THE PRESENCE OF RENIN ACTIVITY IN BLOOD VESSEL WALLS

By ANNE B. GOULD, Ph.D., LEONARD T. SKEGGS, Jr., Ph.D., and JOSEPH R. KAHN, M.D.

(From the Department of Medicine and Surgery, Veterans Administration Hospital, and the Department of Pathology, Western Reserve University, Cleveland)

(Received for publication, October 14, 1963)

The enzyme renin, which has been known to occur only in the kidney cortex, acts upon a plasma protein substrate to form the polypeptide angiotensin I (1). The cleavage of the dipeptide histidylleucine from the carboxyl terminal of angiotensin I results in the formation of the vasopressor hormone angiotensin II (2, 3).

A large increase in angiotensin has been shown to occur in arterial blood of patients suffering from malignant hypertension. Since the increase in circulating angiotensin found in patients with benign essential hypertension was very low in comparison (4), the possibility was suggested (5) that if angiotensin were formed locally by renin occurring in the walls of blood vessels throughout the circulatory system, it could cause an increase in peripheral vascular resistance without ever reaching a significant concentration in the blood. Such a mechanism would explain the slow, insidious onset of hypertension in patients with the benign form of the disease as well as in dogs following application of the Goldblatt clamp.

The purpose of this paper is to describe the preparation of extracts with renin activity from arteries, veins, and various other tissues, and to compare the properties of the artery extracts with those of kidney renin.

Experimental

Materials.—Renin was prepared from hog kidneys by the method of Haas (6) and standardized in Goldblatt units (1).

To prepare antirenin,[1] dogs were injected subcutaneously with 100 units of hog renin per day, 5 days a week for 3 months. At the end of this period, the dogs were bled, and the serum prepared. This serum containing 7.7 units of antirenin per ml (1 unit of antirenin will neutralize 1 unit of renin) was used without any further purification.

Partially purified renin substrate containing 2.7 units/mg protein and purified 270-fold from hog plasma was used as routine in these experiments unless otherwise indicated. Purified

[1] The method of preparing antirenin was obtained from Dr. Harry Goldblatt of Mt. Sinai Hospital, Cleveland.

type A renin substrate (7) of ultimate purity as judged by several criteria including countercurrent distribution, ultracentrifugal analysis and N-terminal determinations, was used in one experiment. In addition, synthetic tetradecapeptide substrate (8) was used in two experiments.

Polyethylene glycol (PEG) was prepared as a 50 per cent (w/v) aqueous stock solution of carbowax[2] 1540, mol wt 1300 to 1600.

Protein Determination.—Protein was determined spectrophotometrically by the Warburg and Christian method (9).

Preparation of Hog Artery Extract.—Frozen hog abdominal aortas with the attached renal, hypogastric, and iliac arteries were obtained from the slaughter house. The length of branching arteries included with the aortas varied from almost none to about 5 inches of iliac and hypogastric. Preparations which included the largest amounts of branching artery provided the most activity. The arteries were cut into small pieces, refrozen, and homogenized with dry ice at top speed in a Waring blendor. Enough dry ice was added to keep the arteries frozen during the 5 minutes of homogenization. The frozen artery powder, 300 gm, was extracted by stirring for 1 hour with 2 volumes of distilled water between 0° and 15°C and centrifuged at 1290 *g* in a refrigerated centrifuge. The residue was washed with 75 ml of distilled water and centrifuged at 13,820 *g* for 15 minutes. The resulting supernatants, after being strained through cheese cloth to remove fat particles, were combined, cooled to 0°C and the pH lowered to 1.6 by the slow addition of 1 N sulfuric acid. The acidification step not only aids in the purification of the extract, but also removes angiotensinase from the preparation. In order to assay at this state of the preparation, a 50 ml portion of the acid-denatured supernatant was dialyzed and concentrated by lyophilization. It was brought to 4 ml with water and a 0.5 ml aliquot was assayed for renin activity. To the remainder of the supernatant (about 550 ml), finely powdered ammonium sulfate was added slowly with mechanical stirring at 0° to 3°C to give a 0.5 M solution; the pH was maintained at 4.3 by the addition of 1 N sulfuric acid. The solution was centrifuged at 1290 *g* and the precipitate was discarded. Ammonium sulfate was added to the supernatant to 2.0 M at pH 4.3. The precipitate was recovered by centrifugation as before, dissolved in about 80 ml of 0.002 M phosphate buffer, pH 7.5, and dialyzed against 11 liters of the same buffer for 15 hours. The dialyzed material was centrifuged, and a 0.2 ml aliquot of the supernatant was assayed for renin activity.

