Theoretical Approaches to Estimation of Plasma Renin Activity: A Review and Some Original Observations

Suzanne Oparil

Performance of accurate, reproducible, and interpretable assays for plasma renin activity and other components of the renin/angiotensin system in the clinical setting requires a clear understanding of the various reactions in the renin/angiotensin cascade and the nature of their interactions. Plasma renin activity, the rate of angiotensin generation from plasma incubated in vitro, is the most commonly used clinical index of function in the renin/angiotensin system. Renin activity is measured by radioimmunoassay of angiotensin I generated in vitro under carefully controlled conditions. The value obtained for plasma renin activity depends on pH and duration of incubation and on the method used to protect the angiotensin I generated. We recommend incubation at neutral pH in buffered plasma for three hours in the presence of either ethylenediaminetetraacetate + 8-hydroxyquinoline + dimercaprol or ethylenediaminetetraacetate + phenylmethylsulfonylfluoride. Addition of a standard preparation of human renin to the plasma incubation step of the renin activity assay serves the dual purpose of permitting measurement of renin activity and furnishing an internal standard for comparison of assay procedures. The many variables among renin assay methods can be cancelled by referring to a common internal renin standard.

Introduction

Measurements of plasma renin (EC 3.4.99.19) activity are widely used in the clinical evaluation of hypertensive patients. Since systemic hypertension is the most common chronic disease treated by physicians in the United States, afflicting 15% of the population, appropriate diagnosis and treatment of these patients is an important health issue (1, 2). Effective drug treatment for hypertension is available and has been shown to reduce the incidence of strokes, heart failure, and renal failure in hypertensive populations (3, 4). Unfortunately, long-term medical treatment of hypertension is often unsuccessful because of excessive cost, inconvenience, and side effects of drugs. For this reason it is desirable to identify potentially curable secondary causes of hypertension when the patient's condition is first evaluated. Many of these secondary causes can be defined by abnormalities in the renin/angiotensin system. Further, recent studies suggest that it may be possible to subclassify “essential” hypertensive patients on the basis of their plasma renin activity and aldosterone excretion, in an attempt to individualize antihypertensive treatment (5, 6). Rational drug therapy based on defined pathophysiological mechanisms is expected to be more effective and free of side effects than existing empirical regimens.

Mechanism of Renin Action

A clear understanding of the clinical importance of plasma renin activity presupposes familiarity with the biochemistry and physiology of the renin/angiotensin system (7). The renin/angiotensin system is an important regulator of blood pressure and volume homeostasis in normal and hypertensive man. Figure 1 summarizes the biochemical events related to the action of renin. Renin is a highly specific proteolytic enzyme produced in specially modified cells in the afferent arteriole of the kidney near the glomerulus, the “juxtaglomerular cells.” Renin cleaves its substrate, an α-globulin synthesized by the liver, to produce the decapeptide angiotensin I. Converting enzyme (no EC no. assigned), a dipeptidylcarboxypeptidase found mainly in lung but also in circulating plasma and in peripheral capillary beds, removes the carboxyl terminal His-Leu from angiotensin I to produce the octapeptide angiotensin II. Angiotensin II, the most potent vasoconstrictor hormone known, also stimulates adrenal aldosterone production and thereby extracellular fluid...
Renin Activity

Plasma renin activity, the rate of angiotensin generation from plasma incubated in vitro, is the most commonly used clinical index of function in the renin/angiotensin system. Plasma renin activity correlates well with circulating angiotensin II concentration, but is much easier to measure because of the 50- to 100-fold increase in plasma peptide concentration after the in vitro incubation step. Renin activity can be measured by either bioassay or radioimmunoassay of the generated angiotensin.

