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THE QUANTITATIVE ASSAY OF ANGIOTONIN

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An active polypeptide known as angiotonin or hypertensin, formed by the reaction of a renal enzyme (renin) with a plasma—α2—globulin (hypertensinogen), causes vasoconstriction in perfused rabbits' ears (Page and Helmer, 1940 ; Page, 1942) and toads' hind limbs (Houssay and Taquini, 1938a, 1938b), and a rise of blood pressure in anaesthetized or pithed dogs (Braun-Ménendez, Fasciolo, Leloir, and Muñoz, 1940) and cats (Dexter, Haynes, and Bridges, 1945). Based on these effects, methods for the assay of angiotonin have been suggested, but these are not suitable for the detection and determination of the angiotonin-like activity of human plasma since they are not quantitative and are not sufficiently specific or sensitive.

A sensitive, and what is believed to be a specific, method for the quantitative assay of angiotonin has been developed and is described below. It is based on the pressor response obtained on injecting the material into the dibenamine-treated rat's blood-pressure preparation described previously by Dekanski (1952).

A comparative study of angiotonin and posterior pituitary extract with respect to both the pressor and the oxytocic actions has been made and is also reported.

METHODS

Pressor Activity of Angiotonin.—This was assayed on the blood pressure of the dibenamine-treated rat. Dibenamine effectively antagonizes the pressor responses to adrenaline, noradrenaline, isoamylamine, tyramine, nicotine and piperidine (Dekanski, 1951) but not those to pituitary extract or angiotonin. In each assay four doses were used, two of the standard and two of the unknown, the ratio of high to low dose being the same (usually 3 to 2) for both the standard and the unknown. Each dose was injected every 3 to 5 min. once in each group of four, and there were six groups in all. Solution Angiotonin (Eli Lilly, U.S.A.), 10 cat units/ml., served for both the standard and the unknown preparations. Rats weighing 250 g. or less were more sensitive to angiotonin than those weighing 300 g. and more. It was found that, in order to maintain a steady base line, the amount of dibenamine could be usefully increased to at least twice that previously suggested (Dekanski, 1952) (six repeated injections of 100 μg. dibenamine per 100 g. body weight intravenously at 5 min. intervals). When plasma was tested the ratio of high to low dose was usually 2 to 1 and there were 3 or 5 groups in all.

The pressor activity of angiotonin was also compared with that of pituitary standard (International Pituitary Posterior Lobe Standard—100 mu./ml. normal saline, freshly made each time). Each dose of pituitary standard was injected intravenously 3 to 5 min. after the preceding injection of angiotonin, and each dose of angiotonin solution was injected 6 to 8 min. after the preceding dose of pituitary. Each dose, either of pituitary standard or of angiotonin solution, was administered once in each group of four doses and there were 5 to 6 groups in all.

Oxytocic Activity of Angiotonin.—This was compared with that of pituitary standard (20 mu./ml. normal saline, freshly made each time) on the rat's uterus suspended at 30° C. in a bath containing modified Ringer–Locke solution with reduced $CaCl_2$ (60 mg./l.) and no Mg (Gaddum, Peart, and Vogt, 1949).

Inactivation of Pituitary Extract and of Angiotonin.—The inactivation of the pressor activity of pituitary extract and of angiotonin was studied by treating each of the preparations or plasma at room temperature (van Dyke, Chow, Greep and Rothen, 1942) with sodium thioglycollate, at *p*H 7.4. M-Sodium thioglycollate was freshly prepared each time from the acid with H_2O and $NaHCO_3$. The solution was added to posterior pituitary extract, angiotonin solution or plasma to give a final concentration of 0.1 M- or 0.01 M-sodium thioglycollate, and the mixture left for varying times before testing.

Action of 5-Hydroxytryptamine (Serotonin).—The action of serotonin on the blood pressure of the anaesthetized rat was studied before and after treatment with hexamethonium, or with dibenamine, or with both.