The preparation of the artery extract through the second ammonium sulfate fractionation and dialysis is a modification of the Haas method (6) for the extraction and partial purification of renin. Fractionation of the acid-denatured extract with ammonium sulfate resulted in a 5-fold concentration, and a 2-fold purification.

The enzyme preparation was further purified by fractionation with polyethylene glycol (PEG). The remainder of the dialyzed supernatant was diluted to 10 mg of protein per ml, and PEG (10) was added dropwise at 0°C and pH 2.0 until a 14 per cent concentration of PEG had been reached. The 0 to 14 per cent fraction was separated by centrifugation (13,820 *g*) and discarded. PEG was added to the supernatant to give a 35 per cent solution. The 14 to 35 per cent fraction was separated by centrifugation as before, and dissolved in about 5 ml of 0.15 M phosphate buffer, pH 7.5. A 0.050 ml aliquot of this preparation was assayed for renin activity. The purity of the 0.5 to 2.0 M ammonium sulfate fraction was increased about 6-fold by PEG fractionation with a 76 per cent recovery. The data obtained from the processing of three batches of frozen hog arteries are summarized in Table I. The volume used in the foregoing method can be proportionately reduced, thus permitting the preparation of an active extract from as little as 5 gm of artery.

Enzyme Assay.—The fractions to be assayed were pipetted into 12 ml siliconized centrifuge

[2] Carbowax is the trademark of Union Carbide Chemicals Co., New York, for its polyethylene glycol products.

tubes containing 0.05 M phosphate buffer, pH 7.5, 2 units of renin substrate and 0.3 μM of phenylmercuric acetate (used as a bacteriostatic agent). The total volumes were adjusted to 1.0 ml. The reaction mixtures were incubated at 37°C from 1 to 6 hours, depending on the amount of enzyme present. Similar control samples were also prepared in which heat-coagulated extracts were used. At the end of the incubation period, 0.5 ml of buffer was added which contained sufficient sodium dihydrogen phosphate and hydrochloric acid to bring the pH to 5.5. The tubes were heated on a boiling water bath for 10 minutes, cooled, and centrifuged. The supernatant was assayed in the rat (11) and compared to a stock solution of angiotensin I, standardized in terms of Goldblatt units (1). The amount of renin in the preparations was estimated from the amount of angiotensin formed as compared to the amount formed under the same conditions when a standard renin preparation was used.

TABLE I

*Partial Purification of Artery Extracts**

Purification step	Protein	Specific activity	Recovery of starting activity
	gm	*units/gm of protein*	*per cent*
Homogenate	20.40		
Acid-denatured extract	2.38	0.148	100
0.5 to 2.0 M ammonium sulfate precipitate	0.873	0.347	86
14 to 35 per cent polyethylene glycol precipitate	0.116	1.99	66

* All values represent the average of three separate fractionations.

RESULTS

A Comparison of the Properties of Renin and Artery Extract.—

Reaction of artery extract with various substrates: Renin reacts with the natural plasma protein substrate (7) or the synthetic active portion of the natural substrate, the tetradecapeptide substrate (8) to form angiotensin I. The effect of incubating 5.4×10^{-4} units of hog artery extract with synthetic tetradecapeptide substrate (1 unit), partially purified substrate (1 unit), or type A purified substrate (2 units) can be seen in Fig. 1. In all cases, the reaction was conducted at 37°C in phosphate buffer, pH 7.5 containing 0.3 μM of phenylmercuric acetate. The total volumes were 1.0 ml. The rate of angiotensin formation is the same from all three forms of substrate.