Bioassay. Sensitive and reproducible methods for estimating renin activity by bioassay have been and are still being used for both investigative and clinical purposes in many laboratories. Most of the bioassays depend on the ability of angiotensin II, generated under standardized conditions in vitro, to cause smooth-muscle contraction. Preparations that have been used include isolated strips of rabbit aorta (16), rat uterus (17), and rat colon (18); intact, nephrectomized rats (19); and nephrectomized and ganglion-blocked rats (20). The measured biological response is smooth-muscle contraction in the in vitro preparations and systemic pressor effect in the intact animals. Responses are related to control injections of standard angiotensin II. The reason that bioassays for renin activity have not been widely used in clinical laboratories relates to problems of inconsistent standardization and reproducibility, the time-consuming sample processing, and the frequent lack of sensitivity and specificity.

Radioimmunoassay. Plasma renin activity is most commonly measured by radioimmunoassay of angiotensin I, generated in vitro as the product of the reaction of endogenous renin with endogenous substrate in the patient’s plasma, under carefully controlled conditions. After the initial incubation step, the reaction is stopped, usually by freezing. In a second step, 125I-labeled angiotensin I and anti-angiotensin I antiserum are added and allowed to equilibrate with the generated angiotensin I. Bound and free 125I-labeled angiotensin I are separated by conventional techniques and quantified by gamma counting. The amount of angiotensin I present is determined by comparison with a standard curve.

Failure to take into account the complexity of chemical events occurring during the incubation of plasma has contributed to the difficulty of developing accurate and reproducible assays for renin activity. The activities of renin, converting enzyme, and a number of other angiotensinases, as well as the availability of substrate and the possible presence of plasma proteases other than renin must be considered when selecting conditions for the initial incubation step (21). In addition, inhibitors and facilitators of the renin–substrate reaction have been described which may play a role in angiotensin generation in man (22–25).

Renin–Substrate Reaction

Renin reacts with substrate in circulating blood and in blood-vessel walls (26, 27) and isolated tissues (28,
cofactor (32, 33), several molecules are associated with the renin-substrate reaction. In the plasma of normal human plasma, the enzyme kinetics appear to be first order in the velocity of the renin-substrate reaction on the substrate concentration (34).

Renin substrate is a glycoprotein synthesized by the liver; several molecular species of it have been described (33, 35). Substrate concentrations vary with the state of hepatic function, glucocorticoid secretion, and estrogen production. Severe parenchymal liver disease in man is associated with depressed substrate concentrations and secondarily depressed plasma renin activity (36). Substrate concentration is increased after bilateral adrenalectomy (37) and in Addison's disease (38), increased after administration of exogenous glucocorticoid (39) or corticotropin (40) and in Cushing's syndrome (41). In contrast, mineralocorticoid administration and the syndromes of mineralocorticoid excess are not associated with increased substrate concentrations (42). Estrogen administration and pregnancy stimulate substrate production (37, 43, 44). Glucocorticoids and exogenous estrogen in the form of oral contraceptives can enhance substrate synthesis sufficiently to increase renin activity in vitro. Bilateral nephrectomy also stimulates increased renin substrate concentrations because of rapid inactivation of endogenous renin by the liver, with consequent failure to consume substrate, and because of increased substrate synthesis (45). The renin-substrate reaction rate is increased in nephrectomized and estrogen-treated animals (46-49). This has been attributed simply to increased circulating substrate concentration (50, 51). Alternatively, changes in the kinetics of the renin-substrate reaction have been described in plasma after bilateral nephrectomy or estrogen treatment (52-54), which could be related to changes in substrate or renin structure, the disappearance of a renin inhibitor, or the appearance of an activator. An analysis of the molecular radius, net charge, or isoelectric point of plasma renin substrate by use of polyacrylamide-gel electrophoresis and isoelectric focusing has shown that these do not change after bilateral nephrectomy or estrogen treatment (55). More extensive molecular characterization of renin substrate and more rigorous studies of renin-substrate kinetics under conditions of increased substrate production are needed before the increased velocity of the renin-substrate reaction after nephrectomy or estrogen administration can be fully explained.

