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MATURATION OF THE RENIN-ANGIOTENSIN SYSTEM: AGE-RELATED TRANSITIONS IN THE RATE-LIMITING REACTION

presented by

Kendall Bruce Wallace

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Physiology

Major professor

Date 6/15/79

MATURATION OF THE RENIN-ANGIOTENSIN SYSTEM: AGE-RELATED TRANSITIONS IN THE RATE-LIMITING REACTION

By

Kendall Bruce Wallace

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1979

ABSTRACT

Maturation of the Renin-Angiotensin System:
Age-Related Transitions in the Rate-Limiting Reaction

by

Kendall Bruce Wallace

The renin-angiotensin system is a composite of several hydrolytic enzymes acting in concert to determine the steady-state concentration of angiotensin II (AII) in plasma. In adults, the stoichiometry of the renin-angiotensin system is such that the steady-state concentration of AII is limited by the rate of generation of angiotensin I (AI). However, age-related differences in stoichiometry of this system suggests that the relative contribution of renin, angiotensin-converting enzyme (ACE), and angiotensinases in regulating plasma AII in newborns is different from that in adults. The purpose of this investigation was to correlate the maturation of individual enzyme activities with the steady-state concentration of AII in an attempt to identify specific factors responsible for regulating plasma AII concentrations throughout development.

Plasma renin concentration (PRC) increased following birth to maximum values at 3-weeks of age. PRC declined thereafter to adult values by 6-weeks postpartum. In constrast, renal renin content increased in a linear fashion between 1- and 6-weeks postpartum, indicating that the changes in PRC were not due to changes in renal renin availability.

The postnatal changes in plasma renin activity (PRA) paralleled that for PRC. The consistently greater PRC than PRA early during development indicated that the relative concentration of angiotensinogen is low in newborns. Convergence of PRA and PRC in older animals suggested a progressive saturation of renin with substrate as reflected by the age-related increase in specific activity of renin.

The renin-substrate reaction in both adult and newborn plasma conformed with that predicted by first-order reaction kinetics indicating that the rate of formation of AI is dependent on both PRC and the concentration of angiotensinogen. Since the endogenous substrate concentration was considerably less than the Km, the age-related increase in angiotensinogen concentration leads to the progressive increase in the specific activity of renin.

The reciprocity between PRA and AII during the first 6 weeks postpartum indicates that the rate of formation of AI is not the predominant factor regulating plasma AII concentration early during development. Furthermore, postnatal changes in AII inactivation are inconsistent with the increase in AII concentration between 3- and 6-weeks postpartum.

Pulmonary ACE activity appeared late during gestation and increased thereafter throughout the first 6-weeks postpartum. The increase in ACE activity was attributed to a greater enzyme concentration rather than further activation of pre-existing enzyme. Correlation of ACE content with lung weight suggested that the increase reflected <u>de novo</u> synthesis of enzyme associated with a general increase in functional mass of the lung. The age-related increase in ACE content of cell-free preparations of lung tissue was reflected

by a decreased ability of intact lungs from 7-day old rats to clear AI from the circulation. However, enzyme density alone could not account for the differences in AI clearance. Since AI metabolism by isolated, perfused lungs was a flow-limited process, the progressive increase in right ventricular output during development further enhances the age-related increase in the rate of liberation of AII into the pulmonary venous and, subsequently, the systemic circulation.

The limited ACE availability and perfusion of immature lungs may explain the dissociation between PRA and AII during the first 6-weeks postpartum. The subsequent decrease in plasma AII concentration after 6 weeks indicates that pulmonary conversion of AI to AII probably does not limit AII production in older animals. This transition of the rate-limiting step of the renin-angiotensin system may be important in determining the effect of physiological and pharmacological intervention on the steady-state concentration of AII in plasma of developing animals. Although the renin-angiotensin system appears to be functional shortly following birth, transitions in the rate-limiting reaction suggest quantitative differences in the responsiveness of this system to alterations in normal body fluid homeostasis during development.

ACKNOWLEDGEMENTS

I wish to express my genuinely sincere appreciation to Dr. Michael D. Bailie for his generous professional counsel and support throughout my graduate program. This dissertation would not have been possible without the alliance entrusted in me by Dr. Bailie. I would also like to thank Dr. Jerry B. Hook for his continued guidance and support which were invaluable to my graduate experience. I especially appreciate the excellent constructive criticisms given by Dr. Robert A. Roth who was instrumental in developing my professional research attitudes. Dr. Jerry B. Scott and Dr. N. Edward Robinson were also very helpful in the preparation of this thesis.

I wish to acknowledge the technical assistance of Mr. Bruce G. Hook, L.E. Dawson, and Miss Sue L. Glashower, all of whom worked diligently to collect the data necessary for completion of this investigation. The superb clerical qualities of Miss Diane K. Hummel provided for the most efficient preparation of this dissertation and facilitated immensely the progress of my graduate work.

I am especially grateful for the never-ending support, encouragement and understanding offered me by my parents Mr. and Mrs. Lynn A. Wallace, my sister Sue Ann and her husband Ronald A. Rosko, my brother Robert L. and his wife Deltha L. Wallace, my younger brother Jeffrey J. Wallace, and my grandmother Mrs. Clara M. Smith. Their steadfast

caring and interest and the security they provided were vital for the efficient completion of this thesis. In view of their significant contribution toward helping me achieve this goal, I dedicate this dissertation to Mom and Dad, Sue and Ron, Bob and Del, Jeff, and Gram as a token of my sincere appreciation for the closeness our family shares.

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INTRODUCTION

The renin-angiotensin system has been implicated in the regulation of peripheral vascular resistance as well as in the control of plasma volume and electrolyte composition (Gross et al., 1965; McGiff and Nasjletti, 1976; Oparil and Haber, 1974; Page and Bumpus, 1961; Peach, 1977; Peart, 1965, 1975; Regoli et al., 1974; Reid et al., 1978; Skeggs et al., 1977). Although angiotensin II (AII) does not appear to be involved in the control of resting blood pressure, inhibition of AII production significantly reduces the blood pressure response to stressful stimuli (Laffan et al., 1978; Laragh et al., 1977; Ondetti et al., 1977; Rubin et al., 1978; Thurston and Swales, 1978; Vollmer et al., 1978; Williams et al., 1978).

The contribution of this system to the regulation of blood pressure and body fluid composition appears to be greater in newborns than adults (Mott, 1975). Removal of small fractions of the total blood volume caused proportionately less reductions in arterial blood pressure of immature rabbits compared to adults (Mott, 1965). This greater ability of newborns to sustain hemorrhage was not dependent on baroreceptor innervation or α-adrenergic nerves, but was abolished by bilateral nephrectomy. In contrast, nephrectomy in adult rabbits did not affect their response to blood-letting (Mott, 1969). Bilateral nephrectomy of rabbits less than 1-day old reduced arterial pressure

by 54% (Pipkin et al., 1971). In adult rabbits, removal of both kidneys produced a similar decrement in blood pressure, however, the percent reduction was less in adults due to the increased mean arterial blood pressure. In lambs less than 20-hrs old, nephrectomy resulted in a 25% reduction in arterial pressure whereas similar surgical procedures did not affect resting blood pressure of lambs 1-5 days of age (Pipkin et al., 1974a). It is possible that the greater effect of bilateral nephrectomy in immature animals is related to the enhanced activity of the renal pressor system in newborns compared to adults (Mott, 1975). This postulate is supported by the greater increases in plasma AII concentration of immature animals following hemorrhage compared to that of adults. Removal of 25% total blood volume increased plasma AII concentration in newborn rabbits from 1160 pg/ml to 1680 pg/ml. Plasma AII in adult rabbits increased from 225 pg/ml to 340 ng/ml following 25% blood-letting (Pipkin et al., 1971). The absolute increase in AII was 4-fold greater in newborns compared to adults, however, the percent increase in plasma AII concentration was similar for both ages. Similar results were observed following 25% hemorrhaging in newborn lambs compared to adult sheep (Pipkin et al., 1974a). Reduction of blood volume following furosemide-diuresis also resulted in a greater increase in renin release from kidneys of fetal and newborn lambs compared to adult sheep (Trimper and Lumbers, 1972). In conclusion, evidence concerning the greater ability of immature animals to sustain reductions in blood volume is consistent with a greater reactivity of the renin-angiotensin system of newborns compared to adults. Accordingly, this renal pressor system is

apparently more influential in regulating plasma volume and body fluid composition of immature animals than adults.

In adults, the stoichiometry of the renin-angiotensin system is such that AII availability appears to be regulated primarily by its rate of generation as opposed to the degradation of circulating AII (Hodge et al., 1967; Sambhi and Barrett, 1968). Plasma renin concentration (PRC) has been employed as an index of the overall activity of the renin-angiotensin system. However, recent evidence concerning the renin-substrate reaction suggests that the endogenous angiotensinogen concentration may also limit the rate of formation of angiotensin I (AI) and thus AII (Ayers, 1967; Blaquier, 1965; Eggena et al., 1976; Gould and Green, 1971; Gould et al., 1966; Poulsen, 1971a; Skinner et al., 1975b). In view of this substrate-dependence of renin, plasma renin activity (PRA) has become the more commonly used indicator of AII production. Correlation of PRA and plasma AII concentration supports the postulate that the rate of formation of AI in vivo is the primary factory regulating the steady-state concentration of AII in plasma (Skeggs et al., 1977). Angiotensin I conversion and AII inactivation are not considered to contribute significantly to acute changes in circulating concentrations of AII in adults.

Age-related differences in the individual components of the renin-angiotensin system have been described in several species including man. Plasma angiotensinogen concentration increases following birth in humans and rats (Kotchen et al., 1972; Pohlova and Jelinek, 1974). In contrast, PRA is elevated in newborns and decreases progressively with age (Dillon and Ryness, 1975; Granger et al., 1971; Mott, 1975; Sassard et al., 1975). The enhanced PRA of immature

animals has been attributed to a greater PRC, possibly reflecting both greater rates of renin secretion (Aoi and Weinberger, 1976) and slower rates of renin inactivation (Solomon et al., 1977). Angiotensin-converting enzyme (ACE) activity is low at birth and increases progressively throughout development (Kokubu et al., 1977; Wallace et al., 1978). The changes in ACE content have been correlated with age-related increases in the ability of fetal lungs to remove AI from the circulation (Stalcup et al., 1978; Wigger and Stalcup, 1978). The longer half-life of AII in kidneys and liver of newborns compared to adults suggests that angiotensinase activity increases during development (Pipkin, 1972; Pohlova and Jelinek, 1974).

The immaturity of the renin-angiotensin system in newborns may be such that the relative contributions of angiotensinogen, renin, converting enzyme, and angiotensin-degrading enzymes in limiting the availability of circulating AII may be very much different from that in adult animals. From a mechanistic viewpoint, the elevated PRA of newborns leads to greater rates of AI formation compared to adults. However, the slow rate of conversion of AI to AII in newborns retards the quantitative relationship between PRA and AII formation. Therefore, despite the greater rates of AI production in newborn plasma, the net rate of turnover of AII may be much slower due to both the depressed ACE activity and the longer half-life of circulating AII.

Since all of the recognized biological effects of the renin-angiotensin system appear to be exerted through angiotensin II, knowledge concerning the regulation of circulating AII concentrations is central to understanding the physiological relevance of this system. The overall activity of the renin-angiotensin system is a

composite of the individual activities of the constituent enzymes (Figure 1). Accordingly, accurate predictions concerning the effects of perturbing the system on the steady-state concentration of circulating AII necessitates a complete understanding of the individual enzyme concentrations and the affinity with which these enzymes interact with their respective substrate. Since age-related differences in enzyme activities may influence the regulation of plasma AII concentration, it was the purpose of this dissertation to assemble a basic description of the stoichiometry of the renin-angiotensin system in developing animals. Results of such an investigation may provide evidence not only for identifying specific factors responsible for alterations in homeostasis of neonates, but may also reveal a means by which such anomalies may be treated most efficaciously.

Formation of AII may occur via mechanisms other than the classical renin-angiotensin system, such as through the action of tonin on angiotensinogen (Boucher et al., 1977) or via mechanisms other than the sequential actions of renin and converting enzyme. Because of the incomplete understanding of these alternative pathways for AII generation and a lack of evidence concerning the importance of these pathways in the net turnover of AII in plasma, they are only mentioned here and will not be addressed further in this dissertation.