The effect of substrate concentration on the rate of the renin-angiotensinogen reaction: The rate of angiotensin I formation, using 1.5×10^{-3} units of renin or artery extract and various amounts of substrate, has been studied. The values are plotted in Fig. 2 according to the method of Lineweaver and Burk (12), with the reciprocal of the velocity (units of angiotensin formed per hour) on the ordinate and the reciprocal of the substrate concentration in units on the abscissa. Varying the substrate concentration influenced the rate of angiotensin formation to the same degree whether the enzyme used was renin or artery

extract. That both enzymes react similarly with the synthetic substrate and the natural substrate under the experimental conditions is also evident from Fig. 2.

The effect of temperature on the rate of the renin-angiotensinogen reaction: The effect of temperature on the rate of angiotensin formation from renin substrate by either artery extract or renin can be seen in Fig. 3 where the log of the velocity constant is plotted against the reciprocal of the absolute temperature. The curve is identical for both enzymatic reactions in this temperature range 5° to 37°C. The activation energy of 15,300 cal/mole can be calculated from the slope of the curve (13).

The data necessary for determining the velocity constants in Fig. 3 are presented in Table II. The time of incubation was varied, depending upon the

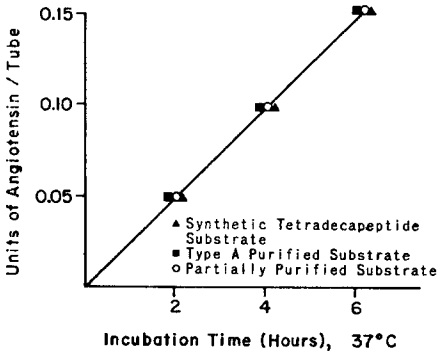

FIG. 1. Hydrolysis of renin substrates by hog artery extract

temperature and amount of enzyme present in the reaction mixture. Each value representing angiotensin I formation is one of a series of determinations made while the reaction was progressing linearly with time. The amount of renin substrate (angiotensinogen) decomposed by the enzyme is assumed to be equal to the angiotensin formed during the experiment.

Raising the temperature from 5° to 37°C caused a 100-fold increase in enzyme activity. This value is high if one considers a 2.5-fold increase in the rate constant for every 10°C rise in temperature as average for enzyme catalyzed reactions (13). Schaffenburg, Haas, and Goldblatt (14) have shown the activity of cat renin to be increased 90-fold by raising the reaction temperature from 2° to 37°C, and the activity of human renin to be increased almost 700-fold for the same rise in temperature.

The effect of preincubation with antirenin on enzyme activity: Antirenin (16), found in the γ-globulin fraction of serum, reacts with renin to prevent the subsequent hydrolysis of renin substrate to angiotensin. The manner by which

the inactivation of renin occurs is unknown: however, all evidence indicates that it is not a precipitin reaction (1).

To determine whether antirenin would react similarly *in vitro* with renin and artery extracts, 1.5×10^{-3} units of each were incubated with 0.77 units of antirenin and 0.05 M phosphate buffer (pH 7.5) at 37°C for 15 minutes. After