Drugs Used.—Urethane (B.D.H.), 25 g. per 100 ml. in distilled water ; dose, 0.7 ml. per 100 g. body weight, subcutaneously. Heparin B.P. (Boots) ; dose, 200 u. per 100 g. body weight, in 1 ml. normal saline,

FIG. 1.—Blood pressure of rat (246 g.). Urethane. Dibenamine 1.49 mg. Pressor responses to different doses of Solution Angiotonin (Eli Lilly)—10 cat u./ml. diluted with saline to give 2 u./ml.—ranging from 0.01 to 0.24 ml. NS, normal saline. From these results the log dose-response graph of Fig. 2 has been constructed.

FIG. 2.—Log dose-response regression line relating the responses to varying doses of angiotonin based on results shown in Fig. 1. b=44.3 mm. Hg.

FIG. 3.—Blood pressure of rat (260 g.). Urethane. Dibenamine 1.5 mg. Pressor effects of angiotonin. A, 0.06 ml., B, 0.04 ml., standard (10 u./ml.). C, 0.09 ml., D, 0.06 ml., "unknown" (7.5 u./ml.). Washed in with 0.2 ml. saline. Interval 3 min. Unknown estimated to contain 7.2 u./ml. Total experimental time 3 hr.

FIG. 4.—Blood pressure of rat (330 g.). Urethane. Dibenamine 2.31 mg. Pressor effects of angiotonin and posterior pituitary standard. A, 6 mu., B, 4 mu. posterior pituitary extract. C, 0.6, D, 0.4 "units" angiotonin. Washed in with 0.2 ml. saline. Interval after pituitary 7 min., and after angiotonin 3 min.

FIG. 5.—Uterus of oestrous rat (155 g.) in 2 ml. bath at 30° C. Comparison of oxytocic effects of angiotonin and posterior pituitary extract. A, 0.3, B, 0.2 mu., posterior pituitary. C, 0.45, D, 0.3 "units" angiotonin. Interval 4 min.

FIG. 6.—Blood pressure of rat (300 g.). Urethane. Dibenamine 1.8 mg. To show that pressor effect of posterior pituitary extract (PLS) is more prolonged than that of equipressor dose of angiotonin. Interval after angiotonin 3 min. Interval after pituitary 8 min.