A. The Renin-Angiotensin System of Adult Animals

1. Angiotensinogen

The formation of AII originates with the synthesis of angiotensinogen (renin substrate) (Figure 1), a protein believed to be associated with the α_2-globulin fraction of plasma (Plentl et al.,

Figure 1. Schematic description of the renin-angiotensin system. Included are the sequential hydrolyses of the major peptide fragments. Numbering of amino acid residues begins at the amino terminus and arrows denote the specific peptide bond cleaved by the respective enzymes. In contrast to renin and angiotensin-converting enzyme, the enzymes responsible for the catabolism of AII represent nonspecific peptidases and thus have not been individually classified by the Enzyme Commission (E.C.).

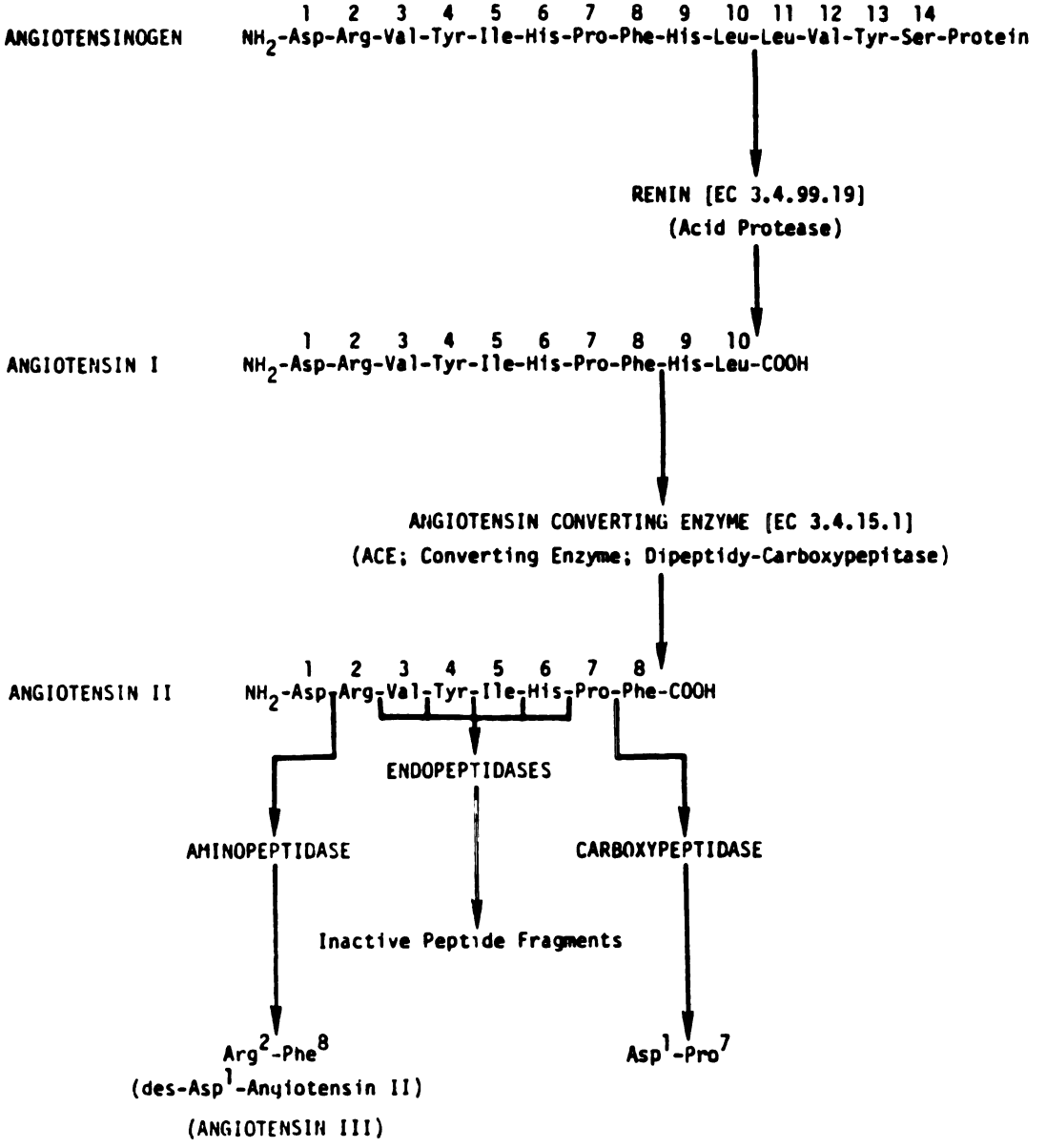

1 2 3 4 5 6 7 8 9 10 11 12 13 14
ANGIOTENSINOGEN
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-Protein
RENIN [EC 3.4.99.19]
(Acid Protease)
1 2 3 4 5 6 7 8 9 10
ANGIOTENSIN I
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH
ANGIOTENSIN CONVERTING ENZYME [EC 3.4.15.1]
(ACE; Converting Enzyme; Dipeptidy-Carboxypepitase)
1 2 3 4 5 6 7 8
ANGIOTENSIN II
NH2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
ENDOPEPTIDASES
AMINOPEPTIDASE
CARBOXYPEPTIDASE
Inactive Peptide Fragments
Arg2-Phe8
(des-Asp1-Angiotensin II)
(ANGIOTENSIN III)
Asp1-Pro7

Figure 1

1943). Experimental hepatectomy provided the first evidence that liver is the principle source of angiotensinogen in plasma (Page et al., 1941). Hepatectomy results in markedly suppressed plasma renin substrate concentrations (Tateishi and Masson, 1972). Direct evidence supporting the hepatic origin of circulating renin substrate was the demonstration of angiotensinogen release from slices of liver tissue (Freeman and Rostorfer, 1972) and the appearance of renin substrate in the venous effluent of perfused rat livers (Nasjletti and Masson, 1972). Weigand and coworkers (1977) isolated hepatocytes from livers of adult rats and demonstrated that these cells were capable of releasing newly formed angiotensinogen into the bathing medium when sufficient amino acids were supplied to the incubation mixture.

Extrahepatic tissue may also produce angiotensinogen. Using cell fractionation techniques, Morris and Johnston (1976a,b) identified angiotensinogen-containing granules in the renal cortex of rats. Angiotensinogen has also been found in cerebrospinal fluid (Reid and Ramsay, 1975), renal lymph (Horky et al., 1971), and human amniotic fluid (Skinner et al., 1975a). Angiotensinogen of lymph and amniotic fluid apparently derives from the circulation whereas indirect evidence suggests that angiotensinogen is synthesized locally within brain. This large molecular weight glycoprotein does not appear to cross the blood-brain barrier.

Addition of puromycin to the perfusing medium completely abolished the release of angiotensinogen from intact, isolated livers of adult rats, indicating that essentially all of the angiotensinogen released into the venous effluent was newly synthesized protein (Nasjletti and Masson, 1971a). Angiotensinsinogen is not believed to

be stored to any great extent prior to release from liver tissue, thus alterations in angiotensinogen secretion by liver appear to be mediated primarily by changes in its rate of de novo synthesis (Bing, 1972; Nasjletti and Masson, 1971a,1972,1973). The steady-state concentration of angiotensinogen in plasma appears to be controlled primarily by its rate of synthesis rather than its consumption by renin (Tateishi et al., 1971).

Plasma renin substrate concentration varies with the state of hepatic function and is influenced by several hormonal factors. The concentration of angiotensinogen is suppressed in humans with severe parenchymal liver disease including cirrhosis and ascites (Ayers, 1967; Gould et al., 1966) and in rats treated chronically with carbon tetrachloride (Loyke, 1965; Loyke and Mackrell, 1967). Sonkodi and coworkers (1976) found that destruction of 80% of the functional liver mass in fasted rats did not affect plasma renin substrate concentration measured indirectly. These investigators postulated extrahepatic sources of plasma angiotensinogen. However, experimentally-induced hepatotoxicity potentiates the pressor response to AI, norepinephrine and epinephrine suggesting a reduction in the metabolic clearance of these vasopressor substances (Vogin and Bokelman, 1971). Thus, elimination of functional liver tissue appears to affect circulating substrate concentrations not only by suppressing the rate of angiotensinogen synthesis but also by prolonging the circulating half-life of angiotensinogen and possibly renin.

The rate of angiotensinogen synthesis is sensitive to regulation by both adrenal cortical and estrogenic hormones. The concentration of angiotensinogen in plasma decreases following hypophysectomy,

adrenalectomy, or adrenal enucleation (Carretero and Gross, 1967a,b; Goodwin et al., 1970; Helmer and Griffith, 1951; Morimoto et al., 1975; Nasjletti and Masson, 1969; Reid et al., 1973). Plasma renin substrate concentration is also suppressed in patients with Addison's disease (Brown et al., 1968). In contrast, plasma angiotensinogen concentration is elevated in Cushing's syndrome but not in patients with primary aldosteronism (Krakoff, 1973). Administration of adrenocorticotropic hormone or adrenocortical steroids increases the concentration of angiotensinogen in plasma (Carretero and Gross, 1967a,b; Haynes et al., 1953; Helmer and Griffith, 1952; Krakoff et al., 1975; Lazer and Hoobler, 1971; Nasjletti and Masson, 1969; Reid et al., 1973).

Administration of estrogens also increases plasma angiotensinogen concentration (Cain et al., 1971; Crane et al., 1966; Helmer and Griffith, 1951; Helmer and Judson, 1967; Hiwada et al., 1976; Nasjletti et al., 1971a,b; Skinner et al., 1969). Accordingly, plasma from pregnant females contains a greater amount of renin substrate than that from non-pregnant animals (Skinner et al., 1972; Symonds and Pipkin, 1975; Weir et al., 1975). The increased plasma angiotensinogen has been attributed to a greater rate of synthesis (Bing, 1972; Nasjletti and Masson, 1971,1972,1973) and appears to result from a direct action of estrogens and glucocorticoids on hepatic tissue. Perfusion of livers isolated from adult rats with a medium containing either stilbestrol or cortisol increases the rate of appearance of angiotensinogen in the venous effluent (Hasegawa et al., 1973a; Nasjletti and Masson, 1972). Furthermore, both slices of liver

tissue and isolated hepatocytes are responsive to stimulation of angiotensinogen synthesis during incubation in the presence of glucocorticoids (Freeman and Rostorfer, 1972; Weigand et al., 1977). It has been suggested that the mechanism of angiotensinogen stimulation is mediated at the tissue hormone receptor level. Eisenfeld and coworkers (1976) correlated the incremental increase in plasma angiotensinogen concentration following estradiol administration with the development of hepatic estradiol-binding protein. Subsequently, these investigators described an age-dependent increase in hepatic estrogen receptors which correlated well with the ability of developing rats to respond to ethinyl estradiol administration by increasing plasma angiotensinogen concentrations (Krakoff and Eisenfeld, 1977). The absence of an age-dependent increase in hepatic glucocorticoid receptors was suggested to be responsible for the lack of a difference in the responsiveness of immature and adult rats to dexamethasone-induced angiotensinogen synthesis (Eisenfeld et al., 1976; Feldman, 1974; Krakoff and Eisenfeld, 1977). The effect of estrogens on hepatic angiotensinogen synthesis appears to be mediated through secondary hormonal mechanisms since hypophysectomy prevents the induction of renin substrate synthesis by estradiol but does not affect the increase induced by dexamethasone administration (Krakoff and Eisenfeld, 1977). Hypophysectomy may suppress the angiotensinogen response by reducing the number of estrogen-binding sites in liver (Chamness et al., 1975).

Plasma renin substrate concentration also increases following nephrectomy, apparently due to the reduction in the rate of substrate

consumption by renin (Bing and Magill, 1963; Blaquier et al., 1962; Carretero and Gross, 1967a,b; Hiwada et al., 1976; Nasjletti and Masson, 1971a; Tateishi et al., 1971). Hasegawa and associates (1973b, 1976) described a factor present in plasma from nephrectomized rats which stimulates the release of angiotensinogen from slices of liver incubated in vitro. The activator appears to be a protein associated with the γ_2-globulin fraction of plasma but has not been further characterized. It is possible that these results may be explained by the increased plasma corticosteroids induced by the stress of surgery. Indeed, the concentration of angiotensinogen in plasma is elevated for several days following sham operations in rabbits (Campbell et al., 1973; Morris et al., 1977; Romero et al., 1970). Prior adrenalectomy reduces the incremental increase in plasma angiotensinogen following nephrectomy (Carretero and Gross, 1967a,b; Freeman and Rostorfer, 1972; Lazar and Hoobler, 1971). However, nephrectomy also has an undefined direct effect on hepatic tissue since the rate of release of angiotensinogen into the venous effluent from perfused livers of bilaterally nephrectomized rats is greater than that from livers of intact rats (Nasjletti and Masson, 1972).

