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Research Article

THE RENAL HUMORAL PRESSOR MECHANISM IN MAN.

I. PREPARATION AND ASSAY OF HUMAN RENIN, HUMAN HYPERTENSINOGEN, AND HYPERTENSIN [1,2]

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Human renin and hypertensinogen differ from those of animal origin in certain properties which make their preparation and assay by current technics unsatisfactory, principally due to the presence of hypertensinase. Their method of preparation, in a form free from hypertensinase but not chemically pure, and their assay are the subjects of this communication.

PREPARATION OF HYPERTENSINASE-FREE HUMAN RENIN

Human renin is prepared by a modification of the method of Katz and Goldblatt (1). Human kidneys are obtained as fresh as possible from autopsied cases. Fatty and fibrous parts are discarded and the remainder ground to a pulp in a flour mill. Two volumes by weight of tap water are added, together with toluol as a preservative, and after thorough stirring, the mixture is stored at a temperature of 5° C. overnight or longer. It is strained through 4 thicknesses of cheesecloth and the remaining pulp squeezed through muslin. The pulp is discarded. The filtrate is adjusted to pH 6.0 (glass electrode) and cooled to 5° C.

When cool, the filtrate is stirred with a power stirrer, and cold 10 per cent trichloroacetic acid is slowly added under the surface of the solution until the pH is exactly 2.9 ± 0.05 (glass electrode). If filtered at this point, the filtrate is brownish and rich in hypertensinase. A 5.13 molar solution of sodium chloride is therefore added slowly under the surface of the solution with constant stirring until its concentration reaches 0.92 molar. This precipitates the hypertensinase but not the renin. The pH is readjusted at once to exactly 2.90 ± 0.05 by the addition of N sodium hydroxide or 10 per cent trichloroacetic acid, and rechecked at the end of 15 minutes. It is then filtered by gravity through fluted filter paper (E & D No. 193) in a cold room at 5° C. The filtrate is clear yellow. Filtration should be complete in 6 hours.

The renin may be precipitated from the filtrate by raising the pH of the filtrate to about 5 and dissolving 400 grams of solid ammonium sulfate per liter of filtrate. The precipitate is filtered by suction using Whatman filter paper No. 1 with a thin coating of kieselguhr, suspended in distilled water, and dialyzed overnight against water at 5° C. with a small amount of toluol added as a preservative. The solution is then filtered. The filtrate has a clear faint brown color. It may be stored with toluol or merthiolate and a small amount of phosphate buffer (pH 6.0) for many months at 5° C. It may be dried from the frozen state and stored as a dry powder at room temperature apparently indefinitely.

This material, though not chemically pure, contains no hypertensinase and is satisfactory for routine assay work. The yield seems to be excellent, although difficulties in determining the amount of renin present in the original crude kidney extract are such as to make yield figures unreliable. The yield per gram of human kidney has averaged about 1.5 cat units of renin. Wide variations have been noted, however, and have been attributed to the period of time elapsing between the death of the patient and extraction of the kidneys, the mode of death of the patient, and the like. The potency of this renin has varied from 15 to 23 cat units per mgm. of nitrogen.

PREPARATION OF HYPERTENSINASE-FREE HUMAN HYPERTENSINOGEN

It has been shown (2) that beef plasma may be freed from hypertensinase by lowering the pH to between 3.8 and 4.0 for 20 minutes at 37° C. or for 1 hour at 17° C. There is, however, some degree of destruction of hypertensinogen by this method, in our experience. When human plasma is subjected to this treatment, both hypertensinase and hypertensinogen are almost completely destroyed. Beef plasma may be freed from hypertensinase without appreciable loss of hypertensinogen activity and human plasma with only minimal loss by the following procedure:

Three hundred grams of solid sodium chloride are added per liter of plasma and stirred until dissolved. A small amount of toluol is added as a preservative. The pH is lowered to 4.0 and the creamy solution is kept at 37° C. for 4 hours. The pH is raised to approximately 6 and the solution dialyzed against water at 5° C. until essentially chloride-free. The solution may be clarified by centrifu-

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] This study was aided in part by a grant from the John and Mary R. Markle Foundation.

gation. If kept at a temperature of 5° C. or less, with toluol as a preservative, this solution of hog hypertensinogen remains potent for months. Further purification may be carried out if desired by fractional salt precipitation as has been described (3 to 5).

PREPARATION OF HYPERTENSIN

Hypertensin is prepared by incubating renin with hypertensinogen and extracting for hypertensin.