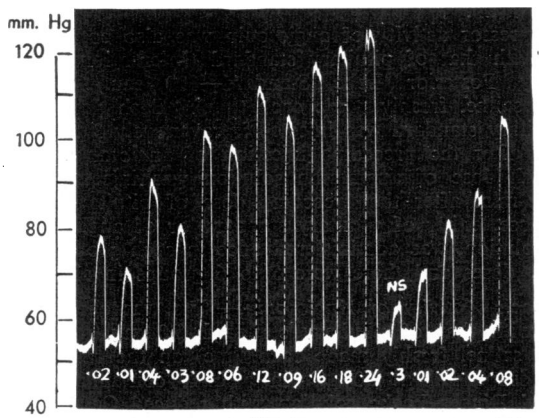

FIG. 1

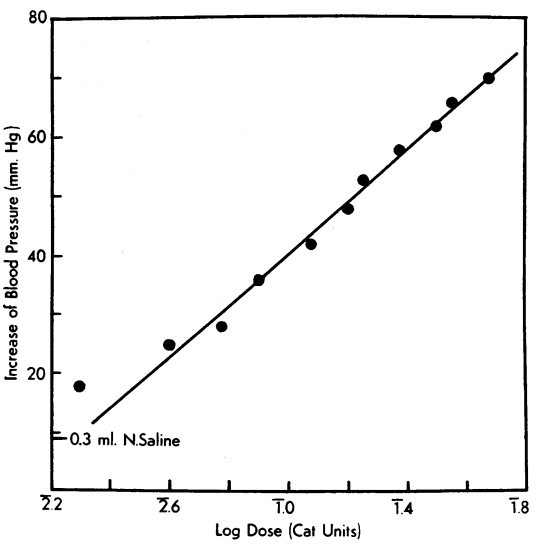

FIG. 2

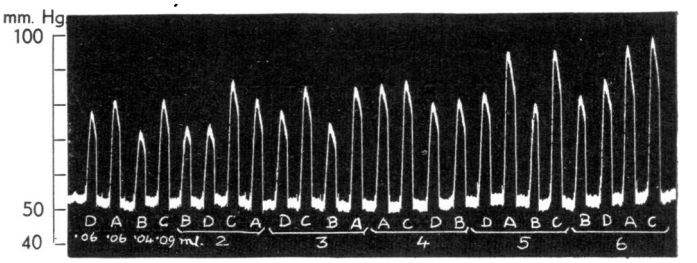

FIG. 3

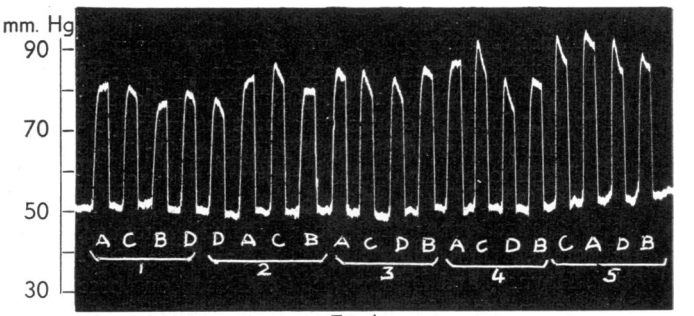

FIG. 4

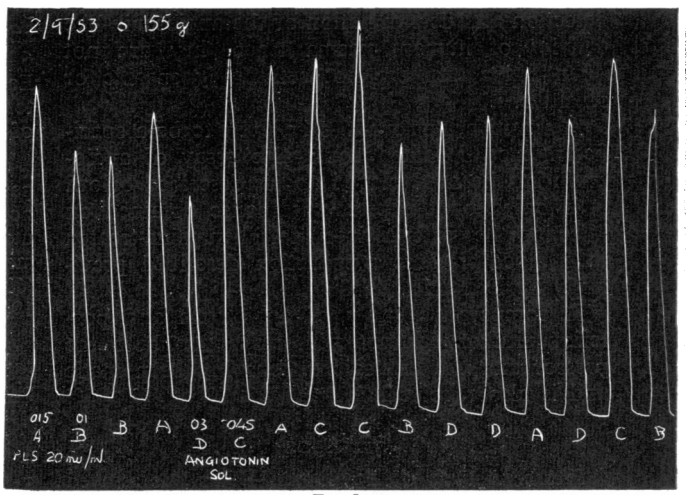

FIG. 5

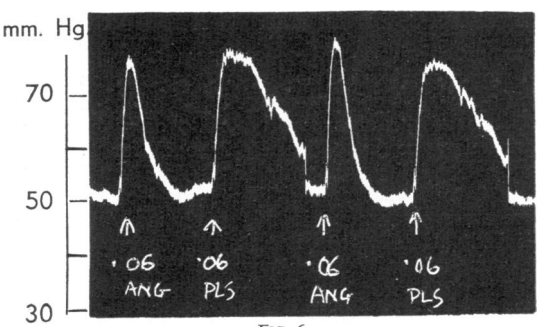

FIG. 6

intravenously. Dibenamine HCl or *NN*-dibenzyl-β-chloroethylamine (Smith, Kline, and French), 1 mg./ml. in faintly acid saline, prepared by dissolving 5 mg. dibenamine in 0.1 ml. 95% ethanol made acid (approx. 0.05 N) with conc. H_2SO_4 and then diluted up to 5 ml. with normal saline. Hexamethonium bromide, 2 mg./ml. in normal saline; dose, 1 to 2 ml. per rat, over a 10 min. period, intravenously. 5-Hydroxytryptamine creatinine sulphate, 20 μg./ml. solution in faintly acid saline (*p*H 4.5), freshly made each time from stock solution (100 μg./ml.); doses are expressed in terms of the base.