Hemodilution and severe hypoxia also increase plasma renin substrate concentrations (Bing and Poulsen, 1969; Gould and Goodman, 1970; Reid et al., 1973). Although the specific mechanisms have not been fully elucidated, it is possible that the enhanced angiotensinogen synthesis results from an increased concentration of adrenal corticosteroids (Reid et al., 1974).

The synthesis of angiotensinogen does not exhibit direct product inhibition (Hackenthal et al., 1976,1977). On the other hand, administration of AII results in a significantly elevated angiotensinogen concentration in plasma of dogs and rats (Blair-West et al., 1974; Carretero and Gross, 1967b; Khayyall et al., 1973; Nasjletti and Masson, 1973). This increase in plasma renin substrate is not believed to result from the inhibition of renin secretion by AII since similar effects are produced by the injection of renin (Carretero and Gross, 1967b). Although adrenalectomy has been reported to prevent the increased secretion of angiotensinogen during AII administration (Blair-West et al., 1974) others have found that hypophysectomy does not affect this response (Reid, 1977). Since addition of angiotensin to medium perfusing isolated rat livers results in an enhanced rate of secretion of angiotensinogen into the venous effluent (Nasjletti and Masson, 1973), circulating AII appears to have a direct stimulatory effect on angiotensinogen synthesis by hepatic tissue. The specific mechanisms responsible for this direct effect have not yet been elucidated. Adrenocortical steroids may play a permissive role in the stimulation of angiotensinogen synthesis by AII.

This positive feedback of AII on the production of angiotensinogen may function to prevent depletion of plasma renin substrate concentration during conditions of elevated renin secretion. It appears that the maintenance of plasma angiotensinogen concentrations during hemorrhagic hypotension results from stimulation of angiotensinogen synthesis by the elevated circulating AII concentrations (Beaty et al., 1976). A similar function for this positive feedback

by AII may be involved in stimulating angiotensinogen production during sodium depletion and in renal and malignant hypertension (Blair-West et al., 1974; Gould and Green, 1971; Gould et al., 1966; Rosset et al., 1973).

Angiotensinogen has been purified to varying degrees from plasma of several species (Eggena et al., 1976; Printz et al., 1977a,b; Schioler et al., 1976; Skeggs et al., 1963; Skinner et al., 1975b; Tewksbury et al., 1976,1977). The active component of the α_2-globulin is a tetradecapeptide attached by an ester bond between the hydroxyl group of the carboxy-terminus serine residue and the carboxyl group of an arginine or lysine residue of the protein moiety. The protein molecule is believed to prevent hydrolysis of the tetracdecapeptide by non-specific peptidases (Skeggs et al., 1967). Multiple forms of angiotensinogen have been identified by some investigators (Faiers et al., 1977; Lentz et al., 1978; Printz et al., 1977a,b; Skeggs et al., 1963) while others report a single homogenous form of substrate (Dorer et al., 1978; Eggena et al., 1976, 1978; Tewksbury et al., 1976,1977). The profile of the different forms of angiotensinogen appears to change under conditions of enhanced substrate synthesis such as renovascular hypertension and uremia (Eggena et al., 1978), administration of glucocorticoids or estrogens (Eggena et al., 1977,1978; Lentz et al., 1978) or during pregnancy (Printz et al., 1977b). In contrast, Menard and coworkers (1974) were unable to detect alterations in the physiochemical properties of angiotensinogen following either bilateral nephrectomy or estrogen administration. The various forms of angiotensinogen have been distinguished by isoelectric focusing, immunochemical cross-reactivity and by DEAE-cellulose chromatography.

The identification of different forms of angiotensinogen may, however, be an artifact of the purification process (Eggena et al., 1976). Each of the different forms of substrate appears to be a glycoprotein with similar amino acid contents (Skeggs et al., 1963). It is possible that differences in the sialic acid, glucosamine, and neutral hexose content may account for the differences in mobilities of these proteins on gel electrophoresis. Angiotensinogen purified from human plasma appears homogenous immunochemically and on disc gel electrophoresis but displays heterogeneity on DEAE-cellulose chromatograms or by polyacrylamide electrophoresis (Dorer et al., 1978; Tewksbury et al., 1976). In view of the similar amino acid composition and rates of reaction with renin displayed by the different forms of substrate (Skeggs et al., 1963,1964), it is possible that loss of carbohydrate moieties from the protein during purification may explain the heterogeneity of such preparations. Accordingly, the physiological relevance of multiple forms of angiotensinogen remains to be determined.

The concentration of angiotensinogen in plasma is conventionally determined indirectly from the amount of angiotensin I formed following complete hydrolysis of endogenous substrate (Eggena et al., 1974; Gould et al., 1966; Krakoff, 1973; Nasjletti et al., 1969). Methods used to expediate the exhaustion of endogenous angiotensinogen include the addition of partially purified homologous or heterologous renin to the reaction mixture (Menard and Catt, 1972). Several methods are available for the quantification of newly generated angiotensin I and II including radioimmunoassay (RIA) and bioassay

(Boucher et al., 1967; Gould et al., 1966; Haber et al., 1969; Helmer and Judson, 1963; Krakoff, 1973; Oparil, 1976; Pickens et al., 1965; Poulsen, 1969; Ryan and McKenzie, 1968).

Methods have also been developed whereby estimates of angiotensinogen are calculated from kinetic parameters of the renin-substrate reaction (Campillo et al., 1976; Quesada et al., 1975). This method does not require addition of exogenous renin to the incubation mixture and is based on simple kinetic measurements of the rate of generation of AI from angiotensinogen. Maximal AI generation is estimated from the double reciprocal plot of AI concentration versus time of incubation (Campillo et al., 1976). This method also offers a simple means for estimating renin concentration as calculated from the specific velocity constant of the reaction. Although the kinetic estimation of angiotensinogen appears to be precise, it is restricted by the same limitations as those described for other indirect measurements.

A direct radioimmunoassay for angiotensinogen has recently been described using antibodies to angiotensinogen purified from human plasma (Eggena et al., 1977). The close agreement between measurements of angiotensinogen obtained by direct radioimmunoassay and those obtained indirectly suggest great potential for this direct method in future determinations of plasma angiotensinogen concentration.

2. Renin

Renin (EC 3.4.99.19) is a glycoprotein which functions as a very specific carboxyl protease cleaving AI from angiotensinogen (Figure 1). Renin was first described by Tigerstedt and Bergman

(1898) as a pressor substance in saline extracts of rabbit kidneys. The role of the kidneys in hypertension remained controversial until renewed impetus was given by the classical experiments of Goldblatt and his associates (1934) who observed that chronic constriction of the renal arteries in dogs resulted in persistent diastolic hypertension. Page (1939) first addressed the question of whether renin itself was the pressor substance. Subsequent investigations demonstrated that renin does not possess a direct pressor effect on smooth muscle (Braun-Menendez et al., 1940; Page and Helmer, 1940) but rather is an enzyme which catalyzes the hydrolysis of angiotensinogen to generate the decapeptide, angiotensin I (Skeggs et al., 1954a). Skeggs and his associates (1954b) subsequently resolved the vasoactive substance into two components, hypertensin I and hypertensin II, by countercurrent distribution. Both peptides produced pressor responses in vivo, however, Helmer (1955, 1957) identified the second component as the true vasoconstrictor material by its effect on isolated rabbit aortic strips. These peptides are now referred to as angiotensin I (AI) and angiotensin II (AII), respectively. The amino acid sequence of AI and AII were first determined by Skeggs and associates (1954b, 1955, 1956a,b,c).

Enzymes with renin-like activity have since been identified in the uterus and placenta (Anderson et al., 1968; Capelli et al., 1968; Carretero et al., 1971; Ferris et al., 1967; Gross et al., 1964), brain (Ganten et al., 1971a,b,c), brainstem (Fischer-Ferraro et al., 1971), hypothalamus (Fischer-Ferraro et al., 1971; Haulica et al., 1975), pineal gland and hypophysis (Haulica et al., 1975), adrenal glands (Ryan, 1967), large arteries and veins (Ganten et al.,

1970; Gould et al., 1964), submaxillary glands (Cohen et al., 1972; Inagami et al., 1974; Taylor et al., 1971) and liver (Gould et al., 1964).

Although these extrarenal enzymes possess activities similar to that of renin, the specificity with which these enzymes cleave angiotensinogen is questionable and it is possible that these enzymes represent non-specific acid proteases. The enzyme from salivary glands has been classified as an isoenzyme of renin (Taylor et al., 1971). Furthermore, since both isoproterenol administration and restriction of dietary sodium suppress submaxillary gland renin activity and release, the function of this enzyme and the mechanisms controlling its release appear to be different than renin of renal origin (Takeda et al., 1969). Brain renin-like enzymes appear to be synthesized locally and respond to stimulation in a reciprocal fashion to that of renal renin (Fischer-Ferraro et al., 1971). The existence of a local renin-angiotensin system in the brain has been implicated from indirect observations and has been suggested to participate in fluid and electrolyte homeostasis through dipsogenic effects and stimulation of the release of antidiuretic and corticotropic hormones. The physiological relevance of a central renin-angiotensin system has recently been reviewed (Barker, 1976; Bennett and Snyder, 1976; Fuxe et al., 1976; Ganten et al., 1975; Severs and Daniels-Severs, 1973). Renin-like enzymes of the chorion and uterine myometrium and those located in the adventitia of blood vessel walls are released in response to hemorrhagic hypotension, ischemia, and norepinephrine in a manner similar to that of renal renin (Capelli et al., 1968; Ferris et al., 1972; Ganten et al., 1970; Ryan and Ferris,

1967). Despite these apparent similarities in renin from different tissues, Potter and coworkers (1977) found differences in the catalytic properties of dog renin prepared from kidney compared to that derived from uterine tissue. Inasmuch as bilateral nephrectomy does not suppress renin-like activity in uteri of pregnant rabbits or in mesenteric blood vessels of dogs (Ganten et al., 1970; Gordon et al., 1967) it has been suggested that these tissues possess the capacity to synthesize and release renin locally.

It has not been determined whether these extrarenal enzymes contribute to the regulation of plasma AII concentrations. The renin associated with blood vessel walls may generate AII locally and thus not require release of the enzyme into the systemic circulation. However, splanchnic renin activity has been reported to be greater in portal vein blood than in arterial blood suggesting that renin is released into the circulation from blood vessel walls (Ganten et al., 1970). Similarly, the uterine enzyme has been implicated in maintaining plasma renin concentrations of nephrectomized pregnant dogs under conditions of enhanced renin secretion (Carretero et al., 1972). Furthermore, Bing and Poulsen (1976) found that gentle manipulation followed by removal of the submaxillary glands from previously nephrectomized mice resulted in a greater than 10-fold increase in plasma renin concentration. Subsequent experiments demonstrated that this elevated renin content was due to increased concentrations of both the inactive and active form of the enzyme (Bing et al., 1977). It was suggested from the time-course of the response that the increase in plasma renin resulted from transfer of the enzyme from submaxillary lymph and/or interstitial fluid to the circulation. It

therefore, appears that renin derived from extrarenal tissues may contribute to the regulation of circulating enzyme, however, no definitive evidence concerning this postulate exists. The physiological relevance of extrarenal renin remains to be further defined.

In the kidney, renin is synthesized and stored within the juxtaglomerular apparatus (JG apparatus) consisting of specialized cells of the afferent arteriole and the epithelial lining of the distal tubule (Davis, 1973; Davis and Freeman, 1976; Oparil and Haber, 1974). The specialized epithelial cells of the distal tubule situated in the angle between the afferent and efferent arterioles supplying the glomeruli have been termed the macula densa cells of that glomerulus. The cells of the afferent arteriole appear to be specialized myoepithelial cells derived from smooth muscle lining the vascular epithelium and have been refered to as the juxtaglomerular (JG) cells (Barajas and Latta, 1967). These JG cells contain numerous membrane-bound granules which presumably represent storage granules of newly synthesized renin (Cook, 1968; Edelman and Hartroft, 1961). The number of these storage granules varies in parallel with the plasma renin activity and renal renin content (Dauda et al., 1968; Dunihue, 1946; Tobian, 1962).