Chemical alteration of the substrate molecule in vitro affects renin activity. For example, acidification of human plasma to pH 3.9 in an attempt to inhibit angiotensinases before angiotensin generation at pH 5.5 produced a 20% decrease in renin activity (16). Similarly, selective denaturation of human-derived substrate has been demonstrated on warming plasma to 32 °C for 1 h at pH 3.3 (56). In another study, the progressive acidification of human substrate (either in plasma or partially purified) before incubation with renin led to an irreversible loss of activity proportional to the pH decrease (57). Activity was decreased below pH 5.0; the decrease amounted to 40% at pH 4.0 and 80% at pH 3.0. The effect seemed to be greater when strong acids were used to lower pH than when buffer was used. Porcine renin and the purified tetradecapeptide substrate were less sensitive to acid denaturation. Acidification of renin alone produced no change in activity until the pH was less than 3.0. These studies emphasize the importance of substrate configuration in determining renin activity and the importance of the method of pH adjustment in preparation of plasma for radioimmunoassay.

Most current evidence indicates that renin attacks all forms of substrate at similar rates to produce the same angiotensin I. The amino terminal tetradecapeptide portion of the molecule is an adequate substrate for renin, which specifically hydrolyzes the Leu10-Leu11 bond in the substrate. A number of peptide analogs of portions of the tetradecapeptide sequence have been synthesized and shown to competitively inhibit the renin-substrate reaction (58). These are being used to explore in greater detail the structural requirements for renin action and the role of the renin/angiotensin system in maintaining pressure and volume homeostasis in vivo. Their potential use as reagents in in-vitro assays of components of the renin/angiotensin system has not yet been exploited.

Angiotensin I Metabolism in Plasma

The product of the renin-substrate reaction, angiotensin I, is rapidly removed from plasma by the combined action of converting enzyme and angiotensinases. The half-life of tracer amounts of 125I-labeled angiotensin I in heparin-treated plasma incubated at 37 °C in the absence of enzyme inhibitors is 3-5 min. The in vivo half-life of 125I-labeled angiotensin or native angiotensin I is even shorter—about one circulation time (15 s). Angiotensin I has little or no biological activity when circulating at physiological concentrations. It may be thought of as a circulating prohormone that is converted to angiotensin II by converting enzyme, which is found in highest concentration in the pulmonary capillary bed and in lesser concentrations in circulating blood, kidney, and various other systemic capillary beds (59-61). The production site of the converting enzyme in plasma is not yet certain, but it is assumed to be released into the circulation from lung and other tissues in which it is present in large quantities. Physiological regulation of its activity has not been described in man. Plasma converting enzyme activity is reportedly increased in patients with untreated active sarcoidosis and decreased in other forms of chronic lung disease (62), but the decrease was not quantitatively related to the severity of the lung disease, and steroid treatment of sarcoidosis restored activity to normal. Presumably these phenomena relate to alterations in quantity or
function of pulmonary converting enzyme, which is found in association with endothelial cell membranes (63). It is not known whether these alterations in converting enzyme activity cause changes in circulating angiotensin II concentration or circulatory homeostasis.

The substrate specificity and cofactor requirements of converting enzyme have been determined in considerable detail, both in vivo (64, 65) and in vitro (66–69). The kinetic properties of the enzyme partially purified from plasma and from mammalian lung are similar (70), but it is not certain whether the enzyme from the two sources is the same. The pH optimum for converting enzyme acting on angiotensin I is about 7.5. The reaction is chloride dependent (optimal Cl− concentration, 0.1 mol/liter) and divalent-cation dependent. Converting enzyme is stable to repeated freezing and thawing, but enzyme activity is lost if the pH of the medium is decreased to less than 4.5 (69). The enzyme is inhibited by a variety of peptides derived from the venoms of several species of snakes (71). One of these, the nonapeptide pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (pyr = pyrrolidone carboxylic acid), a long-lasting competitive inhibitor of converting enzyme, has been used in studies of the role of the renin/angiotensin system in blood pressure and volume homeostasis and as a diagnostic and therapeutic agent in angiotensin-dependent hypertension (72).