RESULTS

Quantitative Assay of Solution Angiotonin (Eli Lilly).—Fig. 1 demonstrates the blood pressure responses to increasing doses of the angiotonin solution which was used as reference standard. Fig. 2, which has been constructed from Fig. 1, shows the log dose-response regression line relating the responses (mm. Hg) to varying doses of angiotonin. A straight log-dose-response regression line could always be obtained by the method used. The preparation treated with dibenamine maintains a constant basal pressure and readily discriminates between doses differing by approximately 15%. The injections of small doses (0.02 to 0.6 cat units) can be made every 3 to 5 min. without tachyphylaxis developing, and the responses are satisfactory for about 50 injections.

The results of a pressor assay of the angiotonin are given in Fig. 3, from which data the value of M was calculated to be $\bar{1}.9693$; hence R=0.9317. After correcting for the dose levels injected, the ratio of unknown to standard was found to be 0.72: the true value was 0.75 (0.75 ml. of standard diluted with 0.25 ml. normal saline). The potency of the unknown was therefore estimated as 7.2 cat units/ml., with fiducial limits 6.64–7.70 cat units/ml. (7.4% error), instead of the known value of 7.5 cat units/ml. The slope b=56.1 mm. Hg, and the index of precision λ=0.036. Analysis showed that the variance due to regression was significant (P=<0.001), as was that due to the groups (P=<0.001).

Comparison of Pressor and Oxytocic Effects of Angiotonin with those of Posterior Pituitary Standard.—The results of a comparison of the pressor effect of angiotonin and of pituitary standard are given in Fig. 4. By calculation it was found that the Solution Angiotonin, 10 cat units/ml., contained pressor activity equivalent to 101.8 mu. of pituitary/ml. (approximately 1 mu. of pituitary ≡ 0.1 cat units) with fiducial limits 88.3 to 117.3 mu./ml. (14% error). The slope b=26.1, and the index of precision λ=0.064. Analysis of variance for deviation from parallelism showed that there was no significant difference of slope between the curves for pituitary and angiotonin (P=0.05–0.1). Part of the results of a comparison of the oxytocic effect of angiotonin and of pituitary standard is given in Fig. 5. It was found that the Solution Angiotonin, 10 cat units/ml., contained oxytocic activity equivalent to 7.5 mu. of pituitary/ml. (approximately 1 mu. of pituitary≡1.33 cat units) with fiducial limits 6.65 to 8.35 mu./ml. (11% error). The slope b=96.9, and the index of precision λ=0.064.

Effect of Thioglycollate (TG) on the Pressor Activity of the Posterior Pituitary, Angiotonin, and Plasma.—The pressor and oxytocic effects of the angiotonin and the pituitary standard seem to be qualitatively similar, the only difference being a rapid fall in blood pressure to the initial level after angiotonin (Fig. 6). With minute doses of angiotonin and pituitary standard the difference in responses may be undetectable. The possible occurrence of vasopressin in plasma samples or extracts makes it necessary to eliminate effects due to this substance when carrying out angiotonin assays of such samples. The differentiation between effects due to vasopressin and those due to angiotonin is readily effected by treatment of the plasma or extract with sodium thioglycollate before assaying. This treatment inactivates vasopressin but not angiotonin. Samples of plasma for these tests were obtained from patients with renal hypertension, at the onset of acute haemorrhagic nephritis. To serve as controls, several plasmas from non-nephritic and normotensive patients were

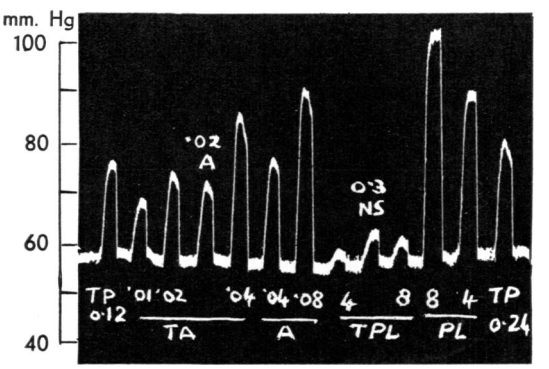

FIG. 7.—Blood pressure of rat (260 g.). Urethane. Dibenamine 2.08 mg. Pressor effects of pituitary standard (100 mu./ml.) and angiotonin standard (10 cat u./ml.) before and after 24 hr. treatment with 0.01 M-sodium thioglycollate at room temperature, and those of plasma pretreated with TG under similar conditions. TP, treated plasma; TA, treated angiotonin; TPL, treated pituitary standard; PL, pituitary standard; A, angiotonin standard; NS, normal saline. The numerals associated with the code letters indicate, for pituitary standards the dose in mu., for angiotonin the amount of standard solution in ml., and for plasma and saline the vol. in ml.