There are three or four generally accepted mechanisms regulating the secretion of renin by the JG apparatus (Davis, 1971; Davis and Freeman, 1976; Peart, 1978; Reid et al., 1978; Schrier, 1974; Zanchetti et al., 1976). One mechanism involves the juxtaglomerular cells of the afferent arteriole acting as pressure or volume receptors. A decrease in intravascular or transmural pressure in the afferent arteriole stimulates the release of renin (Oparil and Haber,

1974; Skinner et al., 1963). Conversely, increases in afferent arteriolar wall tension inhibit renin release. A second mechanism controlling renin release is modulated by changes in the transport of sodium or possibly chloride across the macula densa cells of the distal tubule (Davis, 1973; Nash et al., 1968; Vander and Carlson, 1969). The third mechanism is neurogenic, mediated by sympathetic innervation of the juxtaglomerular cells (Schrier, 1974; Vander, 1965; Wogermark et al., 1968). Whether the effect of the sympathetic nervous system on renin secretion is direct via the sympathetic fibers which terminate in the JG cells (Barajas, 1964; Barajas and Muller, 1973; Dolezel et al., 1976; Nilsson, 1965) or is an indirect response mediated by the neurogenic tone of the vascular cells in the afferent arteriole is disputed. Sympathetic stimulation of renin release, whether direct or indirect, is mediated by beta-adrenergic receptors in the JG apparauts (Assaykeen et al., 1970; Davis, 1973; Vandongen et al., 1973; Winer et al., 1969). A fourth and not yet universally accepted mechanism postulated for controlling renin release involves secondary effects mediated by the central nervous system (Reid et al., 1978). This neuronal control influences renin secretion either directly via renal nerves, or indirectly via effects on the adrenal medulla and posterior pituitary. The different mechanisms controlling the rate at which renin is secreted into the circulation from the JG apparatus are integrated in such a way that the net effect on renin secretion relies on the sum of the individual stimuli (Davis and Freeman, 1976; Zanchetti et al., 1976).

A final mechanism regulating release of renin from kidneys is mediated by the biologically active product of the renin reaction. Intravenous infusion of physiological doses of angiotensin II suppresses renin secretion in dogs under conditions of basal or elevated renin release (Vander and Geelhoed, 1965). Naftilan and Oparil (1978) demonstrated direct inhibition of renin release from rat kidney slices incubated in the presence of high concentrations of AII. This negative feedback effect of AII may function to limit the release of renin and therefore decrease the rate of AII generation under conditions of elevated plasma AII concentrations (Beckerhoff et al., 1972; Cain et al., 1971; Low and Oparil, 1975). This inhibitory effect of AII on renin secretion has been implicated in the pathogenesis of hypertension. Infusion of AII into normotensive patients resulted in a dose-dependent decrement in renin activity, however, similar infusions of AII did not affect plasma renin activity of normal-renin or high-renin hypertensive subjects (Williams et al., 1978). Subsequent infusion of a converting enzyme inhibitor into the hypertensive patients reduced mean systemic blood pressure and AII concentrations but did not affect plasma renin activity, suggesting that the loss of the negative-feedback inhibitory affect of AII on renin secretion may predispose these subjects to excessive rates of AII generation and thus the development of hypertension.

Renin secretion has been correlated with renal renin content (Park et al., 1978). When dogs were subjected to renal denervation, salt-restriction, or two-kidney Goldblatt hypertension, the rate at which renin was secreted into the renal venous blood was found to be proportional to the content of renin in that kidney. Similarly, the

rate of appearance of renin in the bathing medium during incubation of renal cortical slices *in vitro* also parallelled the renin content of the slice. These data were interpreted to suggest that perhaps the rate of synthesis of renin is mediated in part by the basal rate of renin secretion.

Renin purified from several sources appears to exist in 3 general forms having molecular weights of approximately 40,000, 60,000 and 140,000 (Skeggs *et al.*, 1977). Prior to 1970, renin was thought to be a homogenous enzyme of molecular weight (MW) 37,000-43,000. The only variants were slight differences in isoelectric points (Skeggs *et al.*, 1967). The first indication of multiple forms of renin was in 1970 when Lumbers presented evidence for the activation of renin in human amniotic fluid following acidification of the sample to pH 3.0. Acid activated renin has since been described in plasma, amniotic fluid, and extracts of kidney (Day and Leutscher, 1974, 1975; Day *et al.*, 1975; Lumbers, 1971). In most instances, the major component of purified renin preparations has a molecular weight near 40,000 and is the most active species of the enzyme (Boyd, 1973, 1974; Levin *et al.*, 1970; Murakami and Inagami, 1975; Rubin, 1972; Waldhausl *et al.*, 1970). The minor components are less active and have MW 58,000-63,000. Acid activation of renal, amniotic and plasma renin has been interpreted to suggest that renin may be stored by tissues in an inactive form prior to its release into the circulation (Boyd, 1974; Leckie, 1973; Lumbers, 1971; Rubin, 1972). Indeed, Morris and Johnston (1976b) presented evidence for inactive renin within storage granules isolated from renal cortical tissue of rats. In accordance with this postulate is the observation that when the enhanced rate of

renin secretion induced in rats by adrenalectomy and salt restriction is abruptly blocked by desoxycorticosterone (DOCA) substitution treatment, a net increase in renal renin content occurs which is revealed only by prior acidification of the kidney extracts (DeSenarclens et al., 1977). This doubling of the inactive renal renin content was accompanied by a simultaneous appearance of numerous granules in the epithelioid cells of the juxtaglomerular apparatus. Administration of furosemide to adrenalectomized and salt-depleted rats resulted in a 20-fold increase in plasma renin activity compared to similar rats given DOCA (Banichahi et al., 1976). This enhanced rate of renin secretion was accompanied by depletion of the acid-activatable renin in secretory granules of the JG apparatus.

James and Hall (1974) reported that acute stimulation of renin release induced by administration of furosemide results in elevated concentrations of the active form of renin in plasma, whereas chronic stimulation of renin during hemorrhage was accompanied by increases in both active and inactive forms of plasma renin. These results provided the first evidence suggesting that the enhanced secretion of renin is mediated initially by transformation of inactive to active renin. During periods of chronic stimulation, the de novo synthesis of the enzyme is believed to be in a steady-state which may explain the increase in both forms of renin. In accordance with this postulate is the finding that the increased concentration of active renin in plasma following acute stimulation of renin release with isoproterenol is accompanied by a decrease in the amount of the inactive form of the enzyme in plasma (Derkx et al., 1976). In rabbits, acid-activatable renin accounted for 10% of the total plasma

renin (Grace et al., 1977). Restriction of dietary sodium initially resulted in increased concentrations of active renin and the virtual disappearance of inactive renin from plasma, however, with time the proportion of acid activatable renin increased progressively until it again represented 10% of the elevated plasma renin activity. This time course for changes in the profile of the different forms of plasma renin suggests that under conditions of stimulated renin secretion, the additional enzyme initially derives from intrarenal stores of the inactive form of the enzyme, whereas increased rates of synthesis of new enzyme accounts for the prolonged phase of enhanced rates of secretion of renin.

Inasmuch as the affinity of renin for angiotensinogen was not altered by activation of the enzyme, it was concluded that the activation process involves a change in the number of active renin molecules, most likely through derepression of inactive renin (Boyd, 1973, 1974; Morris and Lumbers, 1971, 1972). In support of this postulate was the separation of an inhibitor protein (MW 13,000) from the high molecular weight form of renin (Boyd, 1974; Leckie and McConnell, 1975). Addition of the protein back to active renin of lower molecular weight resulted in a dose-dependent inhibition of the enzyme along with the appearance of greater amounts of the high molecular weight form of renin (Leckie and McConnnell, 1975). Acidification destroyed the inhibitor protein resulting in irreversible activation of renin.

Activation of renin in vivo would be assumed to involve mechanisms similar to those induced by acid-activation in vitro. If activation occurs through the hydrolysis of an inhibitor protein from

the renin molecule, a proteolytic enzyme would be a likely candidate in mediating the activation of renin *in vivo* (Morris and Lumbers, 1971, 1972). Activation of renin during incubation with proteolytic enzymes has been demonstrated (Boyd, 1974; Day and Leutscher, 1975; Morris and Lumbers, 1971, 1972). This activation is induced by tryptic or peptic digestion of inactive renin and is usually accompanied by a reduction in molecular weight of the enzyme (Boyd, 1974; Leckie, 1973). Recently, Potter and coworkers (1978) purified renin from renal cortical tissue of dogs. These investigators found that tryptic digestion of the enzyme increased renin activity by 40% and was accompanied by complete conversion of the high molecular weight form of the enzyme to a smaller protein having MW of 41,000.

Rubin (1972) demonstrated that acidification of the enzyme prior to centrifugation of the homogenate was required for activation of renin, suggesting that a soluble enzyme was involved in the activation process. Morris and Lumbers (1971, 1972) separated a protein substance from amniotic fluid which was capable of activating renin in a manner similar to that by acidification of the sample. This activating factor, unlike renin, was stable at pH 1.5 and could enhance renin activity when incubated with amniotic renin at pH 4.5 but not at pH 7.5. Pepsin, an acid protease which is stable at pH 1.5, could also activate renin when incubated at pH 4.5 but not at pH 7.5. It was therefore proposed that the activation of the inactive, high molecular weight renin during acidification may result from hydrolysis by pepsin. The fact that renin activity did not increase following incubation of amniotic fluid with carboxypeptidase enzymes suggests

that cleavage of internal peptide bonds is required for activation. Inasmuch as trypsin can activate renin at pH 7.5 (Morris and Lumbers, 1972), increased hydrogen ion concentration alone does not appear to be the sole prerequisite for the activation process. The requirement of low pH for this process may be due to the autocatalytic activation of pepsinogen by high concentrations of hydrogen ions. The proportion of active renin in plasma may be determined by the extent of reaction of active renin with protease enzymes which may be mediated in turn by the rate of fusion of renin granules with intracellular lysosomes (Morris and Johnston, 1976b).

In contrast to the activation process involving hydrolysis of an inhibitor protein from renin with a reduction in the molecular weight of the inactive form of enzyme, several investigators did not observe a reduction in molecular mass of renin following activation (Day and Luetscher, 1974, 1975; Day et al., 1975, 1976). The increase in renin activity of human amniotic fluid following acidification to pH 3.5 apparently did not involve the dissociation of an inhibitor from renin since no change in molecular weight was observed (Lumbers, 1971; Morris and Lumbers, 1972). These investigators suggested that activation of renin involved the conversion of a precursor or protein-bound form of the enzyme to active renin via yet undefined structural changes in the enzyme molecule. Sealey and Laragh (1975) have proposed the term "prorenin" to describe the inactive form of the enzyme in plasma. These investigators observed a 50% increase in plasma renin activity after prolonged storage of the sample at -20°C for several months. The greater renin activity was not accompanied by

changes in the concentration of angiotensinogen or of activators or inhibitors. They therefore concluded that activation most likely involved the passive interconversion of a precursor, prorenin, to the active form of renin. Slater and Haber (1978) estimated the molecular weight of renin extracted from human renal cortex to be approximately 58,000. If, however, this extract was incubated in the absence of inhibitors of proteases, or the homogenate frozen and thawed several times, or if the extract was acidified to pH 2.8, the majority of the enzymatically active protein migrated with a molecular weight near 42,000. Inasmuch as antibody prepared against the higher MW form of the enzyme cross-reacted with the active renin, it was concluded that the larger form of the protein may represent a precursor of active renin, either in the form of a zymogen or a protein-bound enzyme complex. In contrast, Funakawa and Yamamoto (1978) found that renin prepared from dog kidneys migrated on Sephadex columns with a MW 43,000 and was not activated by acidification to pH 3.0 suggesting that renin is stored in kidneys in the active form.