Methods heretofore described (3, 6 to 8) have not usually eliminated hypertensinase from the solutions of both renin and hypertensinogen. The yield of hypertensin by these methods in our experience has been of the general order of 20 or 25 per cent of that expected. In order to improve the yield and lessen the amounts of renin used in incubating, hypertensinase-free solutions of hog renin and beef hypertensinogen have been prepared as described. The solution of hypertensinogen is incubated with renin, at 37° C. at a pH of 7.3, usually for 2 hours, 2 units of renin being used for each unit of hypertensinogen. When using small volumes, a 100 per cent yield of hypertensin is obtained, but when larger quantities (several liters) are prepared, the yield has been 50 to 70 per cent of that anticipated from the amount of hypertensinogen originally present in the plasma.

The reaction may be stopped by 95 per cent alcohol or by heat. Since losses are encountered in the vacuum distillation of large volumes of the protein-free alcoholic solution, the latter method is preferable. The incubated hypertensin solution is heated to 70 to 90° C. in a boiling water bath for 5 to 10 minutes at approximately pH 4. A crude hypertensin solution may be obtained by adjusting the pH to about 6 and removing the precipitated proteins while still hot by filtration through fluted filter paper (E & D No. 192). If a purer hypertensin solution is desired, the heated solution at pH 4 may be placed with toluol in a dialysis sac suspended in a glass column. Distilled water is allowed to trickle slowly between the column and the sac and a water clear dialysate containing hypertensin is obtained. This procedure should be carried out at a low temperature. To obtain the best yields this must be continued for a number of days. The hypertensin solution is concentrated from the frozen state without loss of potency. One cat unit of this material contains 0.03 mgm. nitrogen.

Aqueous or alcoholic solutions of hypertensin may be concentrated by evaporation at an acid pH under reduced pressure. With volumes of 200 ml. or more, losses occur during evaporation even when the temperature remains 40° C. or less. We are able to confirm one report (3), however, that hypertensin is stable at 100° C. without boiling for 2 hours in acid solution, although it is readily destroyed by alkali at this temperature. When volumes of less than 200 ml. are evaporated under reduced pressure, no destruction of hypertensin has been noted, even with the temperature rising to 70° C. The solution may be concentrated by lyophilization without loss. The concentrated solution may be filtered and extracted with ether at pH's 5 and 9 to remove fat and other impurities and further purified by any one of the procedures previously described (3, 9), using phenol, glacial acetic acid, ammonium sulfate, or 70 per cent alcohol. It is much less pure than the ultrafiltrate.

ASSAY OF HYPERTENSIN

Various methods have been described for the assay of hypertensin including the isolated rabbit's ear (10), frog's or toad's hind limb (11, 12), guinea pig ileum (13), and isolated arterial rings (14). These methods lack specificity when plasma samples are employed and are not particularly quantitative although they have a high degree of sensitivity. A method which is of the same order of sensitivity and which has the advantages of specificity and quantitation has been described by Leloir and his collaborators (2). It depends on the pressor effect of hypertensin administered intravenously to anesthetized dogs. Renin, hypertensinogen, and hypertensinase may likewise be determined by this method by measuring the amount of hypertensin formed from the interaction of renin with its substrate, hypertensinogen, or by measuring the amount of hypertensin destroyed from the action of hypertensinase on hypertensin.

a. *Technic of assay:* Dogs anesthetized with nembutal (0.04 gram per kgm. intraperitoneally), cats anesthetized with dial-urethane (Ciba) (0.88 ml. per kgm. intraperitoneally), or pithed dogs or cats make satisfactory assay animals. It is commonly stated that nephrectomy increases the sensitivity of the preparation, but this has not been our experience in most cases unless the animal is uremic (15). It has been our custom to use animals with intact kidneys. Cats are about 4 times more sensitive than dogs.

A cannula is inserted into the jugular vein for intravenous injections and into the carotid artery for blood pressure determinations. The latter is connected to a mercury manometer with a suitable graphic recorder.

Injections are made briskly. The presence of hypertensin is denoted by a rapid rise in blood pressure and a fall to the initial level in 2 to 4 minutes. After another minute, another injection may be made. Although some workers (16, 17) have claimed that tachyphylaxis to hypertensin takes place, others (3, 9, 18 to 20) have repeatedly stated that tachyphylaxis does

not occur. The latter belief has recently been confirmed (21). We have never seen any evidence of tachyphylaxis to hypertensin in either dogs or cats. We have given as many as 50 injections of hypertensin to both dogs and cats over the course of several hours without observing the development of tachyphylaxis.

