as great as that found in several other tissues. The kidney had many years ago been found to possess a high angiotensinase activity, according to Dr. Fasciolo. Dr. Bumpus remarked that the high angiotensinase activity of kidney probably accounted for the fact that angiotensin can never be detected in the urine. The point was emphasized by one speaker who had failed to find angiotensin in the urine even after infusion of high concentrations of the peptide into the renal artery. Dr. Morris found high angiotensinase activity in the vascular bed itself. He had infused angiotensin into the femoral artery of the isolated hind limb of a dog and assayed the effluent coming from the femoral vein. Despite the fairly high concentrations, most of the angiotensin had been destroyed before reaching the vein.

Dr. Gross discussed the experiments of Brunner, Regoli and Riniker, who had synthesized and examined β-angiotensin. This isomer of angiotensin which is resistant to angiotensinase *in vitro* did not produce a much prolonged pressor response. However, when it was injected intramuscularly, its action was found to be twice as great as and more prolonged than that of angiotensin. This finding is consistent with the idea that a high angiotensinase activity may be present in tissues other than blood. It was suggested that, in hypertensive patients, hypertensinase activity may be reduced.

SESSION 4

Chairman: Dr. Hubert Bloch

New Procedures for Measurement of Human Plasma Angiotensin and Renin Activity Levels

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THE need for specific, accurate and sensitive methods for the determination of angiotensin and renin activity in plasma and other biological fluids has become more acute since the demonstration of the powerful stimulating effect of angiotensin on aldosterone secretion and excretion and because of the accumulating evidence concerning the effect of angiotensin on sodium regulation and excretion.[1-5] Such methods are essential for a better understanding of the role of the renin-angiotensin system in human hypertensive disease and edematous conditions.

In this paper, we would like to describe, firstly, a much shorter method for the isolation and determination of free circulating plasma angiotensin and, secondly, a new method for the measurement of plasma renin activity.

MATERIALS AND REAGENTS

(i) Dowex resin 50W-X2, 100-200 mesh: Baker's Analyzed Reagent. Before use, 500 g. of resin is washed with 2 litres (l.) of 4N NaOH. The sodium salt of the resin is then washed with 1 l. of water, 1 l. of 2N HCl and again with 2 l. of water. Finally, the resin cake is washed with 0.2N ammonium acetate solution, pH 6, until the pH of the eluent reaches pH 6.

(ii) Angiotensin: Valine-5 angiotensin II, aspartic β-amide, synthetic Ciba Company preparation 19990-A. A solution of 1 microgram (μg.) per ml. in 20% aqueous ethanol kept at 4° C. is used as standard solution in all bioassays. This solution is prepared every two weeks.

(iii) Whatman No. 2 paper: "Especially selected for chromatography". This paper is washed in a soxhlet apparatus for 72 hours with a mixture of 95% aqueous ethanol:secondary butanol:isopropanol:water, 1:1:1:1 v/v.

(iv) Chromatographic jars: 24 x 12 inches lined with blotting paper and containing about 1 l. of the upper phase of n-butanol:water:acetic acid, 45:50:10 v/v.

(v) Palmer Manometer: Palmer, London, England. A capillary tube replaces the original manometer.

ISOLATION AND DETERMINATION OF FREE CIRCULATING PLASMA ANGIOTENSIN

Methods for measurement of blood angiotensin have been proposed in the past by Skeggs and by Paladini and their co-workers.[6-9] Our first procedure,[10] based on that of Paladini *et al.*,[8] was an attempt to improve the specificity, recovery rate and accuracy of previous methods. It had the advantages of quantitative recovery, high degree of

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*Research Fellow of "La Fondation pour Bourses en Médecine et en Biologie" of the Swiss Academy of Medical Sciences.
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specificity and sensitivity. However, the technique was laborious and time-consuming. The new procedure described below has the same degree of specificity and sensitivity as the previous one and has the great advantage of being more rapid. One technician may do six determinations within three days. The main differences from the previous method are: (1) the rapid withdrawal of blood and its separation at low temperature (0-5° C.), (2) the elimination of the ether-water partition, (3) a new form (NH_4+) of Dowex resin and different eluents, and (4) a different paper chromatographic system for the purification of the angiotensin fraction obtained from the column.

PURIFICATION OF PLASMA ANGIOTENSIN (Fig. 2)

Fifty to 100 ml. of arterial blood is rapidly cooled through a siliconized copper coil (internal diameter: 2 mm.; length: 140 cm.), immersed in crushed ice and drawn into a heavy wall glass flask containing 10 ml. of ammonium citrate* (3.8% solution, pH 6.5) or the same volume of the ammonium salt of EDTA (3.8% solution, pH 6.5) under partial vacuum.