Regardless of whether the inactive form of renin exists as an inhibitor-enzyme complex or as a protein-bound precursor to renin, the common function of the binding protein is to suppress enzyme activity. It therefore appears that both schools of thought concerning the inactive form of renin are compatible in that the enzyme is immobilized in some fashion such that the catalytic site(s) are repressed by the binding protein. Under such conditions, it is possible that the binding sites for the antibody are accessible which may account for the cross-reactivity of antibody to active renin with the high molecular weight form of the enzyme (Slater and Haber, 1978).

Storage of renin at 4°C for 3 days was reported to result in up to a 6-fold increase in plasma renin activity (Osmond et al., 1973). Sealey and Laragh (1975), however, found that renin activity did not change appreciably during storage at -20°C and required a full year of storage for significant activation to occur. Contradictory studies by these same investigators (Sealey et al., 1976) demonstrated that cryoactivation of renin did not occur in frozen plasma, but proceeded with decreasing rapidity at temperatures from -5°C to +4°C. Prolonged storage of plasma at -20°C did not alter angiotensinogen concentration and therefore the increased renin activity most likely reflected increased amounts of active enzyme. More recent reports confirm the absence of cryoactivation of renin when human plasma is stored at -20°C for 50 weeks (Lijnen et al., 1976, 1977a; Roulston and MacGregor, 1978). Storage of human plasma at 0°C for 7-days has been reported to cause a 250% increase in renin activity (Lijnen and Amery, 1978), however, the results were highly variable (19-735%). Contradictory results have been reported which demonstrate storage of plasma at either 4°C or at -20°C for up to 1 year does not alter renin activity (Emanuel and Williams, 1978; Fyhrquist and Puutula, 1978). Although the mechanisms involved in cryoactivation of renin and its physiological relevance remain obscure, its possible technical implications are profound.

A more recent mechanism by which plasma renin has been shown to be activated is mediated by renal kallikreins (Sealey et al., 1978). Addition of human urinary kallikrein to plasma at pH 7.4 or to plasma stored at -4°C for 2 days results in a doubling of renin

activity in these samples. Kallikrein, a trypsin-like enzyme, has been localized to the macula densa cells of the distal tubule and thus may serve as the physiological factor in the activation of inactive renin stored in the JG apparatus (Nustad and Vaaje, 1975; Scicli et al., 1976a,b).

Quantification of renin, whether prior to or following activation, is typically performed by measuring the amount of angiotensin I generated during in vitro incubation of the sample with angiotensinogen. Angiotensin is measured either by radioimmunoassay or bioassay. Inasmuch as the rate of AI formation is dependent on both enzyme concentration and substrate availability, the incubation conditions can be modified to provide measurements of either the activity or the concentration of renin in the sample.

Renin activity is the most commonly employed measurement and is almost exclusively performed when using plasma as the source of enzyme. Since plasma contains substrate for the enzyme, incubation of plasma without the addition of exogenous substrate provides a measurement of the rate of AI formation from endogenous angiotensinogen and thus reflects most nearly the plasma renin activity (PRA) as it occurs in vivo. Accordingly, PRA is expressed as the rate of AI formation per unit volume of sample.

Failure to account for the complexity of chemical events occurring during the incubation of renin has contributed to the difficulty of developing accurate and reproducible assays for renin activity. The activities of renin, converting enzyme and several nonspecific angiotensinases as well as proteases must be considered when

selecting conditions for the initial incubation step. Inasmuch as renin activity is conventionally expressed as the rate of AI formation, most assays for renin require the addition of inhibitors of AI degradation to the incubation mixture to provide for the quantitative accumulation of product. The most widely used inhibitors of angiotensinases are ethylenediaminetetraacetic acid (EDTA), diisopropylfluorophosphate (DFP), dimercaprol (BAL), and 8-hydroxyquinoline (8-HQ). Most investigators suggest a mixture of these inhibitors to be most effective in blocking AI degradation (Delorme et al., 1976; Oparil, 1976; Oparil et al., 1974).

In contrast to PRA which is measured from the rate of generation of AI from endogenous substrate, plasma renin concentration (PRC) is determined following addition of exogenous angiotensinogen to the reaction mixture. If angiotensinogen is present in concentrations saturating to renin, the reaction will proceed at the maximum possible rate, independent of substrate concentration (Favre et al., 1973; Poulsen and Jorgensen, 1974; Stockigt et al., 1971). Under such conditions, the rate of formation of AI is directly proportional to the concentration of renin present in the incubation mixture, and thus reflects PRC. Several investigators have found that the concentration of endogenous angiotensinogen in human and rat plasma is not sufficient to saturate renin (Gould and Green, 1971; Haas and Goldblatt, 1967; Poulsen, 1968, 1971a; Rosenthal et al., 1971). Therefore, addition of exogenous angiotensinogen is performed in an attempt to saturate the enzyme with substrate and thus obtain measurements of AI generation dependent solely upon renin concentration. It therefore,

becomes apparent that if angiotensinogen is not saturating *in vivo*, values of PRA are less than those of PRC. On the other hand, if substrate is saturating, PRC will theoretically be equal to PRA.

Plasma renin concentration is generally measured from the rate of AI formation during incubation of renin with excess angiotensinogen and inhibitors of AI degradation (Gould *et al.*, 1966; Lever *et al.*, 1964; Menard and Catt, 1972; Nasjletti and Masson, 1971b; Skinner, 1967). However, PRC can also be estimated from the incremental increase in AI generated during incubations with increasing concentrations of exogenous renin (Haas *et al.*, 1968; Poulsen, 1971b, 1973) or from a kinetic analysis of the time-dependent disappearance of substrate (Campillo *et al.*, 1976; Poulsen, 1968, 1969; Quesada *et al.*, 1975). Finally, Galen and coworkers (1978) recently described a fluorimetric assay for renin wherein fluorescamine reacts with the free amino group of the tetrapeptide product of angiotensinogen hydrolysis to induce a fluorogenic reaction. This fluorimetric assay may provide a more simple and direct method of measuring the renin-substrate reaction. However, the validity of this method and the specificity with which the fluorescent label attacks the tetrapeptide as opposed to other peptides remains to be further characterized.

Plasma renin activity is classically employed as an index of the activity of the renin-angiotensin system as a whole. This application of PRA assumes, however, that the renin-substrate reaction in plasma is the rate-limiting step in the overall regulation of circulating AII. Indeed, changes in plasma AII concentrations have been found to be accompanied by proportional changes in PRA (Barrett *et*

al., 1977; Lijnen et al., 1978; Soveri and Fyhrquist, 1977). Swales and Thurston (1977) demonstrated that PRA correlated well with plasma AII concentrations in both normotensive and hypertensive human subjects having PRA values ranging from 2 to 20 ng/ml·hr. Therefore, in adults, PRA may be a valid index reflecting plasma AII concentrations.

Changes in PRC, on the other hand, are not paralleled by changes in plasma AII (Lijnen et al., 1977b), suggesting that the concentration of renin in plasma is not the sole determinant of plasma AII concentrations. It may be inferred that angiotensinogen is not saturating to renin under these conditions and thus the renin-substrate reaction proceeds via first-order kinetics. Therefore, PRC does not appear to be a valid measurement reflecting plasma AII concentrations. The concentration of renin, however, is a necessary measurement for determining renin secretion rates and the clearance of renin from the circulation.

Renin is cleared from the circulation primarily via hepatic inactivation (Johnson et al., 1971; Peters-Haefeli, 1971; Schneider et al., 1970), the half-life of renin in plasma being 30-90 min. Houssay and coworkers (1942) provided the first evidence for the role of liver in clearing the blood of circulating renin. These investigators found that hepatectomy significantly prolonged the circulating half-life of renin in plasma of nephrectomized and anesthetized dogs. Inactivation of renin has since been demonstrated using intact liver perfused in situ (Schneider et al., 1968) and in isolated, perfused livers (Tapia et al., 1972). Subsequent experiments have suggested that circulating renin is cleared from the plasma almost exclusively via metabolism by

the liver (Haecox et al., 1967; Schneider et al., 1968). The close agreement between values for the metabolic clearance and hepatic clearance rates suggests that the in vivo clearance of renin can be attributed almost exclusively to its inactivation by liver (Schneider et al., 1970). Less than 5% of the circulating renin is metabolized by the kidneys or excreted in the urine of dogs or rats (Houssay et al., 1942; Rappelli and Peart, 1968). Inasmuch as the percent extraction of renin by the liver (approximately 20%) is not affected by changes in hepatic plasma flow (Schneider et al., 1969, 1970), it appears as though the hepatic clearance of renin is a flow-limited phenomenon. Therefore, the circulating half-life of renin in plasma is prolonged under conditions of low output heart failure and decreased under conditions of elevated cardiac output (Schneider et al., 1970).

The disappearance of renin from plasma has been described as consisting of two exponential components having half-lives of approximately 11 and 70 min (Assaykeen et al., 1968; Hannon et al., 1969; Michelakis and Mizukoshi, 1971; Schaechtelin et al., 1964; Schneider et al., 1968). Approximately 20-30% of the circulating renin is cleared from plasma during the initial rapid phase whereas the majority of the renin is inactivated during the slower phase (DeVito et al., 1977). Although these results could be explained by differences in the compartmental distribution of the enzyme, DeVito and coworkers (1977) suggested that the two components may represent different molecular forms of renin in the circulation.

3. Angiotensin I (AI)

Angiotensin I (AI), the product of the renin-substrate reaction (Figure 1), has historically been considered to be a biologically inactive precursor to AII. These conslusions were based on the relatively weak activity of AI on isolated smooth muscle strips (Aiken and Vane, 1970; Helmer, 1955, 1957). Superfusion of myocardial strips demonstrated that AI possesses one-third to one-half the constrictor effects of AII. This activity was abolished by pretreatment with specific inhibitors of converting enzyme suggesting that the response was due to conversion of AI to AII within the myocardial muscle (Britton and DiSalvo, 1973; Gerlings and Gilmore, 1973). Administration of AI centrally stimulates thirst, sodium appetite, and release of ADH from the neurohypophysis (Chiaraviglio, 1976; Fitzsimons, 1971; Severs and Daniels-Severs, 1973; Sirois and Gagnon, 1975; Swanson et al., 1973). However, these effects appear to be dependent upon the conversion of AI to AII since administration of inhibitors of converting enzyme into the brain blocks the central effects of AI (Casner et al., 1976; Chiaraviglio, 1976; Severs et al., 1973; Sirois and Gagnon, 1975; Swanson et al., 1973). In contrast, systemic injection of inhibitors of AI conversion do not block the central effects of AI (Bryant and Falk, 1973), suggesting that the inhibitors do not gain access to the local sites of AII formation within the brain. Sirois and Gagnon (1975) found that when posterior pituitaries dissected from rat brain were incubated in vitro, the stimulatory effect of AI on ADH release was completely blocked by pretreatment with inhibitors of converting enzyme. It therefore

appears that the biological activity of AI on vascular smooth muscle and in the central nervous system is mediated by AII generated locally within the tissue.

Administration of AI also causes renal vasoconstriction and enhanced rates of synthesis of adrenal catecholamines and glucocorticoids (Hofbauer et al., 1973; Itskovitz and McGiff, 1974; Keim et al., 1972; Peach, 1971; Peach et al., 1971; Saruta et al., 1972). In the isolated perfused adrenal, AI was equipotent to AII in stimulating catecholamine release and the effect of AI was not reduced by coperfusion with inhibitors of converting enzyme (Ackerly et al., 1976; Peach, 1971; Peach et al., 1971). Similarly, incubation of adrenal cortical slices or suspensions of isolated zona glomerulosa cells with inhibitors of converting enzyme did not attenuate the steroidogenic effect of AI (Ackerly et al., 1977; Larner et al., 1976; Saruta et al., 1972). The renal vasoconstrictor effect of AI appears to be mediated by the induction of prostaglandin synthesis and is not blocked by inhibitors of converting enzyme (Itskovitz and McGiff, 1974; Lonigro et al., 1973). In the presence converting enzyme inhibitors, AI is about one-tenth as active as AII in releasing prostaglandins into the effluent medium of isolated, perfused kidneys, suggesting a direct stimulatory effect of AI independent of its conversion to AII (Blumberg et al., 1976, 1977, 1978; Needleman et al., 1973).