also tested. Blood was taken off into heparin and centrifuged at once; the separated plasma was chilled to 0° C. and tested as quickly as possible thereafter.

Fig. 7 demonstrates the effect of treating pituitary standard, angiotonin solution, and pathological plasma containing angiotonin-like activity, with 0.01 M-sodium thioglycollate for as long as 24 hr. The results show that TG in the stated concentration completely inactivates the pressor effects of posterior pituitary extract (100 mu./ml.) but not that of angiotonin (10 units/ml.). The results with plasma under similar conditions seem to demonstrate that the pressor action of the plasma also survives treatment with TG.

Fig. 8 provides more evidence that pituitary added to normal plasma (100 mu./ml.) has pressor activity about equal to that of pituitary standard

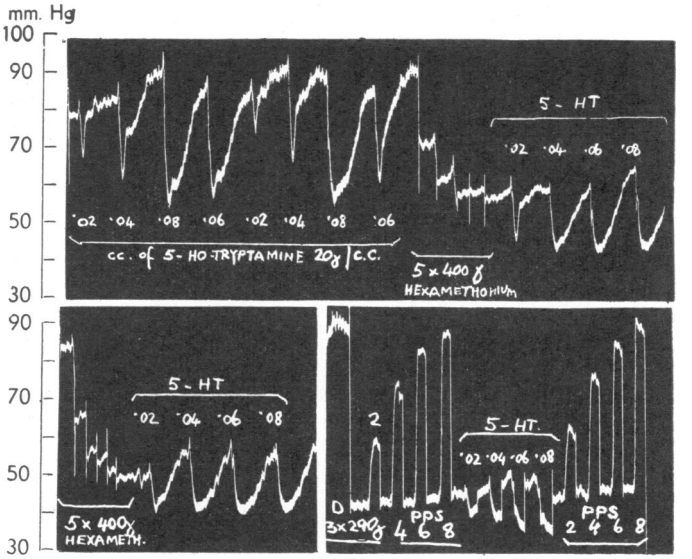

FIG. 10.—Blood pressure of rat (290 g.). Urethane. Hexamethonium 4 mg. Dibenamine 0.87 mg. Depressor effects of 5-hydroxytryptamine (0.4 to 1.6 μg.) before and after treatment with hexamethonium (twice) and dibenamine (D). PPS 2, 4, 6, and 8 mu. pituitary standard.

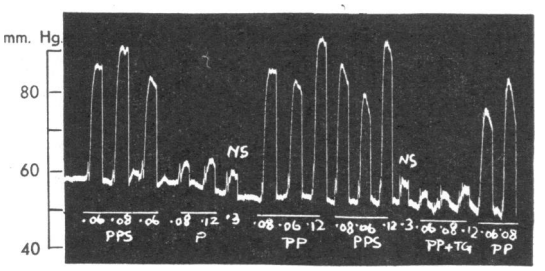

FIG. 8.—Blood pressure of rat (270 g.). Urethane. Dibenamine 1.89 mg. Pressor effects of pituitary standard (PPS 100 mu./ml.), normal plasma (P) and plasma with added pituitary standard (PP 100 mu./ml.). Note the absence of pressor responses to plasma containing added pituitary (PP 100 mu./ml.) after treatment with 0.01 M-sodium thioglycollate (PP+TG) for 40 min. at room temperature. NS, normal saline. Washed in with 0.2 ml. saline. Interval 8 min.