Angiotensin I is destroyed in plasma by a variety of proteolytic enzymes, the angiotensinases (EC 3.4.99.3). The best characterized of these are an aminopeptidase (angiotensinase A) with a pH optimum of 7.5 (73) and an endopeptidase (angiotensinase B) with a specificity similar to that of chymotrypsin (EC 3.4.21.1) and a pH optimum of 5.5 (30). The aminopeptidase is the predominant angiotensinase in plasma under physiological conditions. Its specificity is the same as that of aminopeptidase A (74), and it requires protein-bound calcium ion for activation. Angiotensinase A is blocked by modifications of the amino terminus of angiotensin I, such as substitution of D-aspartic acid, succinic acid, or sarcosine, or β-rearrangement of aspartic acid.

### Enzyme Inhibitors

Generated angiotensin I must be protected from converting enzyme and angiotensinases if the concentration of the decapptide is to accurately reflect renin activity. This cannot be done for angiotensin II, because conditions favorable for in vitro conversion also permit extensive angiotensinase action, causing simultaneous generation and destruction of angiotensin II. Despite the use of internal standards, this makes the assay of generated angiotensin II a cumbersome and nonreproducible measure of plasma renin activity. When plasma renin activity is assayed as generated angiotensin I, chemical inhibitors of both angiotensinases and converting enzyme are added to the incubation mixture to maximize recovery of the angiotensin I generated. Table 1 lists the most commonly used inhibitors of angiotensinases and converting enzyme and their mechanisms of action. Dimercaprol (BAL), 1 8-quinolinol sulfate, and ethylenediaminetetraacetate (EDTA) act as chelating agents. They are potent inhibitors of plasma angiotensinase A, which is Ca2+ dependent, and of converting enzyme, which appears to be Ca2+, Zn2+, and Co2+ dependent, because these ions—but not Fe2+, Co2+, and Mn2+—reverse EDTA inhibition (75). Disopropylfluorophosphate (DFP) acts as a phosphorylating agent and phenylmethylsulfonylfluoride (PMSF) as a sulfating agent, both binding covalently to enzyme serine. DFP is a potent inhibitor of angiotensinase B (30) but is ineffective against angiotensinase A and the converting enzyme in plasma (76). PMSF has a mechanism of action similar to DFP (77), so presumably has a similar enzyme specificity. It has recently been substituted for DFP in a number of plasma renin activity assay protocols because of its relative lack of toxicity. None of these enzyme inhibitors is known to interfere with the renin–substrate reaction or with the radioimmunoassay for angiotensin I. so all are acceptable reagents in the plasma renin-activity assay.

Alternatively, nonenzymatic methods of protecting generated angiotensin I have been used successfully in renin assays. Prior acidification of plasma to pH 3.5–4.0 destroys angiotensinases but also denatures substrate, and so exogenous substrate must be added (56). Adsorption of angiotensin to a solid support with a large surface area—such as Dowex 50, fuller’s earth, or charcoal—affords effective protection against angiotensinases and converting enzyme. The angiotensin I must then be eluted from the solid support with appropriate reagents, concentrated, and bioassayed or radioimmunoassayed. The anti-angiotensin I antibody molecule is used both for protection against enzymatic conversion and degradation and for quantitation of angiotensin I in the trapping method (78) for analysis of renin activity. This rapid and versatile method can also be used to determine renin and substrate concentration.

### Angiotensin I Survival in Plasma

The efficacy of angiotensinase and converting enzyme inhibitors in immunoassay can best be evaluated by testing the survival of angiotensin I in plasma treated