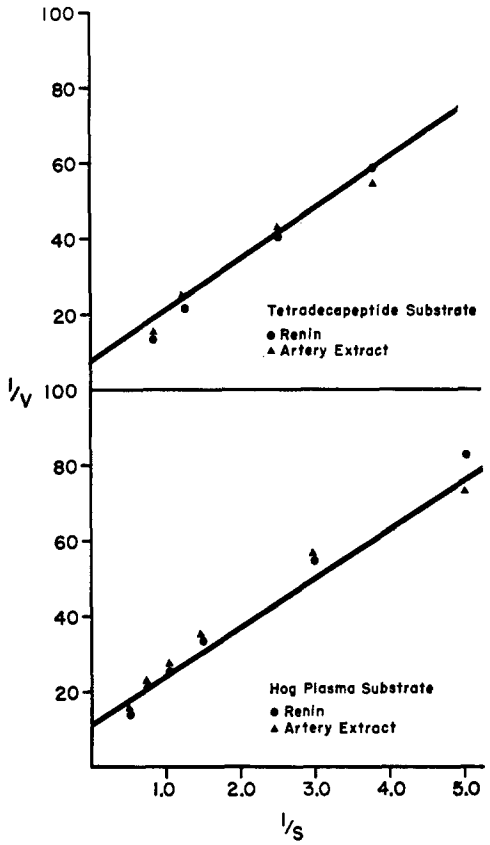

FIG. 2. Effect of renin substrate concentration on the velocity of hydrolysis.

incubation with antirenin, tubes were cooled, 2 units of renin substrate added, and incubation continued. The effect of preincubation of renin or artery extract with antirenin can be seen in the kymograph record in Fig. 4. Only a slight amount of vasopressor activity was found in those reaction mixtures which had been preincubated with antirenin in comparison to an identical sample not preincubated with antirenin. The source of the enzyme had no apparent effect on the inhibition of renin activity by antirenin.

pH Optimum.—Renin and artery extracts were found to hydrolyze renin

substrate most favorably between pH 6.7 and 7.5. Below and above these pH values, the activity began to decline. The influence of pH on the amounts of angiotensin formed by incubating 2.25×10^{-3} units of renin or artery extract with 2 units of renin substrate and 0.05 M phosphate buffer in a volume of 1 ml. for 1 hour at 37°C is shown in Fig. 5. The optimal pH for hydrolysis of renin substrate by either renin or artery extract appears to be the same.

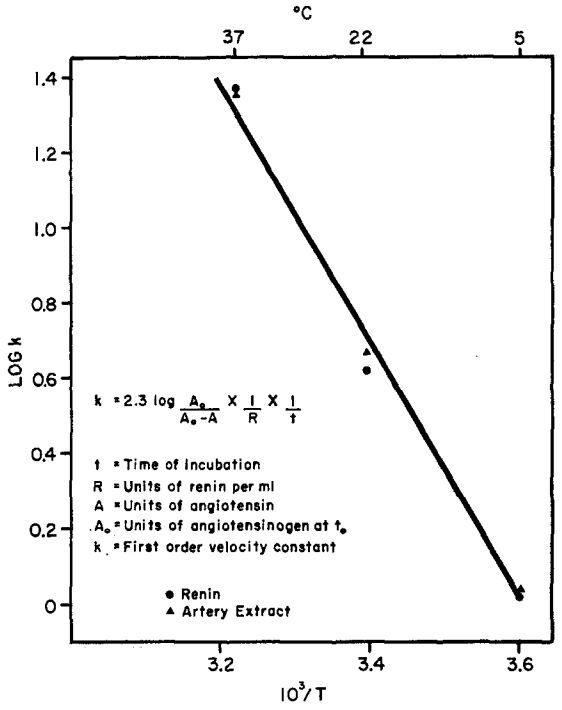

FIG. 3. Log velocity constants (hours^{-1}) of renin activity, as a function of the temperature.

Localization of Renin Activity Within the Artery—To determine the approximate location of renin activity within the artery, hog aortas were trimmed of adhering fat and connective tissue and divided into two groups. One group was unaltered. In the other group, the adventitia was carefully dissected away from the media and intima. Microscopic slides were prepared from representative samples of adventitia to confirm complete separation from the media. Arterial extracts were prepared in the usual way and assayed for renin activity. The amounts of renin activity found in the extracts were as follows: 3.6×10^{-5} units per gm aorta; 5.5×10^{-5} units per gm adventitia; and 1.8×10^{-5} units per gm media and intima. When the aortas were dissected so that instead of having a clean separation between the layers some of the media