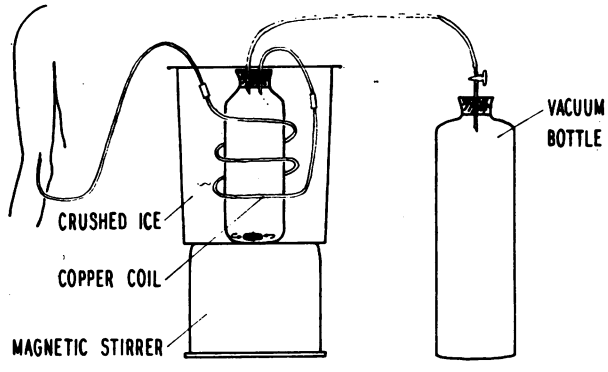

Fig. 1.—Schematic diagram for blood sampling. Arterial blood is taken from the brachial or femoral artery and drawn under vacuum into a bottle immersed in dry ice. A magnetic stirrer is used to cool the blood as rapidly as possible and to mix it with the anticoagulant.

The blood is stirred continuously with a magnetic stirrer during the blood sampling (Fig. 1). The time interval of flow from patient's artery to the sampling bottle varies from four to eight seconds. The temperature of blood at the distal end of the copper coil is 4-8° C. The volume of blood (plus anticoagulant) is measured, and the hematocrit (blood plus anticoagulant) is determined.

After centrifugation for 10 minutes at 3000 r.p.m. at 0-5° C., the plasma is collected, the volume measured and 1N hydrochloric acid is added slowly with continuous stirring until pH 6 is obtained. The plasma is transferred to a Dowex 50W-X2 (NH_4+) chromatographic column (1 cm. x 10 cm.) equilibrated at pH 6 and maintained at 0-5° C.

*Blood collected with heparin often produces a precipitate after 30 minutes at 0-5° C., thus hampering the flow of plasma through the resin column.

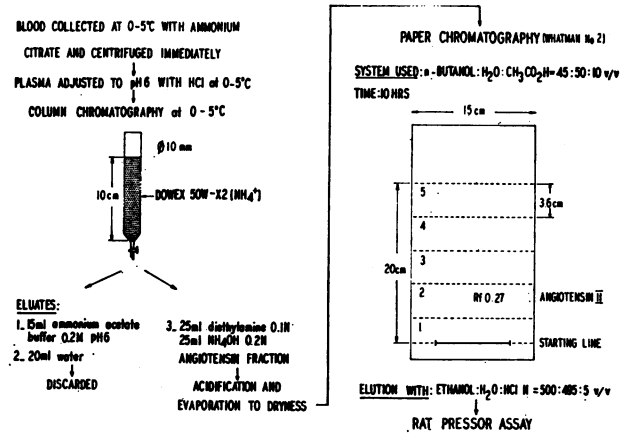

Fig. 2.—Flow sheet for the successive purifications of blood angiotensin.

The column is washed with 15 ml. of 0.2N ammonium acetate (pH 6) and 20 ml. of water. These two eluates are discarded. At this point, the column is transferred to room temperature. Angiotensin is now eluted with 25 ml. of 0.1N diethylamine followed by 25 ml. of 0.2N ammonium hydroxide into a flask containing a sufficient amount of concentrated acetic acid to acidify the eluate (until the appearance of yellow colour of the indicator mixture, two drops of bromethymol blue 0.05%—phenol red 0.05%). The eluate is evaporated to dryness in a conical flask connected to a small rotating evaporator. The temperature during evaporation is kept below 50° C.

The dry residue is dissolved into about 2 ml. of 80% aqueous ethanol and evaporated to dryness. This is repeated five more times to remove all traces of ammonium acetate. The residue dissolved in 0.3 ml. of 80% aqueous ethanol is then transferred on a straight line near one edge of Whatman No. 2 paper strip, 15 cm. wide. Three successive chromatographic washings of the residue-containing flask with 0.4 ml. of 80% aqueous ethanol and three more with 50% aqueous ethanol are made. The polypeptide extract deposited on the starting line is then impregnated with 1N HCl and dried. After a minimum of two hours' equilibration in the chromatographic jar, the ascending chromatographic separation is done with a solution of n-butanol-water-acetic acid, 45:50:10 v/v, during 10 hours, at room temperature (24° C. ± 1). During that time, the solvent front migrates 20 cm. The paper is allowed to dry.

The Rf value of angiotensin standard in this system is 0.27. But since the factors of temperature, humidity and others are critical and since minor variations may alter this Rf value, we always cut a strip of 1.8 cm. above and below the Rf value of 0.27 and two adjacent strips of 3.6 cm., one above and one below, in order to make sure that all angiotensin is recovered. Usually, the angiotensin material from plasma is located in the strip of the Rf value of the standard.

Elution by descending capillary action in a small flask is made with 30 ml. of a mixture of 95% aqueous ethanol:water: 1N HCl in a ratio of 500: 495:5 v/v. The eluates are evaporated *in vacuo* to dryness. The dry residues are then dissolved in about 2 ml. of 80% aqueous ethanol and re-evaporated to dryness. This process is repeated five more times to remove all traces of HCl.