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<th>Table 1. Renin Inhibitors</th>
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<td><strong>Inhibitor</strong></td>
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<td>Dimercaprol</td>
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<td>Ethylenediaminetetraacetate</td>
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<td>Disopropylfluorophosphate</td>
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*a* Covalently bound to serine.
with the inhibitors and incubated under assay conditions. To maximize the sensitivity of this kind of evaluation, we prepared singly labeled 125I-labeled angiotensin I by the method of Nielsen et al. (79). The specific activity of the material was sufficiently high (estimated 750 kCi/mol) to permit its use as a tracer in femtomole quantities. Plasma from male anephric patients that was shown to contain no intrinsic renin activity was treated with EDTA (2.6 mmol/liter final concentration) and adjusted either to pH 5.5 with 0.5 mol/liter HCl or to 7.4 with 4 mol/liter Tris HCl. We used three inhibitor combinations, BAL (1.6 mmol/liter final concentration), 8HQ (3.4 mmol/liter final concentration), and EDTA (80); DFP (1.09 mmol/liter final concentration), neomycin sulfate (2.0 g/liter final concentration) and EDTA (81); and PMSF (15 mmol/liter final concentration) and EDTA. Monoiodinated angiotensin I, 2.0 × 10^5 cpm, or approximately 0.15 pmol, was added to each milliliter of treated plasma and the mixture was incubated at 37°C for 3 h. Aliquots of the reaction mixture were spotted on Whatman 3 MM paper and electrophoresed at 4000 V for 1 h in pyridine-acetate buffer, pH 3.55. The paper was dried, cut into 1-cm strips, and their radioactivity was counted in a gamma counter.

Figure 2 summarizes the results. The BAL/8HQ/EDTA combination resulted in preservation of 90% of 125I-labeled angiotensin I after 3 h at pH 7.4, but was less effective at pH 5.5. In contrast, DFP/neomycin/EDTA resulted in 85% preservation of 125I-labeled angiotensin I after incubation at pH 5.5 and only 10% preservation at pH 7.4. The PMSF/EDTA was nearly 100% effective at pH 5.5 and 85% effective at pH 7.4. Thus, selection of enzyme inhibitors for assay of plasma renin activity depends on the conditions of incubation: BAL and 8HQ are more effective at neutral than at acid pH; DFP is effective only at acid pH; PMSF is effective at both, but is slightly better at pH 5.5 than pH 7.4. These results are consistent with the known enzyme specificities of the inhibitors. It is important to consider the variability of inhibitor effectiveness when comparing the quantity of angiotensin I generated at different pH's in plasma.

Angiotensin I Generation Studies

Inhibitors. The pH/enzyme-inhibitor relationship was further explored by examining the effects of various inhibitor combinations on the shape of the pH "optimum" curves for the renin–substrate reaction in human plasma. Pooled EDTA-treated plasma from male anephric patients was divided into 1.0-ml aliquots and the pH adjusted at intervals of 0.5 pH unit between 4.5 and 9.0 with 0.5 mol/liter HCl or 0.5 mol/liter NaOH. Plasma was treated with the three combinations of enzyme inhibitors in concentrations described previously. After addition of partially purified human renin, 0.2 mU, (sample No. 13; kindly donated by Dr. Erwin Haas, Mt. Sinai Hospital, Cleveland, Ohio) to each tube, the mixture was incubated for 3 h at 37°C and radioimmunoassayed for angiotensin I (80). Figure 3 shows the angiotensin I generation curves. The apparent pH optimum for the reaction was 6.0 in the presence of all three sets of inhibitors. The quantity of angiotensin I generated at optimal pH was greater for PMSF than for DFP or BAL-8HQ. The curves for DFP and PMSF were steeper at high pH values than was the curve for BAL-8HQ, so that at physiological pH, generation of angiotensin I was 40% of maximum in the presence of DFP, 40% of maximum in the presence of PMSF, and 70% of maximum in the presence of BAL-8HQ. The variation in shape of angiotensin I generation curves with choice of enzyme inhibitor suggests that the curves reflect a titration of inhibitor effectiveness as well as a measure of renin activity. The apparent pH optimum was 6.0 with all three sets of inhibitors, confirming several previous studies in human plasma (30, 31, 83). The
amount of angiotensin I generated at optimal pH was 20% higher with PMSF-EDTA than with the other combinations; this presumably reflects its superior inhibitor effectiveness at acid pH under the conditions of this experiment.

To evaluate the effects of the various enzyme inhibitors on the apparent generation of angiotensin I from endogenous renin and endogenous substrate in clinical samples, we performed two kinds of experiments.