TABLE II

Velocity Constants of the Renin Angiotensinogen Reaction*

Renin (R)	Angiotensin (A)	Angiotensinogen ($A_o - A$)	$\frac{A_o}{A_o - A}$	Velocity constant (k)
units/ml	*units/ml*	*units/ml*		
Hog kidney renin				
0	0	2.0	1.0	
0.0004 (37°C for 6 hrs.)	0.105	1.895	1.055	22.5
0.00256 (37°C for 1.5 hrs.)	0.188	1.812	1.103	24.8
0.0004 (22°C for 12 hrs.)	0.036	1.964	1.018	3.76
0.00256 (22°C for 6 hrs.)	0.137	1.863	1.073	4.57
0.0004 (5°C for 143 hrs.)	0.018	1.982	1.009	0.157
0.00256 (5°C for 97 hrs.)	0.107	1.893	1.057	0.224
Hog artery renin				
0	0	2.0	1.0	
0.0004 (37°C for 6 hrs.)	0.115	1.885	1.061	24.6
0.00256 (37°C for 1.5 hrs.)	0.188	1.812	1.103	24.8
0.0004 (22°C for 12 hrs.)	0.042	1.958	1.022	4.44
0.00293 (22°C for 6 hrs.)	0.176	1.833	1.091	4.95
0.0004 (5°C for 143 hrs.)	0.024	1.976	1.012	0.209
0.0032 (5°C for 54 hrs.)	0.085	1.915	1.044	0.249

* The velocity constants were determined according to Haas and Goldblatt (15).

FIG. 4. Effect of preincubation of renin and artery extract with antirenin on the production of angiotensin. Kymograph record of the rat pressor assay. 1, injection of 0.01 of a Goldblatt unit of angiotensin I. 2 and 5, injections of 0.13 ml of boiled supernatant from reaction mixtures containing renin and hog artery extract respectively. 3 and 6, these are the same as 2 and 5 except for preincubation with antirenin. 4 and 7, boiled enzyme controls. The time marks represent minutes.

was included with the adventitia, the amounts of renin then found in the extracts were as follows: 3.4×10^{-5} units per gm aorta; 6.1×10^{-5} units per gm adventitia and adjacent media; and 1.1×10^{-5} units per gm remaining media and intima. These determinations suggest the presence of a larger amount

of renin activity in the outer media. In summary, since there are about equal amounts of media and adventitia in hog aortas, nearly 75 per cent of the renin activity found in hog aortas is located in the adventitia and the remaining 25 per cent in the media and intima.

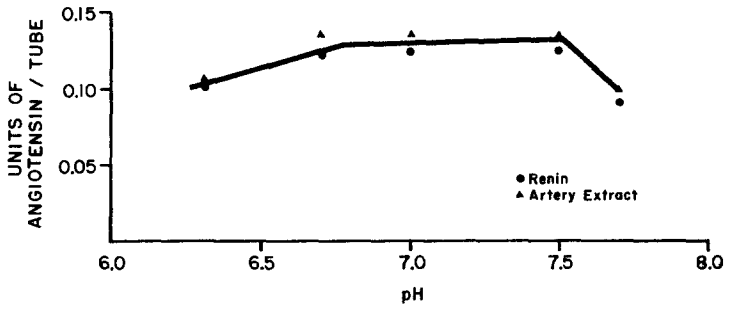

FIG. 5. pH optimum of renin and artery extract.

TABLE III

Extrarenal Sources of Renin Activity

Tissue	Amount of tissue represented in the assay	Vasopressor activity
	gm	*renin units/gm wet tissue*
Liver	4.4	11.0×10^{-4}
Artery	2.2	10.0×10^{-4}
Vein	2.8	6.14×10^{-4}
Lung	4.9	0.94×10^{-4}
Placenta	2.9	0.62×10^{-4}
Pregnant uterus	4.0	0.37×10^{-4}
Spleen	5.4	0.18×10^{-4}
Blood	8.0	0.11×10^{-4}
Skeletal muscle	4.7	None found
Heart	15.0	None found
Uterus	6.4	None found
Brain	9.0	None found

Hog kidney, extracted according to Haas (6), yielded 1 unit of renin/gm wet tissue.