1. *Bioassay Measurement*

The dry residues of the paper chromatographic eluates are dissolved in 0.4 ml. 20% aqueous ethanol (to prevent adsorption of angiotensin on glass) and assayed separately for pressor activity in a rat preparation described in the first procedure[10]—with the exception that rats nephrectomized 24 hours previously were used. More experience with this rat assay made it advisable to modify our assay and use nephrectomized rats which permit a more prolonged anesthesia and a more stable blood pressure baseline to be obtained. This preparation, with sensitive rats, enables us to detect 0.2 nanogram of standard angiotensin. This amount, when injected intravenously in the nephrectomized rat preparation, usually gives an average rise of 2-3 mm. Hg.

Only rats (120-150 g.) responding by a 10 mm. Hg rise or more to the intravenous injection of 5 nanograms of standard angiotensin are used. The unknown solution to be assayed is then injected in increasing amounts until a rise of 5 mm. Hg is obtained. Responses to the unknown solution and to the standard are compared at two dosage levels according to the principle of the 4-point assay. If repeated measurement can be made, the accuracy of the assay is of the order of 90%. But this precision may fall down to 20-25% if only one measurement is possible. Rises of less than 5 mm. Hg are reported as 0.

Since the volume and hematocrit value of blood and plasma volume are determined after the addition of anticoagulant, it is necessary to employ the following formula for determination of angiotensin in undiluted plasma:

$$\text{Angiotensin (nanograms/100 ml. undiluted plasma)} = \frac{\text{Angiotensin found in the extract} \times 100}{\text{ml. of diluted plasma used} \left[1 - \frac{1000}{\text{ml. blood (100-Ht)}}\right]}$$

For instance, 70 ml. (including 10 ml. of anticoagulant) of blood was the volume obtained from subject S.B. (Table VI). The hematocrit of this blood sample (containing the anticoagulant) was 50%. The volume of plasma used was 25 ml. The total amount of angiotensin found in the extract, using the above formula, was 10 nanograms.

$$\frac{10 \times 100}{25\left[1 - \frac{1000}{70\ (100 - 50)}\right]}$$

2. *Recovery Experiments*

Angiotensin added in amounts varying from 0.1 to 1 μg. to plasma (previously adjusted to pH 6 with 1N HCl and cooled to 0-5° C., and subsequently transferred on a Dowex 50W-X2 (NH_4+) resin column, at 0-5° C.) is quantitatively recovered (Table I).

TABLE I.—RECOVERY EXPERIMENTS (PLASMA)

No. of experiments	*Plasma (ml.)*	*Angiotensin added to plasma (μg.)*	*Recovery (%)*
1	50	1.0	100
2	45	1.0	100
3	50	0.1	80
4	35	0.1	80
5	50	0.1	80
6	30	0.1	95
7	20	0.1	110
Mean recovery			92

Plasma was adjusted to pH 6 with 1N HCl and cooled to 0-5° C., before adding the angiotensin.

In order to check the total recovery of angiotensin by this new procedure, the following experiments were carried out. A known amount of angiotensin varying from 0.01 to 1 μg. was added in the first sample of blood during blood sampling. A second sample was collected immediately after the first one, and if any angiotensin was found in the second, the experiment was discarded. The per cent recovery was calculated with the following formula:

$$\text{Per cent recovery} = \frac{\text{Angiotensin recovered} \times \text{(100-hematocrit)} \times \text{ml. blood}}{\text{Angiotensin added} \times \text{ml. of plasma}}$$

Eight recovery experiments were done and results varied between 71 and 116% (mean recovery: 83%) (Table II). Arginine vasopressin, noradrenaline, bradykinin and tyramine, when added to blood, are not recovered by this method. Bradykinin and most of the arginine vasopressin are lost on the Dowex resin column. In addition, the latter substance stays near the starting line in the chromatographic system used. Tyramine is lost in part in the first column eluate and moves much faster than angiotensin in the paper chromatographic system. Noradrenaline is not found on the paper chromatogram.

3. *Adsorption on Red Blood Cells*

After centrifugation at 0-5° C., the red cells were washed and centrifuged at the same temperature three times with 20 ml. of 0.9% saline solution to eliminate any residual plasma. The red cell fraction

TABLE II.—RECOVERY EXPERIMENTS (WHOLE BLOOD)

Number of experiments	Blood (ml.)	Plasma used (ml.)	Hematocrit	Angiotensin added during blood sampling (μg.)	Recovery* (%)
1.	126	48		1.0	71
Control	130	50	45	—	
2.	120	43		0.5	78
Control	120	48	47	—	
3.	112	40		0.5	92
Control	110	38	45	—	
4.	68	28		0.1	77
Control	70	28	47	—	
5.	120	24		0.1	80
Control	110	24	56	—	
6.	96	35		0.04	70
Control	80	30	53	—	
7.	92	27		0.04	80
Control	104	33	53	—	
8.	115	46		0.01	114
Control	110	43	42	—	
Mean recovery					83

*Results are uncorrected for the 6 to 10% losses due to adsorption on red blood cells.

was agitated with 40 ml. of 95% aqueous ethanol. This mixture was filtered at room temperature and the precipitate washed with 80% aqueous ethanol. The isolation of angiotensin was achieved according to the procedure previously described.[10] In three experiments, the angiotensin extracted following precipitation of red blood cells by 95% aqueous ethanol was 6, 7 and 10% of the total angiotensin added.