Although the biological activity of AI in adrenal and renal tissues appears to be independent of its conversion to AII, it is possible that the lack of effect of converting enzyme inhibitors is

due to the inability of these peptides to gain access to the site of AI conversion. Inasmuch as perfusion of adrenal glands and kidneys with AI does not result in the liberation of AII into the venous effluent (Ackerly et al., 1976; Aiken and Vane, 1970; Bailie et al., 1971, 1972; DiSalvo et al., 1971; Hofbauer et al., 1973; Kreye and Gross, 1971; Ng and Vane, 1967, 1968; Peterson et al., 1977), it has been suggsted that conversion of AI to AII occurs locally within these tissues. The newly generated AII is believed to cause it biological effects and then is degraded within the tissue parenchyma. Therefore, the inability of inhibitors of converting enzyme to block AI induced effects in kidneys and adrenal glands may reflect that the inhibitor does not reach the intramural site of AI conversion. Thus, it is possible that AI has no direct effects on renal and adrenal tissue and that the responses observed following infusion of AI are dependent upon its conversion to AII within the parenchyma of the tissue.

Angiotensin I was initially quantified by bioassay which required large volumes of sample in order to obtain sufficient AII to cause pressor effects (Haas and Goldblatt, 1963). Preparations which have been used include isolated strips of rabbit aorta (Helmer and Judson, 1963), rat uterus (Needleman et al., 1972), and rat colon (Regoli and Vane, 1964); intact, nephrectomized rats (Boucher et al., 1961); and nephrectomized rats after ganglionic blockade (Gunnels et al., 1967). The degree of smooth muscle contraction elicited by the sample is compared to that caused by injections of standard AII. Subsequently, several attempts have been made to increase the sensitivity of the bioassay and therefore require smaller sample volumes. Modifications include dialysis of plasma to concentrate renin (Helmer

and Judson, 1963), adsorption of renin onto diethylaminoethyl cellulose (Brown et al., 1964), extraction and concentration of angiotensin from the incubation mixture (Barrett et al., 1977; Fasciolo et al., 1964), and precipitation of plasma proteins and concentrating angiotensin via evaporation of the aqueous phase (Pickens et al., 1965; Spech et al., 1976). Boucher and coworkers (1964) described a method whereby newly formed AII was absorbed onto Dowex resin present in the incubation mixture. The AII was then washed from the resin and quantified by bioassay. The above modifications involve sometimes complex chemical manipulations of either angiotensin or renin which probably account for the poor reproducibility of such measurements. Most of these assays require long periods of incubation (12-24 hr) to generate sufficient AII to cause pressor effects. Under such conditions small changes in temperature, oxygenation, etc., and bacterial infiltration may contribute to the variability described for such methods.

The development of antibodies to angiotensin I (Haber et al., 1969) greatly improved the sensitivity with which AI could be quantified and circumvented many of the problems associated with extraction of AI from plasma or from the incubation mixture. The increased sensitivity also reduced the volume of sample required. Subsequently, slight modifications of the radioimmunoassay for AI have allowed for reproducible determinations of AI in as little as 20 nl of rat plasma (Johnston et al., 1975), and measurements of renin content in single juxtaglomerular apparatus of rat kidneys (Dahlheim et al., 1970a).

Angiotensin I can also be quantified by radioimmunoassay for AII, however, most antibodies to the octapeptide are nonspecific and crossreact with AI and several peptide fragments of AII (Cain and Catt, 1969; Cain et al., 1969a,b,1970; Catt et al., 1967; Dietrich, 1967; Dietrich and Frishknecht, 1968a,b; Vallotton et al., 1967). Furthermore, measurement of the generated AII by radioimmunoassay or bioassay relies on the complete hydrolysis of AI to AII. Therefore, inhibitors are required to prevent AII degradation by angiotensinases and conditions must be controlled such that newly formed AI is quantitatively converted to AII (Delorne et al., 1976; Oparil et al., 1974). Inasmuch as the activity of angiotensin-converting enzyme is relatively low in plasma (Ng and Vane, 1967, 1968, 1970; Oparil et al., 1970) quantitative conversion of AI to AII probably does not occur. Comparison of measurements using antibodies to AI and AII confirm the relative disadvantages and inaccuracies of the present methods of quantifying AII as opposed to measuring AI (Proszynska, 1978). Angiotensin I concentration as determined by RIA has been reported to be lower (Hollemans et al., 1969), higher (Cohen et al., 1971; Menard and Catt, 1972; Sealey et al., 1972), or comparable (Fukachi et al., 1973; Kotchen et al., 1973) to that determined by bioassay. Lijnen and coworkers (1976) found a direct correlation of AI measurements by RIA and bioassay of pooled plasma samples from human subjects under various states of altered renin activity.

Angiotensin I is rapidly removed from the circulation by the combined action of converting enzyme and angiotensinases both of which are ubiquitous throughout the body. The half-life of ^{125}I-labeled

angiotensin I or native AI in the circulation has been estimated to be between 15 and 200 sec (Lee et al., 1971a; Oparil et al., 1970; Poulsen and Poulsen, 1971). The half-life of trace amounts of radiolabeled AI in heparinized plasma incubated at 37°C is somewhat longer (3-5 min) suggesting that converting enzyme and angiotensinases in tissues other than plasma contribute significantly to the net rate of disappearance of AI in vivo.

4. Angiotensin-Converting Enzyme (ACE)

Angiotensin I is rapidly converted to the biologically potent vasopressor peptide, angiotensin II, via hydrolytic cleavage of the Phe^{8}-His^{9} bond of AI (Figure 1). The enzyme catalyzing this hydrolysis, angiotensin-converting enzyme (ACE) [EC 3.4.15.1], was first isolated from equine plasma (Skeggs et al., 1956a). Converting enzyme has been identified as a carboxy-terminal dipeptidyl peptidase, following experiments where radioactive AII (Ryan et al., 1971) and His-Leu (Igic et al., 1972; Ryan et al., 1970) were isolated from pulmonary effluents following infusion of appropriately labeled AI. Injection of an undecapaptide with an extra histidine residue in position 10 of AI did not yield AII in vivo (Ng and Vane, 1970), suggesting that ACE cleaves the dipeptide from the carboxy terminus of AI. Lentz et al. (1956) isolated stoichiometric amounts of His-Leu from their converting enzyme reaction mixture. When these investigators subjected angiotensin I to carboxypeptidase treatment, leucine was the first amino acid liberated and the residual nonapeptide possessed little vasopressor activity. Histidine was the second amino acid cleaved and pressor activity was restored in the octapeptide, but

was lost when phenylalanine was subsequently released. The residual, inactive heptapeptide was the limit product, as it was when AII was used as substrate for carboxypeptidase. This led to the conclusion that proline was the next amino acid, which was subsequently confirmed when the entire sequence of equine angiotensin II was determined (Skeggs et al., 1956b).

Experiments using an isolated lung preparation demonstrated that 75-99.9% of the injected bradykinin is degraded during a single transpulmonary passage (Alabaster and Bakhle, 1972b; Levine et al., 1973). These findings provided the first evidence for a similarity in the site and rate of AI and bradykinin hydrolysis. Subsequently, both angiotensin converting and bradykinin degrading activities were concentrated in the same subcellular fraction (Alabaster and Bakhle, 1973; Bakhle, 1968; Sander and Huggins, 1971), and have been identified in a preparation of pulmonary plasma membranes (Ryan and Smith, 1971). Ryan and coworkers (1970, 1972) co-infused the vascular marker blue dextran with radiolabeled AI and bradykinin into perfused lungs. The radioactive metabolites emerged quantitatively and simultaneously with the dye suggesting that both AI and bradykinin are metabolized without leaving the pulmonary vascular space. In partially purified preparations, bradykinin and angiotensin hydrolyses have been shown to be inhibited by chelating agents, suggesting that both enzyme activities represent metalloproteins (Alabaster and Bakhle, 1973; Bakhle and Reynard, 1971). The ratio of bradykininase and ACE activities remained constant throughout the purification of the enzyme, suggesting that a similar enzyme moiety is responsible for both processes (Ueda

et al., 1972). The final product of purification, a single protein band, has been shown to hydrolyze both bradykinin and angiotensin (Das and Soffer, 1975; Soffer et al., 1974). Pure converting enzyme has been isolated from human plasma, human seminal fluid, rabbit lung, hog lung, hog kidney and bovine kidney (Depierre et al., 1978; Dorer et al., 1974; Igic et al., 1972; Lanzillo and Fanburg, 1977a,b; Oshima et al., 1974; Soffer et al., 1974). All preparations catalyze hydrolysis of both AI and bradykinin. It is now fairly well established that a single, pure carboxyl-terminal dipeptidyl peptidase catalyzes both angiotensin conversion and bradykinin degradation.

Conversion of angiotensin I is a relatively well defined enzymatic reaction whereas bradykinin inactivation represents the effects of several enzymes. Although hydrolysis by converting enzyme is the major route of inactivation of bradykinin, cleavage of any bond inactivates this peptide (Bakhle, 1974; Igic et al., 1972; Levine et al., 1973). Ryan and coworkers (1970) found that at least five peptide bonds were hydrolyzed when radioactive bradykinin was perfused through isolated rat lungs.

Besides the quantitative differences observed for bradykinin degradation and angiotensin conversion, there remains one qualitative difference between the two activities. The chloride dependence of angiotensin-converting enzyme was first described by Skeggs and coworkers (1956b), and has been used as criterion in the definition of true converting enzyme. Although the hydrolysis of AI is blocked almost entirely in a chloride-free medium, that of bradykinin continues at about half the maximal rate (Dorer et al., 1974; Igic et al., 1972; Nakajima et al., 1973). This anion requirement is best met

with the halide (Bakhle and Reynard, 1971; Piquilloud et al., 1970) but varies with the substrate employed. In cell-free preparations of lung tissue, increasing the concentration of chloride in the incubation medium is accompanied by a similar increase in AI conversion; however, bradykinin inactivation remains unchanged (Alabaster and Bakhle, 1972a; Sander et al., 1971). The presence of chloride has been observed to alter the spectral properties of converting enzyme (Oshima et al., 1974), presumably reflecting a conformational change in the enzyme. Dorer and associates (1974) demonstrated that chloride ion significantly increased the maximal velocity and affinity of the enzyme for the hydrolysis of bradykinin. Thus, chloride has been described as an allosteric modifier of converting enzyme (Cheung and Cushman, 1973). Igic and coworkers (1972a) coupled converting enzyme covalently to Sepharose 4B to form a water-insoluble complex and simulated organ perfusion by using a column packed with the Sepharose-enzyme complex. During a single passage through the column, bradykinin was completely inactivated and 60% of the angiotensin I was converted to angiotensin II. Using this system, these investigators demonstrated that the hydrolysis of neither peptide was affected by the removal of chloride from the buffer system (Igic et al., 1972). Coupling of the pure enzyme to the column may produce a change in protein structure which orientated the active site or sites of the enzyme such that chloride was no longer required for the binding of AI. Accordingly, it has been suggested that ACE may have two possible orientations of binding to AI, corresponding to low and high chloride concentrations (Alabaster and Bakhle, 1973; Oshima et al., 1974).

Only the latter orientation is suitable for AI hydrolysis to proceed. Bradykinin, however, may bind with either enzyme protein configuration.

Converting enzyme has been estimated to constitute approximately 0.1% of the total lung protein (Das and Soffer, 1975). The molecular weight of the native enzyme has been estimated to be approximately 130,000 (Das and Soffer, 1975; Lanzillo and Fanburg, 1977a,b; Soffer et al., 1974). Much higher estimates of molecular weight have been obtained by gel filtration but were attributed to the large oligosaccharide content of the enzyme which greatly influences the migration of ACE on Sepharose or during electrophoresis at suboptimal concentrations of acrylamide (Depierre et al., 1978; Dorer et al., 1972; Hartley and Soffer, 1978; Lanzillo and Fanburg, 1974, 1976a, 1977a; Nakajima et al., 1973; Oshima et al., 1974; Stevens et al., 1972). Experiments using purified ACE indicate that the enzyme contains a single subunit which is highly asymmetric (Soffer et al., 1974). The enzyme is rich in aromatic amino acids, contains a substantial number of half-cystine residues, a high content of hydrophobic residues, and possesses a moderate degree of hydrophobicity (Hatch, 1965). Converting enzyme contains one molar equivalent of bound zinc (Das and Soffer, 1975; Soffer et al., 1974) which can be substituted by other divalent metals, including cobalt or manganese (Alabaster and Bakhle, 1973; Bakhle and Reynard, 1971; Cushman and Cheung, 1971a). The enzyme requires a free carboxyl group of the substrate (Elisseeva et al., 1971), will not act on D-amino acid residues (Erdos and Yang, 1967; Oparil et al., 1971, 1973), and will

not cleave a peptide bond consisting of the imino group of a prolyl residue (Elisseeva et al., 1971), which explains why newly generated angiotensin II is not further degraded by converting enzyme (Yang et al., 1970). Results using various peptide substrates suggest a greater affinity for basic or aromatic residues than for acidic or branched-chain amino acids, which may explain why bradykinin is more tightly bound by ACE than is angiotensin I (Dorer et al., 1974).