Extrarenal Sources of Renin.—The physiological significance of extrarenal renin activity may depend, to a certain extent, on the prevalence of this enzyme throughout the organ systems. Extracts from a number of hog tissues were prepared as previously described for the preparation of artery extracts through the second ammonium sulfate fractionation and dialysis. In those tissues having only a small amount of renin activity, the amount of buffer used to dissolve the precipitate was reduced so that a larger amount was

represented in the assay. An aliquot of the enzyme preparation, varying from 0.2 to 0.65 ml, was incubated with 2 units of renin substrate, 0.3 μM of phenylmercuric acetate, and 0.05 M phosphate buffer in a total volume of 1 ml for various lengths of time up to 6 hours, depending upon the amount of activity in the samples. In this manner, as little as 0.5×10^{-4} units of renin representing as much as 15 gm of tissue can be assayed. Although the results of the tissue assays in Table III are expressed in renin units, no additional proof, other than the hydrolysis of renin substrate, was obtained to indicate that the enzyme was renin.

DISCUSSION

The presence of renin in artery extracts was first suggested by the work of Dengler (17) who recovered a substance from arterial walls which was similar to renin in that it is thermolabile, non-dialyzable, and forms a vasoconstrictive material when incubated with plasma. However, differences from renin were observed. Renin in amounts large enough to exert the same vasoconstrictive action in the rabbit's ear as a given amount of artery extract were far more active in causing an increase in blood pressure in the rat. In addition, cocaine did not enhance enzyme activity of renin and artery extract to the same degree. These dissimilarities were noted by Dengler who did not draw definite conclusions as to the identification of the active principle. Dengler's preparations, which were ammonium sulfate fractions prepared from saline extracts of acetone powders, may have included other vasoactive substances which could account for the differences which were observed.

In the present study, the enzyme activity of renin and artery extracts appear to be identical. The extracts were prepared according to the partial acid denaturation method developed by Haas (6) for the preparation of renin from kidneys, and were further purified by polyethylene glycol fractionation. Lowering the pH of the extract to 1.6 in the acid denaturation step destroys many contaminating enzymes, including angiotensinase. The artery extracts not only hydrolyze the synthetic tetradecapeptide renin substrate, but also purified type A hog plasma substrate. The reaction, in all respects, appears to be identical with the hydrolysis of the substrates by renin. The activation energy of 15,300 cal/mole, as determined from the slope of the curve in Fig. 3 is the same for both reactions. The effect of substrate concentration on the velocity of the reaction catalyzed by either enzyme is also the same. Both renin and artery extract are inhibited by antirenin, and both enzymes have similar optimum pH values.

The renin activity in the tissues that were examined may be localized in the walls of the arterioles and capillaries which supply blood to the organs. Most of the activity in the aorta was located in the adventitia, which is the site of the small capillaries of the blood vessels. The enzyme was found in vascular

tissues such as the placenta and pregnant uterus; however, the non-pregnant uterus contained no activity, or too little to be detected by this method. The liver is a very good source for this enzyme. The lungs and spleen also contained some activity, while none is present in the extracts of heart, skeletal muscle and brain.

The amount of renin activity in the walls of blood vessels and in extrarenal tissues is quite small compared to the kidney. A concentration of renin activity equal to 1 Goldblatt unit per kg of wet weight was found in arteries or liver while hog kidneys contain 1 unit of renin per gm. Although the amount of renin found in blood vessels is small, it must be remembered that it only takes 1.0 unit of renin to raise the blood pressure of a dog 30 mm of mercury mean pressure. A concentration of renin within arteries and tissues, possibly associated with capillaries and arterioles, may have a far greater effect on blood pressure than a similar amount of renin in the circulation.

