase in the plasma. After the injection of the 6 units of renin into the hypertensive dog treated with 3000 units of plant hypertensinase by intravenous injection, 200 cc. of blood contained enough hypertensin to give a rise in blood pressure of 50 mm. Hg (intravenous injection into a normal unanesthetized trained dog). The hypertensinase level of the blood was increased threefold. Four hours after the injection of plant hypertensinase had been stopped, the hypertensinase had returned to its normal level and the blood pressure was up to its previous hypertensive level.

Intravenous Injection of Inactivated Plant Hypertensinase into a Hypertensive Dog.—

The plant hypertensinase preparation was inactivated by incubation at 37°C. for 1 hour at pH 11. The pH was then lowered to 8.1 by the slow addition of 1/10 N HCl. Instead of 30 units of hypertensinase per cc. the solution now contained barely 1 unit per cc.

A hypertensive dog (No. 8–82) weighing 10 kilos, with a blood pressure of 200 mm. Hg received by slow intravenous injection, a quantity of inactivated extract representing 3000 units of plant hypertensinase. Throughout the injection, which lasted 75 minutes, the blood pressure of the dog did not change. After the injection, the dog reacted in a normal way to the intravenous injection of 0.05 cc. of 1:1000 adrenalin, with a rise in blood pressure of 60 mm. Hg, to the injection of 1 unit of hypertensin with a rise of 20 mm. Hg, and to the injection of 1 unit of renin with a rise of 25 mm. Hg. Then 5 units of renin were injected intravenously and, at the height of the rise of blood pressure, 200 cc. of arterial blood was withdrawn, to be treated for the demonstration of hypertensin and hypertensinase. The plasma of this sample contained enough hypertensin to give a rise of 30 mm. Hg in a normal unanesthetized dog. The hypertensinase level of the blood was not increased after the intravenous injection of 3000 units of inactivated plant hypertensinase.

DISCUSSION

There is no proof that the enzyme hypertensinase is involved in the humoral mechanism of renal hypertension. Blood plasma does contain hypertensinase, and the kidney contains a large amount of it, rating only second to intestinal mucosa (3). An indication that the kidney is the main source of hypertensinase in the blood plasma is the almost complete disappearance of hypertensinase from the latter, after bilateral nephrectomy (3); but this finding has been questioned (9). It has been asserted that renal venous blood, from an ischemic kidney, contains less hypertensinase than blood from the renal vein of a normal kidney (10), but this we have not been able to confirm, and it has not been confirmed by other investigators (11). In addition, the importance of hypertensinase is rendered questionable by the fact that the hypertensinase content of the plasma of hypertensive dogs has always been found normal (9).

Because hypertensinase is the only known substance in the blood which has the ability to destroy hypertensin *in vitro*, almost all attempts to treat renal hypertension by injection of organ extracts have been based on the idea of supplying the enzyme which destroys the pressor substance, hypertensin, although a lack of hypertensinase has never been established in renal hypertension. Studies of the action *in vivo* of hypertensinase made from the kidney have not been possible because of the relatively low potency and impurity of the preparations which usually contained more renin than hypertensinase.

With the preparation of a purified and highly potent plant hypertensinase, which does not contain renin, the attempt has been made to gain more knowledge about the antipressor properties of this enzyme. By our simple method for the determination of the potency of the hypertensinase, it has been possible to follow the possible absorption of hypertensinase after injection at various sites in the body.

The properties of the wheat bran hypertensinase used in our experiments were found to be similar to those of the enzyme solution obtained from yeast cells (12). This enzyme destroys hypertensin and pepsitensin, and the authors described it as an aminopolypeptidase. We did not make an exhaustive study of the chemical properties of the enzyme solution used in our experiments. The fact that it did not destroy adrenalin or hydroxytyramin excluded the action of a phenolic oxidase. It is probable that the action is not that of an oxidase but of a proteolytic enzyme acting by hydrolysis.

Failure to raise the blood level of hypertensinase and to reduce blood pressure of hypertensive dogs by the intramuscular injection of large amounts of plant hypertensinase is of interest because the intramuscular injection of renal extract has been used as a treatment for hypertension. Page even used the hypertensinase titer as an indicator of the activity of his renal extracts, which were administered by intramuscular injection. The renal extract of Grollman and his collaborators (13) could not contain hypertensinase, because this was undoubtedly destroyed during the preparation.