Angiotensin-converting enzyme in plasma was thought to account for the conversion of circulating AI to AII in vivo. However, Ng and Vane (1967) demonstrated that conversion of AI in whole dog blood incubated in vitro was very slow, requiring approximately 2 min for conversion of 50% of the peptide to AII. In contrast, when AI was infused intravenously, greater than 80% of the peptide was converted to AII during a single circulation (15-20 sec). These data suggested that conversion of AI occurred rapidly in tissues other than plasma. Inasmuch as the organs used for bioassay of AII were bathed with arterial blood such that the intravenous injected AI traversed the pulmonary vascular bed prior to bioassay, the lungs were a likely candidate for the site of AI conversion. Infusion of AI intravenously resulted in a two-fold greater pressor response than intra-arterial infusions. It was estimated from these data that 50% of the injected dose of AI was converted to AII during a single passage through lung whereas only 30% conversion of AI occurred in all peripheral vascular beds combined. It was concluded that conversion of AI in plasma was insufficient to account for the generation of AII in vivo, and that although conversion of AI to AII occurs to a small extent in most

peripheral vascular beds, lung is the major site of liberation of AII into the circulation (Ng and Vane, 1967).

The rate of production of AII by isolated strips of blood vessels superfused with AI was greatest in the pulmonary artery and occurred to progressively decreasing extents in the strips from more peripheral vascular beds (Aiken and Vane, 1970). The fact that inhibitors of ACE reduced but did not completely abolish the conversion of AI suggested that some of the AII was generated locally within the parenchyma of blood vessel walls. This intramural production of AII was greatest in pulmonary arterial strips, however, peripheral blood vessels were more sensitive to a given dose of infused AII. It was therefore concluded that the pressor effects of AII in peripheral vascular beds were due primarily to AII delivered via the blood stream as opposed to AII generated locally. The possible role of lung in liberating AII into the circulation is complemented by the absence of extraction of this octapeptide by the pulmonary vasculature (Aiken and Vane, 1970; Biron et al., 1969; Hodge et al., 1967; Leary and Ledingham, 1969). In contrast, greater than 50% of the infused AII was extracted by the vasculature of kidneys, hindlimbs and head (Ng and Vane, 1968). When AI was perfused through these tissues, only negligible amounts of the peptide appearing in the venous effluent was identified as AII. Therefore, clearance of AI by extrapulmonary vascular beds does not involve the liberation of newly formed AII into the venous blood. Accordingly, it was concluded that in contrast to lung, extrapulmonary conversion of AI involves the local production of AII utilized and then degraded within the tissue parenchyma. Such a mechanism would account for the negligible amounts of AII appearing in

the venous effluent of extrapulmonary tissues during perfusion with AI (Aiken and Vane, 1970; Ng and Vane, 1968).

Converting enzyme activity has been identified in nearly every tissue (Cicilini et al., 1977; Cushman and Cheung, 1971a; Huggins and Thampi, 1968; Roth et al., 1969) and species (Depierre and Roth, 1972) studied. Incubation of tissue homogenates with synthetic substrates for ACE demonstrates that lung and kidney possess the highest converting enzyme content of all tissues examined. Converting enzyme has been found in the particulate fraction of homogenates (Bakhle, 1968) and in subcellular fractions of lung enriched in membranous particles (Ryan et al., 1972; Sander and Huggins, 1971). The majority of ACE in homogenates of rabbit lung sediments between 1,000 and 25,000 x g. When this pellet was further fractionated by discontinuous density gradient centrifugation, the enzyme appeared in the fraction possessing many membranous particles as identified by electron microscopy (Sander and Huggins, 1971) suggesting that ACE is bound to membranes of lung in vivo. Inasmuch as 5'-nucleotidase, a plasmalemma marker enzyme (Emmelot et al., 1964), co-precipitated with ACE, it was concluded that converting enzyme is bound to endothelial cell membranes (Lanzillo and Fanburg, 1974; Sander and Huggins, 1971). Ryan and Smith (1971) and Lanzillo and Fanburg (1974) have prepared similar fractions of pulmonary tissue rich in vascular membranes which are capable of hydrolyzing AI at rates sufficient to account for AI conversion in vivo. Further evidence that converting enzyme is situated on or near the vascular surface of endothelial cells was obtained by coperfusing lungs with radiolabeled AI or bradykinin and vascular marker blue dextran (Ryan et al., 1970, 1972). The

radioactivity was found to appear simultaneously and quantitatively with the vascular marker in the venous effluent suggesting that the metabolism of AI and bradykinin occurs in close proximity to the vascular lumen. Radioactivity was not retained by the lungs suggesting that AI was metabolized without leaving the vascular space (Ryan et al., 1972). These investigators have subsequently incubated slices of rat lung with antibody to ACE which had previously been conjugated with microperoxidase. Electron microscopy of the slices following incubation with antibody revealed that the oxidized diaminobenzene-antibody conjugate was distributed on the plasma membrane and caveolae of endothelial cells (Ryan et al., 1975). The conjugated antibody was especially prominent in regions identified as capillaries and venules, suggesting that ACE is localized on the luminal aspect of endothelial cells in pulmonary blood vessels of small caliber. Converting enzyme has since been localized to similar membranous structures of endothelium from pig aorta (Ryan et al., 1977). Furthermore, endothelial cells isolated from pig pulmonary artery and aorta (Hayes et al., 1978; Ody and Junod, 1977; Ryan and Ryan, 1977; Ryan et al., 1975, 1976, 1978b) and human umbilical cords (Johnson and Erdos, 1977) possess considerable ACE activity. Subendothelial cells contained negligible ACE activity. This rather specific localization of ACE has recently been proposed for use in isolated cell preparations as a specific endothelial cell membrane marker (Carroll et al., 1978).

Converting enzyme has also been identified in endothelium of extrapulmonary tissues (Caldwell et al., 1976). Fluorescein-labeled antibody to pure rabbit lung ACE was localized in the vascular endothelium of rabbit lung, liver, adrenal cortex, pancreas, kidney, and

spleen. Kidney was the only organ in which the antibody was distributed in epithelioid structures. Renal ACE was found in association with glomerular tufts and the brush border of proximal tubules (Caldwell et al., 1976; Ward et al., 1975; Zimmerman et al., 1973). Accordingly, bradykinin has been shown to be degraded in the proximal but not the distal tubules of intact kidneys (Carone et al., 1975; Hall et al., 1976).

The enzyme of the kidney has been separated with the microsomal fraction of homogenates and hydrolyzes both AI and bradykinin (Erdos and Oshima, 1974; Erdos and Yang, 1967). Despite the presence of large amounts of ACE in kidney, very little AII is liberated into the renal venous blood following injection of AI into the renal artery (Oparil et al., 1970). Therefore, the extraction of AI from the renal circulation has been attributed to the rapid uptake of AI or AII (DiSalvo et al., 1971; Hofbauer et al., 1973). Intrarenally generated AII is then degraded by aminopeptidases prior to its release back into the circulation (Oparil et al., 1970). The inability to completely abolish the vasopressor effect of AI in kidneys by coperfusion with inhibitors of ACE suggests an intrarenal site of AI conversion not accessible to the inhibitors. Bailie and associates (1971) observed that circulating AII was extracted by intact dog kidneys and that the concentration of AII in renal lymph was consistently greater than that in arterial or renal venous plasma. These data support the hypothesis that AII is generated locally within renal tissue and is not transferred back into renal venous plasma. A similar local, intramural generation of AII has also been postulated for uterine smooth muscle (DiSalvo et al., 1976) and adrenal glands (Ackerly et al., 1976).

In addition to the similar morphological localization of converting enzyme in various tissues, several other factors have been described which suggest a marked similarity of ACE from different sources. Lanzillo and Fanburg (1976a) observed that pulmonary ACE from rat, hog and rabbit migrated identically on polyacrylamide gels, suggesting similar molecular weight and subunit structure of these three enzymes. The value they obtained for the molecular weight agrees well with that estimated for ACE from hog, human and guinea pig plasma (Huggins et al., 1970; Lee et al., 1971a; Oshima et al., 1974), from rabbit lung (Das and Soffer, 1975) and from bovine kidney cortex (Oshima et al., 1974). These investigators also compared the kinetic and inhibitory properties of converting enzyme from guinea pig lung and serum (Lanzillo and Fanburg, 1976b). Both enzymes possessed similar affinity for AI. When AI, bradykinin or a tetrapeptide was added to the enzyme preparation, no difference in the inhibitory properties was observed. Immunologic studies have demonstrated cross reactivity between ACE prepared from hog kidney, lung and plasma (Erdos and Oshima, 1974) and between pulmonary converting enzyme of hog and rat (Ryan et al., 1975) and rat, rabbit, guinea pig and dog (Conroy et al., 1976).

Converting enzyme activity has been found to be altered under different physiological conditions. Serum and lung ACE activity are increased following chronic exposure to hypobaric hypoxia (Molteni et al., 1974b), but are reduced during acute hypoxia (Leuenberger et al., 1978). Serum ACE activity is also elevated in infants suffering from idiopathic respiratory distress (Mattioli et al., 1975). Inasmuch as the rate of renin secretion was also elevated during alveolar

hypoxia, it was suggested that the increased serum ACE activity may reflect a general activation of the renin-angiotensin system under these conditions.

ACE activity is also altered under various pathological states. Patients suffering from sarcoidosis possess an elevated serum ACE activity (Lieberman, 1975; Oparil et al., 1976). Converting enzyme activity of lymph nodes is also elevated in these patients (Silverstein et al., 1976). Lieberman (1975) found that daily administration of prednisone to patients with sarcoidosis reduced serum ACE activity to control values. Serum ACE activity is also increased in patients with Gaucher's disease (Lieberman and Beutler, 1976; Silverstein and Friedland, 1977). The elevated serum ACE activity in Gaucher's disease is accompanied by increases in the activity of converting enzyme from splanchnic tissue. Oparil and coworkers (1976) found that serum ACE activity was reduced in pateints suffering from several pathological disorders involving lung tissue, including chronic obstructive pulmonary disease, shock lung, cystic fibrosis, lung cancer, and tuberculosis. Serum ACE activity has also been inversely correlated with the number of arterial lesions developed during experimentally-induced one-kidney perinephritic hypertension in rabbits (Campbell et al., 1978). The pathophysiological relevance of these data is unclear since the origin and physiological importance of serum ACE remains obscure. Altered serum enzyme activities may reflect general damage to lung parenchyma which results in altered rates of sloughing of the pulmonary enzyme into the circulation.

Accordingly, it has been suggested that measurement of ACE activity in serum may be useful in the diagnosis of various pulmonary disorders.

The rate of conversion of AI by isolated perfused kidney, measured by bioassay, is depressed following prolonged DOCA-salt loading (Franklin et al., 1970) and chronic mercuhydrin-salt deprivation (Merrill et al., 1973). However, Allen and Gilmore (1975) observed that salt deprivation decreased the systemic pressor response to AI whereas sodium-loading had the converse effect. Although these observations were not necessarily in accordance with others, they were interpreted to indicate that ACE activity is a labile entity, sensitive to changes in total body sodium. Bell and Bakhle (1975) on the other hand, found no change in plasma or pulmonary ACE activities following administration of oral contraceptives, however, total body clearance of AI was stimulated. This suggests that factors other than ACE activity may be responsible for the altered rates of clearance of AI from the circulation under these conditions; such as changes in vascular sensitivity to AII or altered angiotensinase activities. Therefore, altered rates of conversion of AI following changes in dietary sodium may not be due to changes in ACE activity. When ACE was measured in vitro, the activity of this enzyme was found not to be altered by salt loading or deprivation (Molteni and coworkers, 1976). These investigators concluded that the previously reported changes in the pressor response to injected AI following alterations in dietary sodium most likely reflect changes in the vascular sensitivity or half-life of circulating AII. Indeed, it has been found that restriction of dietary sodium results in an increased number of vascular receptor sites for AII (Brunner et al., 1972) which might account for

the differences in the apparent rate of AI conversion as measured by bioassay (Allen and Gilmore, 1975; Franklin et al., 1970; Merrill et al., 1973).