At this time, the origin of extrarenal renin activity and its function are unknown. It may be produced in the kidney and transported to these various sites, or possibly be formed, used and stored within the blood vessel walls.

SUMMARY

The preparation of an extract of hog blood vessels and organs containing renin activity has been described. The extract hydrolyzes natural or synthetic renin substrate to form a vasopressor material. This reaction could not be distinguished from the hydrolysis of renin substrates by kidney renin. The activation energy, effect of substrate concentration on velocity, inhibition by antirenin, and pH optimum of the extract and of kidney renin are the same. Renin activity is found in the adventitia and media of the aorta, the liver and, to a lesser extent, in other vascular organs.

BIBLIOGRAPHY

1. Goldblatt, H., The renal origin of hypertension, *Physiol. Rev.*, 1947, **27,** 120.
2. Skeggs, L. T., Marsh, W. H., Kahn, J. R., and Shumway, N. P., The existence of two forms of hypertensin, *J. Exp. Med.*, 1954, **99,** 275.
3. Lentz, K. E., Skeggs, L. T., Woods, K. R., Kahn, J. R., and Shumway, N. P., The amino acid composition of hypertensin II and its biochemical relationship to hypertensin I, *J. Exp. Med.*, 1956, **104,** 183.
4. Kahn, J. R., Skeggs, L. T., Shumway, N. P., and Wisenbaugh, P. E., The assay of hypertensin from the arterial blood of normotensive and hypertensive human beings, *J. Exp. Med.*, 1952, **95,** 523.
5. Skeggs, L. T., Angiotensin and allied substances, Proceedings 21st International Congress of Physiological Sciences, Buenos Aires, 1959, 54.
6. Haas, E., Lamfrom, H., and Goldblatt, H., A simple method for the extraction and partial purification of renin, *Arch. Biochem. and Biophysics*, 1954, **48,** 256.
7. Skeggs, L. T., Lentz, K. E., Hochstrasser, H., and Kahn, J. R., The purification

and partial characterization of several forms of hog renin substrate, *J. Exp. Med.*, 1963, **118,** 73.

8. Skeggs, L. T., Lentz, K. E., Kahn, J. R., and Shumway, N. P., The synthesis of a tetradecapeptide renin substrate, *J. Exp. Med.*, 1958, **108,** 283.
9. Warburg, O., and Christian, W., Isolierung und Kristallisation des garungsferments Enolase, *Biochem. Z.*, 1941–42, **310,** 384.
10. Lentz, K. E., Skeggs, L. T., Hochstrasser, H., and Kahn, J. R., Countercurrent distributions of renin substrate and serum proteins in polyethylene glycol-salt systems, *Biochim. et Biophysica Acta*, 1963, **69,** 263.
11. Skeggs, L. T., Kahn, J. R., and Marsh, W. H., A method of assaying small amounts of hypertensin, *Lab. Inv.*, 1952, **2,** 109.
12. Lineweaver, H., and Burk, D., The determination of enzyme dissociation constants, *J. Am. Chem. Soc.*, 1934, **56,** 658.
13. Sizer, I. W., Effects of temperature on enzyme kinetics, *in* Advances in Enzymology, (S. P. Colowick and N. O. Kaplan, editors), New York, Interscience Publishers, Inc., 1943, **3,** 35.
14. Schaffenburg, C. A., Haas, E., and Goldblatt, H., Concentration of renin in kidneys and angiotensinogen in serum of various species, *Am. J. Physiol.*, 1960, **199,** 788.
15. Haas, E., and Goldblatt, H., On the existence of renol, a pressor substance of ischemic kidney, *Lab. Inv.*, 1958, **7,** 336.
16. Wakerlin, G. E., and Johnson, C. A., Reductions in blood pressures of renal hypertensive dogs by hog renin, *Proc. Soc. Exp. Biol. and Med.*, 1941, **46,** 104.
17. Dengler, H., Uber einen reninartigen Wirkstoff in Arterienextrakten, *Arch. exp. Path. u. Pharmakol.*, 1956, **227,** 481.