The sensitivity of the animal may remain quite stable for long periods of time or it may change rapidly as the animal is becoming more deeply or lightly anesthetized. For this reason, the sensitivity of the preparation should be checked after every second or third injection with a solution of hypertensin of known potency. When the sensitivity is changing rapidly, results are not uniform. When minute amounts of hypertensin are being assayed, it is important to test the sensitivity of the cat to normal saline. In some cats, the injection of 10 ml. of saline alone may produce a rise of 10 to 20 mm. Hg. Such animals are not suitable for the assay of small amounts of hypertensin or for the assay of renin in human plasma (*vide infra*).

This method is admirably suited to the determination of relative amounts of hypertensin in a series of solutions of unknown potency, it being a simple matter to determine whether more or less hypertensin is present in one tube than in the next. Thus, the progressive diminution in the concentration of hypertensinogen in plasma after the intravenous injection of renin presents no difficulty in assay. For precise quantitative results, however, a meticulous technic is required, attention being paid especially to the consistency of sensitivity of the assay animal and to the injection alternately of unknown solutions with about the same amount of hypertensin of known potency. The most accurate results are obtained by injecting an amount of hypertensin which will produce a rise of blood pressure between 20 and 35 mm. Hg. It is well, therefore, to inject only a fraction of an unknown tube if it is suspected that excessive amounts of hypertensin are present. By using the most scrupulous technic, however, results are not always satisfactory as certain workers (22) have recently affirmed. This should be appreciated by those using the method and single observations should be interpreted with caution when the absolute values obtained are of importance, as, for example, in the establishment of normal values for the concentration of hypertensinogen in the plasma of patients or animals (*vide infra*) or in the standardization of stock solutions of hypertensin. For such work, we have checked each unknown several times on the same or different cat whenever possible.

b. *System of unitage.* Since the chemical nature of hypertensin is unknown, it is not yet possible to express its potency in mgm. of pure substance. The dog unit has, therefore, been defined by the Buenos Aires workers (9, 19) as that amount which produces an average rise of 25 to 30 mm. Hg when briskly injected intravenously into a 10 kgm. anesthetized dog.

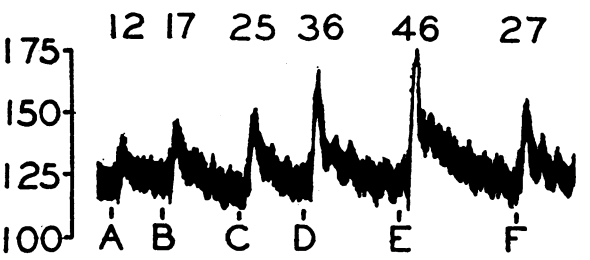

FIG. 1. RELATIONSHIP OF DOSE OF HYPERTENSIN TO RISE OF BLOOD PRESSURE IN 3.5 KGM. CAT UNDER DIAL ANESTHESIA

A. 0.05 ml. (0.25 unit)
B. 0.1 ml. (0.5 unit)
C. 0.2 ml. (1.0 unit)
D. 0.4 ml. (2.0 units)
E. 0.8 ml. (4.0 units)
F. 0.2 ml. (1.0 unit)

Figures above indicate rise in pressure in mm. Hg. It will be noted that quadrupling the dose of hypertensin doubles the rise of pressure.

This amount is equal in its pressor effect to 4 μg. of adrenalin (9) and is equivalent to that obtainable from the hypertensinogen present in approximately 4 ml. of fresh beef plasma.

Since we have used cats for assay purposes, it has been more convenient to utilize a smaller unit. The cat unit has been defined as one-fourth of a dog unit and is equivalent in pressor action to 1 μg. of adrenalin.[3]

As for adrenalin, the rise in pressure after the intravenous injection of hypertensin varies as the square of the amount injected if the rise is not too great, *i.e.*, over 50 or 60 mm. Hg (19) (Figure 1). We have never observed the linear relationship described by some workers (23)

[3] We are indebted to Dr. Eduardo Braun-Menendez for sending us a sample of hypertensin for our standardization.

except in animals in poor condition or with rapidly changing sensitivity. The amount of hypertensin present in an unknown solution may be calculated by comparing the rise in pressure in mm. Hg with that produced by a solution of known potency according to the following formulae (9, 19):

$$X = \left(\frac{Y}{St}\right)^2 \qquad Y = St\sqrt{X} \qquad St = \frac{Y}{\sqrt{X}}$$

where X represents the number of units in the unknown sample, Y represents the elevation of blood pressure in mm. Hg caused by the injection of the unknown solution, and St represents the elevation of blood pressure in mm. Hg caused by the injection of one unit of hypertensin.