If, therefore, 6 to 10% of the angiotensin added to blood becomes adsorbed on red blood cells and cannot be recovered by this procedure, a mean recovery of 83% in eight experiments with a range of 71 to 116% is quite adequate.

4. *High-Voltage Electrophoresis*

In order to confirm the nature of the pressor polypeptide isolated, high-voltage electrophoretic separation was performed. The procedure used is the following. The samples of the "angiotensin material" obtained by this procedure are applied as a 1 cm. spot on a 40 cm. x 5 cm. Whatman No. 2 paper. This "starting spot" is impregnated with 0.1N diethylamine and dried, and the paper is moistened by spraying over it a buffer solution consisting of 0.016N trishydroxymethylaminomethane and 0.0033M cysteine hydrochloride, pH 8.4-8.5. A solution of 0.1M trishydroxymethylaminomethane, 0.01M EDTA and 0.2M boric acid (pH 8.4-8.5) is used in the electrodes vessels. The Whatman No. 2 paper is then placed on a plexiglass plate. The paper is then covered with Saran Wrap. The electrophoresis is conducted at 0-5° C., and a potential of 37V-cm. is applied over a 40 cm. x 5 cm. Whatman No. 2 paper. One hour later, the paper is removed, dried carefully, cut into 1-cm. strips at right angle to the direction of migration, eluted and submitted to the rat pressor assay preparation.

Human angiotensin obtained by incubation of human renin[11] with human plasma and the pressor material extracted from blood by this procedure show the same migration rate as does the standard valine-5 angiotensin II, aspartic β-amide (Table III).

TABLE III.—HIGH-VOLTAGE ELECTROPHORESIS

Sample	Migration (mm.)*
Angiotensin II β-aspartic amide	—10
Human angiotensin II	—10
Angiotensin I decapeptide	+20
Angiotensin II β-aspartic acid	+45

*As determined by the area of maximum pressor activity.

5. *Other Criteria of Specificity*

The "angiotensin material" from human plasma shows the following similarities with the standard synthetic valine-5 angiotensin II, aspartic β-amide: (1) identical Rf values in two different paper chromatographic systems: (a) n-butanol:water: acetic acid, 45:50:10 (Rf angiotensin: 0.27), (b) secondary butanol : isopropanol : water : phosphate buffer, pH 8, 7:7:5:2 (Rf angiotensin: 0.55). (2) Trypsin inactivation. Whenever enough pressor material was available, trypsin inactivation was carried out. In all cases tested, the pressor activity was completely destroyed. (3) Identical pressor response curve in the rat assay. (4) Identical migration rate in one electrophoretic paper system at high voltage, and in another system, at lower voltage.[10]

6. *Effective Angiotensin Plasma Levels*

Constant infusions of angiotensin were administered to three subjects at pressor rates. Arterial and venous blood samples were collected simultaneously for measurement of plasma circulating angiotensin, according to the above procedure, except for subject G.G., in whom two arterial determinations were made.

TABLE IV.—EFFECTIVE ANGIOTENSIN PLASMA LEVELS*

Subject	Average increase diastolic pressure (mm. Hg)	Nanograms/100 ml. plasma	
		Arterial	Venous
G.G.	30	Sample (a) 120	—
		Sample (b) 110	—
S.S.	18	270	90
P.L.	40	80	20

*By constant intravenous infusions of angiotensin at 1.5 μg./min.

The results are described in Table IV and indicate marked variations from 80 to 270 nanograms per 100 ml. of plasma in subjects receiving pressor infusions of angiotensin (1.5 μg./min.) with an increase of 20 to 40 mm. Hg in diastolic pressure. Similar variations have already been reported by Biron[12] with infusions given at a rate productive of a diastolic increase of 30 mm. Hg.

The amounts of angiotensin found in venous blood are considerably lower than those simultaneously found in arterial blood.

7. *Arterial Angiotensin Levels in Normal Subjects*

Arterial plasma angiotensin levels were determined in 20 normal subjects, without a family history of arterial hypertension (except for subject R.P., No. 5) (Table V). The mean angiotensin level in this group of normal subjects is 6.25 nanograms per 100 ml. of plasma. Fourteen of the 20 normal subjects have no detectable angiotensin in their arterial plasma. The upper limit of normal range is 35 nanograms per 100 ml. of plasma.