In the first studies, we pooled samples of EDTA-treated renal venous plasma from three patients with renovascular hypertension who had high endogenous plasma renin activity, and adjusted the pH to 5.7 or 7.4. The plasma was treated with BAL/8HQ or PMSF in the concentrations previously described, (plus EDTA), incubated at 37 °C for 3 h, and radioimmunoassayed for angiotensin I. An anephric plasma pool containing exogenous human renin, 5 U/liter, was processed similarly for comparison. Figure 4 shows that generation of angiotensin I was linear at both pH values with both sets of inhibitors. When incubation time was extended to 18 h, generation became nonlinear under all these conditions. The quantity of angiotensin I measured was greater and the slope of the generation curve steeper at pH 5.7 than at 7.4 with both PMSF and BAL/8HQ. The generation curves for exogenous renin in anephric plasma in the presence of BAL/8HQ or PMSF were superimposable, but the slope of the generation curve in the renal vein pool was flatter with PMSF than with BAL/8HQ at physiological pH, suggesting that the former inhibitor was less effective in the pooled renal venous plasma.

In a second set of experiments the efficacy of inhibition by PMSF and BAL/8HQ at physiological pH was compared in 48 successive unselected plasma samples submitted to our laboratory for renin assay. Incubation was for 18 h, and aliquots were removed at 3 and 18 h for renin activity determination. There was no difference in renin activity between the PMSF (2.8 ± 2.2 ng/ml per hour) and BAL + 8HQ (3.0 ± 2.8 ng/ml per hour) treated samples. During the 18-h incubations generation of angiotensin I was linear for low renin samples treated with PMSF but not for those treated with BAL/8HQ. Generation was not linear for normal- or high-renin samples treated with either inhibitor.

pH. Because the renin-substrate reaction takes place at pH 7.4 in vivo and because of concern about the effects of lowering pH on substrate reactivity and about artifactual generation of angiotensin I from substrate at low pH by action of acid proteases circulating in plasma (84, 85), pH 7.4 has been selected for the initial incubation step in many assays of renin activity (78, 80, 86). Other laboratories have used a pH of 5.5 to 6 for the renin-substrate reaction, based on the belief that this was closer to the pH optimum of the enzyme (81, 87–89). The concentrations of angiotensin I generated under these conditions were two- to fourfold those generated at pH 7.4 (81, 87). The interpretation of these data is complex, because both the apparent pH optimum of the reaction and the amount of angiotensin generated at any pH depends profoundly on the efficacy of inhibitors of angiotensin degradation, the reactivity of substrate, and, to a lesser extent, the contribution of enzymes other than renal renin to the generation of peptides that react with angiotensin I antibody.

To examine the question of whether plasma proteases other than renin contribute to the increased generation of angiotensin I at pH 5.5, we divided plasma samples from seven anephric patients into aliquots, adjusted either to pH 5.5 with 0.5 mol/liter HCl (81) or pH 7.4 with 4 mol/liter Tris HCl (80), and incubated at 37 °C for 18 h with appropriate enzyme inhibitors for each pH as previously discussed. Results are summarized in Figure 5. At pH 5.5, all seven samples generated substantial quantities of angiotensin I in the absence of added renin. At pH 7.4, no angiotensin I was detected in any of the samples. Survival of labeled angiotensin I under both sets of incubation conditions was identical. Thus, enzymes other than renal renin contribute to the generation of angiotensin I in plasma incubated at acid pH. These may be renin isoenzymes produced by other tissues or completely unrelated proteases. Their physiological significance is uncertain. In plasmas containing low concentrations of renin, the non-renin-related
generation of angiotensin I could make a significant contribution to total "renin activity."