ASSAY OF HUMAN RENIN

Two general methods for the assay of renin have been described. For the determination of *large* amounts, intravenous injection into animals as described by a number of investigators (24 to 28), is satisfactory. For the detection of *minute* amounts, such as exist in plasma in certain disorders, renin must be determined by a much more sensitive technic, such as the indirect method of Muñoz *et al.* (29). The direct method of Leloir and co-workers (2) consists of destroying the hypertensinase present and incubating the solution of renin with hypertensinogen and assaying the amount of hypertensin formed. This is not applicable to the detection of human renin, however, because the renin is destroyed along with the hypertensinase (29). With plasma containing pig or dog renin, hypertensinase may be destroyed by lowering the pH to between 3.8 and 4.0 for 20 minutes at 37° C. or for 1 hour at 17° C. (2). Although pig and dog renin are not destroyed by this procedure, human renin is almost entirely inactivated. If a pH of 4.5 instead of 4.0 is used, neither hypertensinase nor renin in human plasma is destroyed. The following method, which is a modification of that mentioned above (2), has been devised for the destruction of hypertensinase in human plasma without destroying human renin. It is also applicable to the detection of minute amounts of pig and dog renin in plasma.

To 30 ml. of human serum or plasma at 5° C. are added 2.7 grams (90 grams per liter) of solid sodium chloride and a few drops of toluol as a preservative. The pH is lowered to 2.0 (glass electrode) with 10 per cent hydrochloric acid and left for 2 hours at 5° C. Whereas the blood hypertensinase is destroyed by this procedure, the renin remains unchanged. The pH is raised to between 5 and 6 with N sodium hydroxide and the solution is dialyzed in a cellophane sac overnight against tap water at 5° C. The contents of the sac are removed and divided into 3 equal parts. To each is added an excess (6 to 8 ml.) of hypertensinase-free beef hypertensinogen, prepared as described, 0.5 ml. M/2 phosphate buffer at pH 7.3, and 0.2 ml. 1:1000 merthiolate (Lilly). The first tube is incubated for 4 hours at 37° C.; the second is unincubated, and the third is incubated for 4 hours at 37° C. with enough hemolyzed fresh human red blood cells (blood hypertensinase) to produce a faint pink color. Longer periods of incubation increase the sensitivity of the method, but the

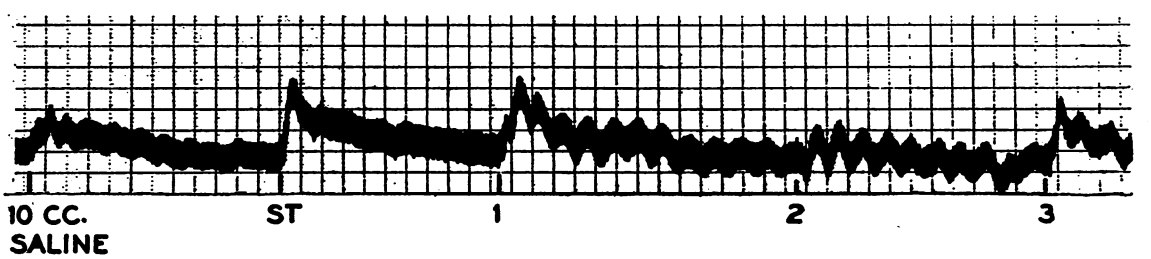

FIG. 2. DEMONSTRATION OF HUMAN RENIN ADDED TO HUMAN PLASMA.

BLOOD PRESSURE RECORDED ON CAT UNDER DIAL ANESTHESIA

St: Injection of 0.5 cat unit of hypertensin.

1 and 2: These tubes were prepared as described in text after adding 0.03 ml. (0.5 cat unit) of human renin to each of two 5 ml. samples of normal human plasma. Tube 1 was incubated and tube 2 unincubated.

3: 0.03 ml. (0.5 cat unit) of hypertensinase-free human renin incubated with hypertensinase-free beef plasma as in tube 1 without preliminary treatment with salt, low pH, and dialysis, *i.e.*, by the direct method of Leloir and co-workers (2), using hypertensinase-free solutions.