Helmer, Kohlstaedt, Kempf, and Page (14) found that most of the material in their antipressor extract which was capable of destroying hypertensin was in the same range of ammonium sulfate precipitability as the material responsible for the antipressor activity, when assayed on hypertensive dogs and rats. The reduction of arterial blood pressure of hypertensive patients and animals with extracts of kidney (15) was explained therefore as due chiefly to their ability to destroy hypertensin (16). It is difficult to evaluate the results of their treatment with kidney extracts because the authors state also that the preparation of active extract is almost a hit or miss problem and that expressions such as "specific" and "non-specific" would only confuse the issue. Goldblatt, Kahn, and Lewis (17), with a kidney extract made in accordance with the details given by Page and collaborators, failed to reduce the blood pressure of hypertensive dogs. The kidney extract prepared by Schales,

Stead, and Warren (18) did contain a goodly amount of hypertensinase, because 1 liter inactivated the amount of hypertensin produced by incubating 1 to 3 liters of beef serum with an excess of renin under optimum conditions. In five of their seven patients, there was a significant lowering of the high blood pressure, which appeared to be related to the severe local and general reactions. Because the intramuscular injection of an extract poor in hypertensinase also produced a fall in blood pressure, similar to that produced by the unmodified extract, the authors concluded that the blood pressure–lowering effect of their renal extract was due to a general toxic effect of the crude tissue extract rather than to specific interference with the renin-hypertensin mechanism. In these experiments, too, the effect cannot be attributed to hypertensinase which, as we have shown above, is not absorbed into the blood stream from an intramuscular injection.

The intravenous injection of large amounts of plant hypertensinase gave us the opportunity to study the direct action of hypertensinase on renin and hypertensin *in vivo*. In the circulating blood, hypertensinase interferes not only with the action of hypertensin, as in the test tube, but it also inhibits the effect of renin. The impression is gained that the action on renin is even greater than on hypertensin. This may be explained by the slow enzymatic action of the renin and hypertensinase, two antagonistic enzymes, which allows better opportunity for the destruction of the end product, hypertensin, formed in the blood. Hypertensin, however, injected intravenously, acts so quickly that it is only slightly affected by the slowly acting hypertensinase of the blood. To produce effects, the level of hypertensinase in the plasma must be greatly increased, and the best results were obtained when it was increased at least threefold above normal. When no increase was produced (as by the injection of inactivated extracts) both renin and hypertensin retained their normal activity.

In brief experiments, the intravenous injection of plant hypertensinase lowered the blood pressure of dogs with experimental renal hypertension to normal levels. Inactivated plant hypertensinase failed to produce this effect. The lowering of the blood pressure was temporary and lasted only as long as the hypertensinase level was increased. Within 4 hours after the injection of 3000 units of plant hypertensinase into a dog of 10 kilos, the blood pressure returned to its original level.

The demonstration that the pressor substance hypertensin is still formed in the blood after the injection of plant hypertensinase can be explained on the assumption that this enzyme does not quickly destroy all the pressor substance formed.

SUMMARY

Many plants contain an enzyme similar in most biological properties to the hypertensinase obtained from blood and some animal tissues, notably kidney and intestinal mucosa.

Wheat bran is a rich source of the plant hypertensinase, and from it a potent, non-toxic preparation was made by the use of isoelectric and ammonium sulfate precipitation as the means of purification.

Hypertensinase derived from bran and administered intramuscularly was not absorbed, or was absorbed only very slowly, into the blood plasma.

Repeated intramuscular injection of large quantities of plant hypertensinase did not reduce the blood pressure of dogs with experimental renal hypertension.

The intravenous injection of large quantities of plant hypertensinase into dogs resulted in an immediate increase in the content of hypertensinase in the plasma.

Dogs with a high hypertensinase level in the plasma failed to react, or reacted much less markedly to the intravenous injection of amounts of renin or hypertensin which had previously proved effective.

The slow intravenous injection of plant hypertensinase into a dog with experimental renal hypertension reduced the blood pressure to the normal level for the period during which the concentration of plant hypertensinase in the blood was considerably elevated. After the return of the hypertensinase of the plasma to normal, the blood pressure rose again to its previously high level.

Inactivated plant hypertensinase did not increase the hypertensinase content of the plasma, did not interfere with the action of renin and hypertensin, and did not reduce the high blood pressure of dogs with experimental renal hypertension.

In a dog with an increased level of plasma hypertensinase, the pressor substance hypertensin could still be detected in the systemic blood immediately after the intravenous injection of renin in an amount to which the animal responded with only a slight rise in blood pressure.

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