To evaluate the effect of pH on endogenous renin activity in clinical samples containing optimal enzyme inhibitors, we subjected to radioimmunoassay plasma from 99 successive unselected EDTA-treated samples submitted to our laboratory for renin determination, with use of three different conditions: (a) pH 7.4, unbuffered, containing BAL/8HQ/EDTA; (b) pH 7.4, buffered with 4 mol/liter Tris HCl, containing BAL/8HQ/EDTA; and (c) pH 5.5, in the presence of DFP/neomycin/EDTA. There was a consistent increase in generation of angiotensin I on buffering the plasma at pH 7.4. This is compatible with the observation that plasma pH can increase by as much as one unit during a 3-h incubation at 37° C, resulting in conditions unfavorable for the renin-substrate reaction (51). Use of the concentrated Tris buffer minimized the dilution of plasma, which decreases the rate of angiotensin I generation. The mean renin activity of samples incubated at pH 5.5 (7.4 ± 1.2 ng/ml per hour) was significantly (P < 0.001) greater than for samples incubated at neutral pH (5.2 ± 0.8 ng/ml per hour). The greatest increases were seen in those samples with high renin activity. Low-renin samples frequently showed no change or a decrease in renin activity on adjusting the pH to 5.5 (21). Thus, although lowering the pH tended to increase apparent angiotensin I generation, there was considerable sample-to-sample variation in magnitude of the response. To the extent that this may represent variable sensitivity of substrate configuration to acid treatment or variable activation of plasma proteases other than renal renin, determination of renin activity in an acidic medium may represent an unphysiological measurement. Downward adjustment of plasma pH offers little advantage in terms of assay sensitivity, because low-renin samples show the smallest increases in apparent angiotensin generation. For these reasons, our laboratory prefers incubation at physiological pH. As long as appropriate enzyme inhibitors or an angiotensin-capture technique is used, incubation at acid pH is acceptable if care is taken not to lower the pH much below 5.0 and thus denature the substrate. A comparison of the most commonly used methods and detailed description of procedures can be found elsewhere (7,90) and are beyond the scope of this discussion.

Comparison of Bioassay with Radioimmunoassay

It is difficult to compare results obtained by bioassay with those obtained by radioimmunoassay of plasma renin activity because of variability in incubation conditions for generating angiotensin and in angiotensin standards. Dr. Bangham will address the problem of standardization in greater detail elsewhere in this symposium (Ed. note: This paper will also appear in Clin. Chem.) Normal values for renin concentration obtained by radioimmunoassay in a number of laboratories are reportedly lower than bioassay values, both based on an international renin standard (91). Other studies have given values for renin activity by radioimmunoassay that are higher than (81, 87, 92), comparable to (93, 94), or lower than (95) bioassay values. Critical comparison of procedures will require measurement of angiotensin generated under identical conditions by both bioassay and radioimmunoassay in the absence of toxic enzyme inhibitors with use of the same angiotensin standard (7).

Plasma Renin Concentration

Plasma renin concentration is the total quantity of renin present per milliliter of plasma. By reference to an internationally acceptable standard preparation of renin, it is possible to calculate the concentration of endogenous renin in serum or plasma (PRC) in terms of Goldblatt units (GU/ml of plasma). The plasma renin concentration is measured indirectly by adding graded amounts of renin to the first incubation step of the renin activity assay and determining the increase in reaction rate caused by the exogenous renin (78, 91, 97, 98). Concentration measured in this way correlates well with plasma renin activity in most clinical situations. Homologous, endogenous substrate, normally present in plasma, was used in some of these studies, while in others purified substrate was added in high concentration to enhance the rate of angiotensin generation and thereby to improve the precision of the assay. Under these conditions the angiotensin generation rate becomes independent of the substrate concentration and proportional to the renin concentration [zero-order kinetics (57, 78, 96)].

Excess ovine substrate has been used in the measurement of plasma renin concentration in man because at comparable substrate concentrations human renin reacts fivefold faster with ovine than with human substrate (56, 96). The incubation of human renin/ovine substrate is done at pH 7.4, the pH optimum for this combination. Potential interference from endogenous human substrate has been eliminated by preincubation of plasma at pH 3.3 to denature substrate (56). The most recent demonstration that addition of plasma from an anephric subject (high endogenous substrate) did not alter the rate of formation of angiotensin I from standard human renin reacting with sheep substrate suggests that the acid-inactivation step may be unnecessary (96). Because several times as much angiotensin I is generated after incubation with exogenous (ovine) substrate, the procedure has been applied to patients.
with low plasma renin activity, particularly the syndromes of mineralocorticoid excess, to increase the sensitivity of renin measurement (96).