Note that tubes 1 and 3 produce approximately the same pressor response while the unincubated control tube (tube 2) has no significant influence on the blood pressure.

danger of bacterial contamination and the appearance of toxic side-products make prolonged incubation unsatisfactory as has been observed (2). At the end of incubation, the tubes are heated for 5 minutes in boiling water and 3 volumes of 95 per cent alcohol are added, the precipitated proteins removed by filtration, the filtrate made slightly acid, and the alcohol removed by evaporation under reduced pressure over boiling water to a volume of 2 to 3 ml. The contents of the flask are taken up in two 5 ml. washings with normal saline. The pH is brought to neutrality and the tubes are ready to be assayed for their content of hypertensin.

The specificity of the method is indicated by a pressure rise from injection of the first tube and a lack of pressor response from the second and third tubes (Figure 2). It is to be emphasized that any results obtained without the use of one or more control tubes are of no significance since nonspecific pressor responses not infrequently occur. Adrenalin, tyramine, guanidine, pituitrin, spätgift, etc., are not recovered by this method. As some workers (30) have observed and as we have confirmed, pepsitensin is not appreciably destroyed by the hypertensinase of red blood cells. It is destroyed by renal hypertensinase, however, as reported by others (31).

The approximate limit of sensitivity of this method is 0.2 cat unit of renin added to 10 ml. of plasma, depending on the sensitivity of the cat preparation. The *cat unit of renin* is defined as that amount which yields 1.0 cat unit of hypertensin when incubated for 2 hours at 37° C. with an excess of hypertensinogen.

ASSAY OF HUMAN HYPERTENSINOGEN

One *cat unit of hypertensinogen* is the amount which is capable of giving rise to 1.0 cat unit of hypertensin in the presence of an excess of renin. The amount of hypertensin formed is in proportion to the amount of hypertensinogen employed (20). For the assay of human hypertensinogen, the method of Leloir and co-workers (2) has been modified as follows:

Two cc. of human plasma are incubated for 25 minutes at 37° C. with an excess of hypertensinase-free human renin (50 cat units), the pH being maintained at 7.3 with 0.5 ml. M/2 phosphate buffer. The reaction is stopped by the addition of 3 volumes of 95 per cent alcohol. Further preparation of the tube is the same as that described for renin.

In calculating the potency of plasma samples a correction must be made for the dilution by citrate or oxalate. The use of hypertensinase-free renin is important since the tubes are incubated for 25 minutes instead of 5 to 10 minutes as for beef hypertensinogen and pig renin.

Certain investigators (22) have recently studied this method in great detail from the point of view of each variable. Their method is similar to ours except that they incubate 100 dog units (400 cat units) of human renin with 2 ml. of human plasma for 7 minutes. The shorter period of incubation has the theoretical advantage of avoiding the destructive action of plasma hypertensinase on the hypertensin formed. Actually, however, the amount of hypertensinase in nonhemolyzed human plasma has no appreciable action on hypertensin in a 20-minute incubation period at 37° C. and our values have been almost identical with theirs (32, 33). The only advantage of our method over theirs is that it requires the use of only an eighth the amount of human renin.

In the establishment of absolute values for the concentration of hypertensinogen in plasma of human beings, a fairly wide range of values has been obtained by this method by both ourselves (32, 33) and the above mentioned workers (22), in part due, we believe, to the inherent difficulties of the method already discussed and in part to normal variations in titer. In order to obviate as much as possible errors in interpretation in such a study, it has been our custom to test each plasma on several occasions in the same or different assay animal, and to compare the values of pathological plasmas with that of a normal control subject.

ASSAY OF HYPERTENSINASE

A unit of hypertensinase has been previously defined (34) as the amount which, in a volume of 10 ml. containing one dog unit (or 4 cat units) of hypertensin, destroys 0.5 dog units (or 2 cat units) of hypertensin in 4 hours at a temperature of 37° C. and pH 7.3. The method of assay of hypertensinase as previously described for dog plasma (34, 35) can be directly applied to human plasma using either the cat or the dog as an assay animal. We have regularly used a 2-hour incubation period and converted the units of hypertensin destroyed to units of hypertensinase

(4 hours) by means of curves previously published (35). Since the hypertensinase content of human plasma is lower than that of dog plasma (36), 1 to 3 ml. of plasma are used for man instead of 0.125 to 1.5 ml. for dogs.

SUMMARY

Methods for the preparation of hypertensin and of human renin and hypertensinogen free from hypertensinase have been described, together with their assay by modifications of the methods of Braun-Menendez, Fasciolo, Leloir, Muñoz, and Taquini.[4]

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[4] Since this article was sent to press, a complete description of the original methods of the Buenos Aires investigators has come to our attention in the book by Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., Muñoz, J. M., and Taquini, A. C., Hipertension arterial nefrógena. El Ateneo, Buenos Aires, 1943.

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