TABLE V.—ARTERIAL ANGIOTENSIN LEVELS IN NORMAL SUBJECTS

Patient	*Age*	*Blood (ml.)*	*Plasma (ml.)*	*Blood pressure (mm. Hg)*	*Angiotensin (nanograms per 100 ml. plasma)*
1. P.B.	20	122	46	128/70	0
2. C.C.	23	88	27	150/82	10
3. J.H.	22	110	39	120/60	15
4. A.M.	35	136	68	120/74	0
5. R.P.	36	120	45	120/72	30*
6. W.M.	26	138	38	140/72	0
7. J.T.	39	104	37	120/70	0
8. Y.D.	20	50	18	120/62	0
9. A.B.	27	90	33	120/80	10
10. E.Mc.P.	27	86	52	120/60	0
11. E.B.	52	120	47	120/70	0
12. R.N. (a)	19	100	40	120/70	0
(b)		100	41		0
13. P.P.	29	124	40	120/60	0
14. J.G.B.	23	92	32	118/60	0
15. J.B.	42	100	35	110/62	0
16. R.C.	18	122	34	120/80	0
17. A.D.	25	40	16	130/70	0
18. C.G.	32	84	30	132/70	25
19. C.D.	31	102	37	115/55	0
20. M.L.	23	110	35	110/60	35

*Mother hypertensive.

Plasma angiotensin levels were determined in peripheral venous blood of nine normal subjects (Table VI). All except one had no detectable angiotensin in their peripheral venous blood. The one exception showed a level of 55 nanograms per 100 ml. of plasma.

TABLE VI.
PERIPHERAL VENOUS ANGIOTENSIN LEVELS IN NORMAL SUBJECTS

Patient	*Age*	*Blood (ml.)*	*Plasma (ml.)*	*Blood pressure (mm. Hg)*	*Angiotensin (nanograms per 100 ml. plasma)*
1. S.B.	23	70	25	144/74	55%
2. R.C.	34	104	35	118/74	0
3. J.McC. (a)	37	112	40	118/54	0
(b)		106	35		0
4. J.G.P.	27	112	35	120/64	0
5. F.S.	35	116	37	124/76	0
6. W.S.	39	110	40	120/80	0
7. W.D.	53	92	27	140/72	0
8. A.S.	37	80	30	136/92	0
9. R.C.	33	88	37	120/70	0

II. MEASUREMENT OF RENIN ACTIVITY

It is of paramount importance to devise a method in which angiotensinase(s) will not interfere in the reaction of renin substrate, by inactivating part of the angiotensin as it is liberated, and to carry the incubation without first using procedures which, by destroying these proteolytic enzymes, may also markedly alter the state of the plasma. If this can be accomplished, the angiotensin liberated during incubation from the renin substrate reaction would reflect the true renin activity, as it probably occurs in blood. Furthermore, very low levels of renin activity could be detected simply by prolonging the time of incubation. Attempts to find adequate chemical inhibitors of angiotensinase(s) have been unsuccessful, but we have found the Dowex resin 50W-X2 (NH_4+) to be most suitable in protecting angiotensin from proteolytic enzymes during incubation.

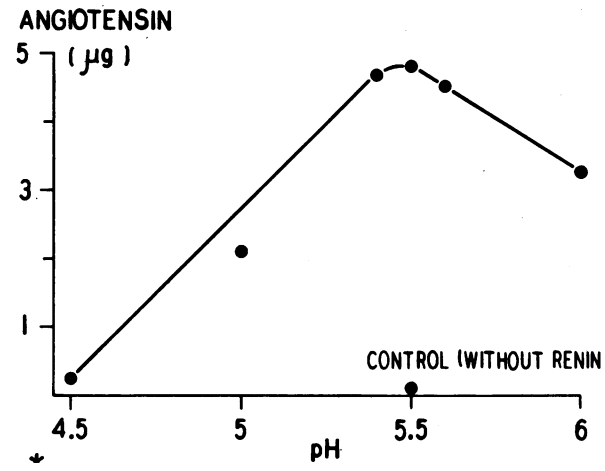

Fig. 3.—Influence of pH on the liberation of angiotensin (10 ml. of plasma, 0.04 dog unit of human renin, three hours, 37° C.).

Optimal conditions for determinations of renin activity were first studied. Results of the study of factors which may influence renin activity, such as pH and human renin concentrations, are shown in Figs. 3 and 4. A linear relation is obtained when amounts of plasma varying between 5 and 15 ml. are incubated with human renin (0.04 dog unit) at pH 5.5 for three hours at 37° C. This confirms essentially observations made previously by other workers.