Addition of a standard preparation of human renin to the first incubation step of the renin activity assay serves the dual purpose of permitting measurement of renin concentration and furnishing an internal standard for comparison of assay procedures. The many variables among renin assay methods can be cancelled by referring to a common internal renin standard. The principle of this renin-addition method is that under conditions of complete angiotensinase and converting enzyme inhibition, generated angiotensin I increases linearly with the amount of renin added. The concentration of endogenous plasma renin can be calculated from the slope of the line, which represents the quantity of angiotensin I produced per Goldblatt unit (see below) of added renin, and the intercept, which represents the amount of angiotensin I produced with no added renin (Figure 6). The human renin standard currently in use for this method is a moderately purified, angiotensinase-free preparation purified by Dr. Erwin Haas and associates (99) and distributed by the British Medical Research Council. The renin standard has been calibrated in Goldblatt units (G.U.) (1 G.U. = the quantity of renin or angiotensin which, when injected intravenously into a conscious, trained dog, raises the direct mean femoral arterial blood pressure by 4kPa [30 mmHg]) (100); 1 G.U. of standard renin generated angiotensin I at a rate of 0.56 µg/min during incubation in vitro with isologous substrate in angiotensinase-free human serum (101).

1 G.U. is equivalent to 1 IUB unit (Dr. Erwin Haas, personal communication). A sample calculation of endogenous renin concentration as units of renin in reference to Figure 6 follows:

\[
\text{Endog. renin acty. (ng of angiotensin/ml of serum)} = \frac{42.8}{44.5 \times 10^4} G.U. \text{ of renin/ml of serum}
\]

Assuming that angiotensin I generation from endogenous renin is linear with time, for a 16-h incubation:

\[
\text{Renin activity} = \frac{42.8}{16} = 2.68 \text{ ng of angiotensin/ml per hour}
\]

For routine purposes, assays can be done with only two samples (no added renin and a single concentration of added renin). The slope then becomes the difference between the two angiotensin concentrations divided by the concentration of added renin (97). If standard human renin can be supplied in adequate quantity, the use of an internal renin standard is recommended for general use in quality control of renin assays and in standardization of investigative results (91).

In general, plasma renin activity and plasma renin concentration are very highly correlated, so it is not necessary to measure both for clinical purposes. There are some exceptions. Conditions of estrogen excess such as oral contraceptive pill administration are associated with increases in substrate concentration sufficient to cause elevations in plasma renin activity. Plasma renin concentration remains normal under these circumstances, presumably due to feedback suppression of renin release by angiotensin II (102–104). Renin concentration far in excess of renin activity has been reported in the plasma of a hypertensive child with Wilm's tumor (105). The tumor was shown to produce a high-molecular-weight form of renin, which was activated in the acidification step of the renin concentration assay. The "big renin" secreted by the tumor was considered to be a possible renin prohormone. Substantial increases in renin activity and content over time in frozen stored plasma have recently been reported and attributed to activation of a circulating prorenin (106). Further investigation is needed before the status of renin prohormone(s) can be assessed.

Circulating Angiotensin I, Angiotensin II, and Renin Substrate Concentrations

Assay procedures with sufficient sensitivity to measure circulating concentrations of angiotensin I (107) and angiotensin II (108–114) in normal man, if plasma-extraction procedures are used, are available for general use. Values for angiotensin II correlate well with those for renin activity (80, 81), but the assay for angiotensin II is seldom performed in clinical practice because the extraction procedures required are time consuming and nonreproducible. Development of a satisfactory method for circulating angiotensin I was delayed by inability to stop the renin-substrate reaction in vitro. Collection of blood into cold ethanol provided a satisfactory solution to that problem (107), but has not eliminated the tedious concentration steps. For that reason, neither circulating angiotensin I nor angiotensin II is commonly measured in the clinical setting.

Renin substrate can be measured indirectly by quantifying the angiotensin generated from the renin-substrate reaction done in the presence of excess homologous renin. Many techniques are available for measurement of generated angiotensin I and II, (16, 30, 115–117), angiotensin II (118), or angiotensin I (41, 119)
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