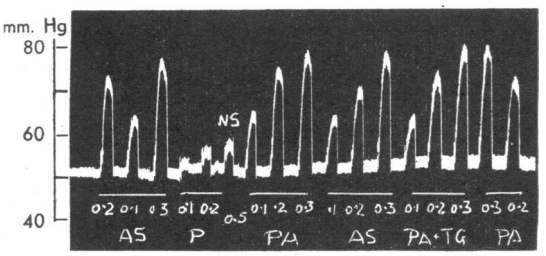

FIG. 9.—Blood pressure of rat (266 g.). Urethane. Dibenamine 1.6 mg. Pressor effects of angiotonin (AS, 2.5 u./ml.), normal plasma (P) and plasma with added angiotonin (PA 2.5 u./ml.). Note the presence of almost equipressor responses to plasma containing added angiotonin before and after treatment with 0.01 M-sodium thioglycollate (PA+TG) for 40 min. at room temperature. NS, normal saline. Washed in with 0.2 ml. saline. Interval 3 min.

(100 mu./ml.) and that the addition of 0.01 M-sodium thioglycollate to the plasma pretreated with pituitary completely inactivates its pressor effect within 40 min. On the other hand, angiotonin added to the same plasma survived the treatment with 0.01 M-sodium thioglycollate as shown in Fig. 9.

Effect of 5-Hydroxytryptamine on the Blood Pressure of the Hexamethonium-treated and Dibenamine-treated Rats.—Other pharmacologically active substances which may be present in plasma and which might interfere with the pressor assay are the vasotonins (Spätgift-Freund, 1920) released when blood is collected. It is likely that the most important of these is 5-hydroxytryptamine (Rapport, Green, and Page, 1948; Rapport, 1949).

Fig. 10 shows that 5-hydroxytryptamine exerts a depressor effect on the rat's blood pressure. This effect may be reduced by intravenous injection of hexamethonium, or dibenamine, or both, but it cannot be abolished.

It is unlikely that, if care is taken in the collection of the plasma, the small quantities of serotonin or histamine possibly present would be sufficient to interfere with the pressor assays. Landgrebe, Macaulay, and Waring (1946) have shown that relatively large doses of histamine are required to depress the blood pressure in the rat preparation. On the other hand, if present in sufficient amount, both serotonin and histamine

would tend to lead to an underestimate of the pressor activity.

Discussion

These results seem to demonstrate that the rat blood-pressure preparation, treated with dibenamine, is a simple, sensitive, and relatively specific method for the quantitative assay of angiotonin, and of the angiotonin-like activity of pathological plasma or plasma extracts. The pressor response is not interfered with by the possible presence of small amounts of pressor organic bases, nor by that of small amounts of vasopressin. Possible interference in the assay by the presence of small amounts of pressor organic bases is eliminated by the use of dibenamine, while possible interference by the presence of vasopressin can be avoided by inactivation of this substance by treatment of the sample with sodium thioglycollate.

Summary

1. A four-point procedure for the pressor assay of angiotonin, and the angiotonin-like activity of various samples of plasma and plasma extracts, has been developed using the rat blood-pressure preparation treated with dibenamine.

2. The pressor actions of the angiotonin standard and of the plasma samples were not due to adrenaline, noradrenaline, isoamylamine, tyramine, piperidine, or nicotine, since they survived the injection of dibenamine. The presssor actions were also not due to pituitary, since they survived treatment with 0.01 M-sodium thioglycollate.

3. The angiotonin solution contained pressor activity equivalent to about 100 mu. pituitary standard/ml., and oxytocic activity equivalent to about 7.5 mu. pituitary standard/ml.

4. The sensitivity of the rat blood-pressure preparation is about 20 times that of the cat preparation and about 80 times that of the dog preparation.

This work was carried out at the suggestion of Dr. G. C. Arneil of the Royal Hospital for Sick Children, Glasgow, whom I wish to thank for co-operating in the investigation of clinical aspects of this research which we are publishing separately. I am grateful to Dr. O. M. Helmer of the Eli Lilly Laboratory for Clinical Research, U.S.A., for a generous supply of "Solution Angiotonin" for this investigation. I am also grateful to Miss Margaret Harvie for her valuable assistance with the statistical work.

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