PROCEDURE

Blood is obtained in the same way as for the angiotensin method. It is collected with the ammonium salt of ethylene dinitrotetraacetic acid

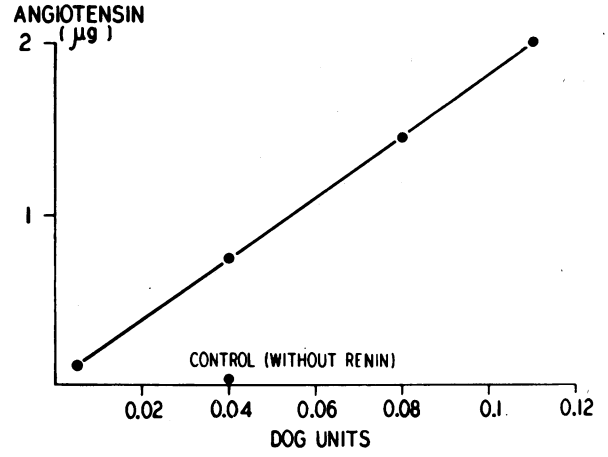

Fig. 4.—Influence of the concentration of human renin on liberation of angiotensin (10 ml. of plasma, pH 5.5, three hours' incubation at 37° C.). A linear relationship between angiotensin liberated and human renin in the range of 0.004 to 0.12 dog unit is obtained.

(EDTA) as anticoagulant, and is centrifuged immediately at 0-5° C. If the plasma is not processed at once, it can be kept frozen.

Cold plasma is adjusted to pH 5.5 by addition of 1N HCl and filtered on glass wool. Four millilitres of moist Dowex 50W-X2 (NH_4+) resin* is added in a siliconized Erlenmeyer flask (50 ml.) to which 10 ml. of plasma is added. The sample is incubated for three hours at 37° C. with vigorous shaking (about 200 strokes per minute). Following incubation, the mixture is transferred on a glass column (10 cm. x 1 cm.) already containing 1 ml. of Dowex 50W-X2 (NH_4+). The column is washed first with 15 ml. of ammonium acetate (pH 6) and secondly with 15 ml. of water. These two eluates are discarded. Angiotensin is eluted with 15 ml. of 0.1N diethylamine followed by 15 ml. of 0.2N ammonium hydroxide into a flask containing a small amount of concentrated acetic acid (see method for angiotensin determination). The eluate is evaporated to dryness in a conical flask. After sublimation of the ammonium acetate, the dry residue is dissolved in 1 ml. of 20% aqueous ethanol and assayed for pressure activity in the rat preparation described previously (see above). Renin activity is therefore measured in terms of the amounts of angiotensin formed under the conditions described.

The sensitivity of this procedure permits the detection of amounts of renin of less than 0.0004 dog unit.

1. Reproducibility

Results of six simultaneous determinations of renin activity on the same plasma indicate the high degree of reproducibility of the procedure (Table VII).

TABLE VII.—Reproducibility*

Experiment number	*Human renin added (dog unit)*	*Angiotensin found (μg.)*
1	0.04	2
2	0.04	2
3	0.04	2
4	0.04	1.7
5	0.04	2
6	0.04	2

*Human renin was added to six identical samples (10 ml.) of plasma from the same subject, and incubated for three hours at pH 5.5, 37° C.

2. Protection of Angiotensin by the Dowex Resin

First, angiotensin added in amounts varying from 0.1 to 5 μg. in nine different experiments to a mixture of 10 ml. of plasma and human renin (0.04 dog unit) is recovered in an average of 84%, after three hours' incubation, at 37° C., at pH 5.5 (Table VIII).

TABLE VIII.—Protection of Angiotensin by Dowex 50W-X2 (NH_4+)*

Experiment number	*Amount of angiotensin added (μg.)*	*Recovery (%)*
1	0.1	72
2	0.1	73
3	0.25	70
4	0.25	95
5	0.5	80
6	1.0	90
7	1.0	85
8	5.0	96
9	5.0	98
Mean recovery		84

*Angiotensin was added to plasma + resin, and incubated at pH 5.5 for three hours, at 37° C.

Secondly, in order to determine the protective efficiency of the resin, parallel incubations of plasma samples (with heparin used as anticoagulant), with a crude preparation of human renin (containing large amounts of angiotensinase), were done in duplicate under the same conditions, except that one of the samples was carried without the resin throughout the procedure. A substantial difference was obtained. Four micrograms of angiotensin was recovered in the experiment done in the presence of the resin, whereas only 0.2 μg. was obtained in the experiment without the resin.

Thirdly, a series of plasma incubations conducted in parallel with or without the Dowex resin, but with ammonium EDTA used as anticoagulant, showed a partial inhibition of angiotensinase in some plasma and a complete inhibition in others (Figs. 5 and 6). Such observations are in accord with the hypothesis that at least two different angiotensinases are present in some plasmas.[13] They also emphasize the importance of the use of the Dowex

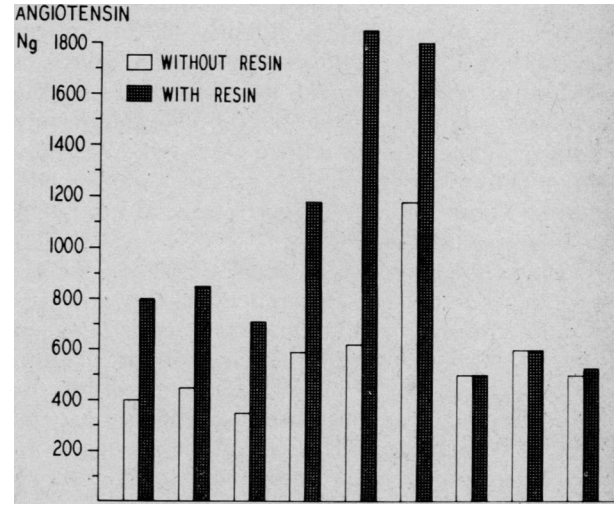

Fig. 5.—Effect of EDTA, with or without Dowex (NH_4+) resin, on angiotensinase(s) inhibition. 10-ml. samples from different patients (0.1M EDTA as anticoagulant), incubated with human renin (0.04 dog unit) for three hours at 37° C.

*A 5-ml. disposable syringe (TOMAC) is cut at the 0 mark to give the full opening of the cylinder. Such a device was found to be very convenient in the measurement of the 4 ml. of moist resin.

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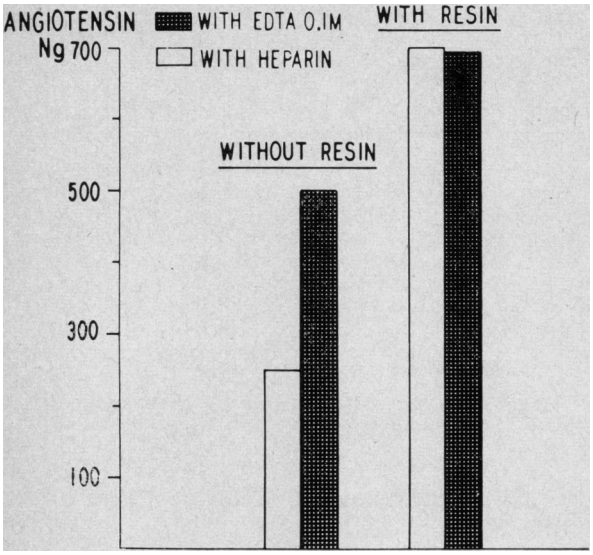

Fig. 6.—Effect of EDTA, with or without Dowex (NH_4+) resin, on angiotensinase(s) inhibition. 10-ml. samples of plasma from the same patient, incubated with human renin (0.04 dog unit) for three hours at 37° C.

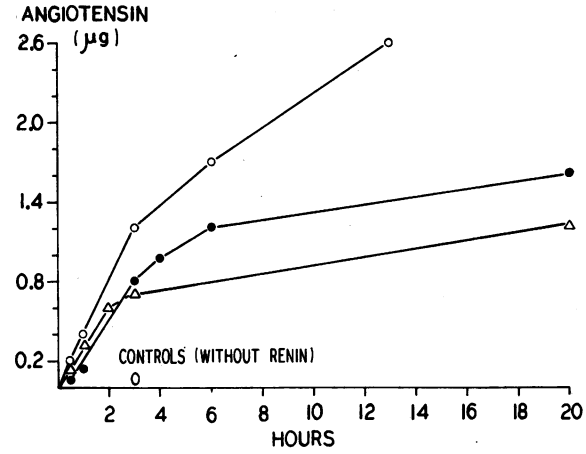

Fig. 7.—Effect of incubation time on angiotensin formation. 10 ml. plasma, incubated with human renin (0.04 dog unit), at pH 5.5, 37° C.

resin (NH_4+) for a more adequate protection of the angiotensin liberated during incubation.

3. *Criteria of Specificity*

The angiotensin obtained following incubation of plasma for measurement of renin activity showed the same criteria of specificity as those described in the method for angiotensin determination (see above).

4. *Factors Modifying the Amounts of Angiotensin Formed*

The possible effect of EDTA, used as anticoagulant, on the renin substrate reaction was studied. Two successive blood samples were collected from the same patient, one with heparin as anticoagulant and the other with EDTA. Parallel incubations were carried out with and without the protection of the Dowex resin, as illustrated in Fig. 6. In the presence of the resin, the same amounts of angiotensin were formed, independently of the anticoagulant used. This suggests that EDTA does not interfere in the renin substrate reaction. From the incubations without the resin, 500 nanograms were obtained in the sample with EDTA, whereas only 250 nanograms were found in the sample with heparin. These findings suggest a partial inhibition of angiotensinase by EDTA.

During the course of our work on renin activity, we noticed significant differences in the amounts of angiotensin released at various intervals between different plasmas, using the same amount of renin as illustrated in Fig. 7. The factors responsible for these differences are not entirely clear. Six experiments were done by adding 1 ml. of plasma from different sources (rabbit or patient) to one of two equal aliquots of plasma taken from the same normotensive subject. The two plasma aliquots were incubated simultaneously in the presence of human renin (0.04 dog unit), using the procedure described above. In two experiments out of six, only half the amount of angiotensin obtained in the parallel incubation (with the added plasma from rabbit or from another patient) was formed. These preliminary findings suggest that some plasmas contain a factor, or factors, which inhibits angiotensin formation, and would therefore be in agreement with the findings of Brunner.[14]

5. *Normal Values*

Twenty determinations in normal subjects showed a mean liberation of angiotensin of 154 nanograms ± S.E. 30 nanograms during the three hours of incubation. The range varied between 0 and 570 nanograms, with 17 determinations (85%) having levels less than 250 nanograms.

DISCUSSION

The polypeptide fraction eluted from the Dowex resin column is directly assayed for pressor activity in the nephrectomized anesthetized rat, without any further purification. The high concentrations of angiotensin generally present and the small amount of plasma used justify such a procedure. However, if only a small amount of angiotensin is released, interfering substances may be more prominent in their effects on the pressor response curve of the rat. In these instances, the unknown material should be submitted to chromatographic separation (see procedure for angiotensin) and, if enough is available, to trypsin inactivation.

The vasopressor lipid, recently reported by Khairallah and Page,[15] is not adsorbed on the Dowex resin column in the conditions described.

By incubating identical aliquots of the same plasma from a given patient for different periods of time (15, 30, 60 and 120 minutes), it is possible to obtain an index of the speed of the renin substrate reaction. At the present time, further work is being carried out along these lines in our laboratory.

Since renin has not yet been obtained in the pure state, little is known concerning its behaviour. Several factors, such as amounts of substrate, presence of different types of substrate, inhibitors, pH, ionic strength and other variables, influence the reaction catalyzed by this enzyme. Some of them are easily controlled; others, unfortunately, are not well known at the present time. Also, little is known concerning the kinetics of the converting enzyme catalyzing the transformation of angiotensin I into angiotensin II. In our opinion, until renin is isolated in pure crystalline form, we should speak of renin activity rather than renin concentration.

SUMMARY

We have described: (1) a new procedure for angiotensin isolation and determination that is much more rapid than the procedure previously described, and which has the same criteria of high degree of specificity, accuracy and sensitivity; and (2) a new method for the measurement of renin activity that permits detection of amounts of renin equivalent to less than 0.0004 dog unit.

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Variations in Plasma Renin Concentration in Several Physiological and Pathological States

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A METHOD has been developed for the estimation of renin in plasma, based upon the initial velocity of angiotensin formation when renin is incubated with a prepared substrate under standard conditions in the absence of angiotensinase. This method was first developed for the measurement of renin in rabbit plasma[1a, b, 2, 4] and has subsequently been adapted for use with man.[3, 10]

Renin is extracted from plasma by adsorption on diethylaminoethyl cellulose (DEAE), eluted, and then acidified to remove traces of substrate and angiotensinase. Substrate is prepared from ox serum, angiotensinase being removed by acidification at high molarity. A standard renin is made from kidneys of the appropriate species, and freed from angiotensinase and substrate.[4, 3] When renin is incubated with the prepared ox serum substrate under standard conditions of pH, temperature and substrate concentration, angiotensin is formed at an initial velocity which is proportional to the renin concentration. Samples are removed from the incubation mixture at frequent intervals, and their angiotensin content is estimated by bracket assay against synthetic valine-5 octapeptide angiotensin on the rat blood pressure preparation[5] (Fig. 1). By

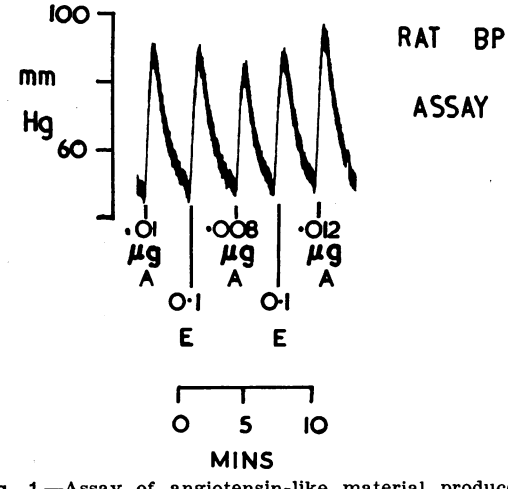

Fig. 1.—Assay of angiotensin-like material produced by incubating plasma extract with substrate. Comparison with synthetic asparaginyl valine-5 octapeptide (Hypertensin, CIBA). E = sample of incubation mixture; A = synthetic angiotensin.

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