In addition to catalyzing the hydrolysis of AI and bradykinin, ACE has also been shown to cleave the carboxy terminus dipeptide from des-Asp^1-angiotensin I (des-Asp^1-AI) to yield the biologically active heptapeptide des-Asp^1-angiotensin II (AIII) (Chiu et al., 1976; Tsai et al., 1975). Since the affinity of converting enzyme for des-Asp^1-AI is greater than that for AI (Chiu et al., 1976) it has been suggested that under specific circumstances where ACE is limiting and des-Asp^1-AI and AI are present in equivalent concentrations, AIII will be the major biologically active product formed. ACE has also been shown to be capable of liberating AII from angiotensinogen via sequential hydrolysis of carboxy terminal dipeptides (Dorer et al., 1975), however, the physiological importance of this function of converting enzyme is most likely negligible.

Several different methods have been employed to assay converting enzyme activity. Bioassays using isolated smooth muscle or the pressor response to AII in intact animals and radioimmunoassays have been widely used to study conversion in vivo. However, bioassays for AII are complicated by possible changes in factors such as receptor sensitivity and number, angiotensinase activity, and AII-induced production of other vasoactive substances, all of which would tend to alter the pressor response to AII in the absence of changes in ACE. Radioimmunoassays for AII are not widely used because most antibodies prepared against pure AII crossreact with several peptide fragments of angiotensin (Bailie et al., 1971; Cain and Catt, 1969a,b; Catt et al.,

1967; Dietrich, 1967; Vallotton et al., 1967). Chemical measurement of His-Leu liberated from angiotensin I has also been used to quantify ACE activity in broken cell preparations. The His-Leu liberated by ACE can be quantified as a radioactive product of appropriately labeled AI (Conroy and Lai, 1978; Huggins and Thampi, 1968; Rohrbach, 1978; Ryan et al., 1977, 1978a), fluorimetrically (Carmel and Yaron, 1978; Cheung and Cushman, 1973; Friedland and Silverstein, 1977; Hayakari and Kondo, 1977; Piquilloud et al., 1970; Russo et al., 1978), or by an increase in ninhydrin-reactive material (Dorer et al., 1970). The advantage of these assays is that they employ a physiological substrate and therefore provide a meaningful comparison of pure enzymes from different sources. However, a major disadvantage is that these methods do not give a valid estimate of converting enzyme content in crude fractions that contain degrading activities for AI or His-Leu. Such activities are widespread and often high (Lee et al., 1971a). Chemical assays employing as substrate a model tripeptide protected at its amino-terminus are more accurate methods for measuring ACE in vitro in that the blocking group protects the peptide from degradation by contaminating aminopeptidase activities. Furthermore, most assays designed for measuring ACE activity using synthetic peptide substrates are simpler and more rapid than are current methods for quantifying AI or His-Leu. However, since the model compound is an artifical agent, it is necessary to compare its hydrolysis by ACE with that of AI in order to establish it as a representative substrate and thus obtain biologically meaningful results.

5. Angiotensin II (AII)

The decapeptide product of the ACE-catalyzed reaction (Figure 1), angiotensin II (AII), is the most potent vasopressor substance known (Haddy et al., 1962; DeBono et al., 1963). Vasoconstriction in response to AII is most evident in precapillary segments of vascular beds as opposed to post-capillary vessels (Jarhut, 1971; Folkow et al., 1961). Rose and coworkers (1962) did not observe venoconstriction following AII whereas DePasquale and Burch (1963) reported that injection of AII enhanced venous tone. Angiotensin II acts mainly at the arterioles of systemic capillary beds (Abell and Page, 1942). Increased vascular resistance in response to angiotensin has been identified in coronary vessels (Drimal, 1968), cutaneous vessels (DeBono et al., 1963; Peart, 1959), umbilical artery (Dyer, 1970), uterine artery (Gough and Dyer, 1971; Greiss and Van Wilkes, 1964) and the placenta (Ward and Gautieri, 1969). Redistribution of blood flow away from the mesenteric, hepatic and splanchnic regions is also a common response to AII infusions (Barer, 1961; Forsyth et al., 1971; Segel et al., 1963). The redistribution of blood flow from the splanchnic area, liver and skin is a result of the direct vasoconstrictor effect of AII whereas dilation of the vasculature of skeletal muscle, brain, heart and uterus is passive, perhaps mediated through the elevated perfusion pressure. Cardiovascular effects of AII include a positive inotropic effect which has been attributed to increased diastolic pressure (Koch-Weser, 1964; Krasney, 1968), decreased cardiac output via a reflex bradycardia (Segel et al., 1960; DeBono et al., 1963) and a positive chronotropic effect mediated

by β-adrenergic receptors (Nishith et al., 1962; Krasney et al., 1965).

Aiken and Vane (1970) observed pulmonary artery constriction when AII was administered in high doses, however, no changes in resistance of the pulmonary vein have been observed (Hyman, 1969). Rose and coworkers (1962) and Chimosky et al. (1962) attributed the pulmonary effects to be secondary, mediated through changes in left atrial or systemic arterial pressure.

Angiotensin II administered intravenously causes a decrease in renal blood flow and glomerular filtration rate and an increase in filtration fraction (Bonjour and Malvin, 1969b; Herrick et al., 1941; Hughes-Jones et al., 1949). A dose-dependent effect has been described for AII-induced diuresis and natriuresis. Merrill et al. (1938) observed renal hypertrophy following injection of renin. Vander (1963) suggested a direct inhibition of sodium and water reabsorption by AII acting at a distal tubular site. This effect appears to be independent of renal innervation (Bonjour and Malvin, 1969a; DiSalvo and Fell, 1970; Geller and Kendrick, 1967). However, the direct action of AII on renal tubules was not detectable by short-circuit current studies using toad bladder, toad skin and isolated proximal tubules (Burg and Orloff, 1968; Coviello and Crabbe, 1965; Healy et al., 1969).

Much controversy exists over the involvement of the sympathetic nervous system in mediating the vasopressor effect of angiotensin. Many investigators suggested a ganglionic stimulating effect of AII (Felberg and Lewis, 1964; Lewis and Reit, 1965; Trendelenburg,

1966). McCubbin and Page (1963) reported an enhanced pressor response to ephedrine and tyramine following administration of AII, whereas AII did not alter the pressor response to exogenous norepinephrine. These investigators interpreted their results to suggest that AII enhances the release of norepinephrine from sympathetic nerve terminals. Conversely, Khairallah et al. (1971) and Panisset and Bourdois (1968) suggested that AII inhibited uptake of norepinephrine by sympathetic nerve fibers. In contrast to the evidence suggesting that a β-adrenergic influence was involved in the cardiovascular response to AII (Krasney et al., 1965; Nishith et al., 1962), the response of vascular smooth muscle and renal tissue to angiotensin is not altered by experimental sympathectomy (Bonjour and Malvin, 1969a; DiSalvo and Fell, 1970; Geller and Kendrick, 1968).

Radiolabeled angiotensin II is bound by specific physiologically active receptor sites in vascular smooth muscle (Baudouin et al., 1971; Goodfriend and Lin, 1970). The specific nature of AII receptors has been examined by comparing the activities and binding affinities of various angiotensin agonists and antagonists in an attempt to identify specific amino acid residues of AII involved in binding to the receptors (Bumpus, 1977). Fractionation of aortic tissue has demonstrated that only the subcellular fraction rich in plasma membranes possessed significant capacity to bind ^{14}C-AII (LeMorvan and Palaic, 1975). The binding characteristics of these fractions was consistent with the physiological data since the time required for maximal binding was similar to that for the development of maximal tension. Furthermore, relaxation of vascular smooth muscle

paralleled the transient unloading of the binding sites of aortic plasma membranes. In the rabbit aorta, saturation of the binding sites is accomplished with a concentration of AII of 10^{-7}M; a concentration capable of eliciting a maximal pressor response. Glossman and coworkers (1974) and Sraer et al. (1974) determined the dissociation constant of this binding site to be 4.5×10^{-11}M for adrenal cortical tissue and 6.0×10^{-11}M for isolated glomeruli. The relative binding affinity of AII and several of its analogs was proportional to their respective biological activity (Douglas et al., 1976). Baudouin and coworkers (1972) suggested the binding of AII at the receptor causes an enhanced efflux of calcium from the tissue. Paiva and associates (1974) reported an enhanced response of guinea pig ileum to AII by increasing the calcium concentration in the medium. These investigators speculated as to the possible importance of calcium flux in AII-tachyphylaxis. However, several groups of researchers noticed diminished tachyphylaxis following treatment with a solution rich in angiotensinase activity or with Dowex-50 which absorbs angiotensin (Bohr and Uchida, 1967; Khairallah et al., 1966; Walter and Bassenge, 1969). The results suggest that tachyphylaxis is the result of prior receptor occupancy. Khairallah et al. (1966) found that the angiotensinase activity of blood vessel walls was inversely related to the tendency of that particular tissue to develop tachyphylaxis to AII. These investigators provided evidence that the receptor site is associated with the luminal aspect of the vascular endothelium when they observed decreased tachyphylaxis to AII following injection of solubilized angiotensinase.

Angiotensin I and II have been shown to share a common binding site (Aiken and Vane, 1970), however, differences have been demonstrated between the vascular binding site and that of the adrenal cortex (Beckerhoff et al., 1975; Steele and Lowenstein, 1974; Williams et al., 1974). These results were obtained through assessing the pressor and steroidogenic effects of AII in the presence of structural analogs or by determining the effect of antibodies on the biological activity of AII.

Increased numbers of receptor sites on vascular smooth muscle and in adrenal cortical tissue have been observed following salt deprivation, bilateral nephrectomy or ganglionic blockade (Brunner et al., 1972; Douglas and Catt, 1976). Palaic and Lemorvan (1971) found that guinea pig aorta made tachyphylactic to AII was capable of binding a greater amount of AII compared to non-tachyphylactic aortic tissue. These data can be explained by the theory of prior occupancy of receptor site proposed by Swales et al. (1975) and Thurston (1976). This hypothesis states that the decreased response to AII during sodium-deprivation or body fluid volume reduction results from prior saturation (or near saturation) of receptor sites by endogenous AII. When specific adrenal binding sites from rats maintained on a low sodium diet were incubated in vitro with radiolabeled AII, a greater concentration of binding sites per adrenal cell was observed compared to that of controls (Douglas and Catt, 1976). Since the binding capacity of adrenal cells was inversely related to the plasma AII concentration, these investigators attributed the changes in apparent receptor number to reflect the degree of receptor occupancy prior to infusion of AII. The increase in AII binding

following bilateral nephrectomy was not accompanied by an augmentation of adenylcylcase, 5'-nucleotidase, or Mg-ATPase suggesting that the response does not reflect a general hypertrophy or hyperplasia of target cells (Devynck et al., 1976; Glossman et al., 1974; Pernollet et al., 1977). This increase in AII binding capacity was not accompanied by changes in the affinity of the receptor for the peptide and was thus interpreted to reflect an apparent increase receptor number, which most likely reflected a smaller fraction of receptors occupied by AII in the nephrectomized animals compared to controls (Devynck and Meyer, 1978). Inasmuch as inactive analogs of AII were also bound to a greater extent following nephrectomy, this response appears to be independent of biological activity. Chronic administration of AII prevented the increase in AII binding capacity following bilaterally nephrectomy which further supported the receptor occupancy theory in explaining these results.

The morphological changes which occur in vascular smooth muscle during angiotensin-induced vasoconstriction have been studied by both electronmicroscopic and histochemical techniques. Angiotensin II induces contraction of individual endothelial cells resulting in a flattened appearance of the cells and separation of interendothelial cell junctions (Constantinides and Robinson, 1969; Robertson and Khairallah, 1971, 1973). Karnovsky (1967) using cellular markers demonstrated an increased permeability of the vascular endothelium which was attributed to the widened intercellular spaces following injection of AII. It was noted that the change in permeability was apparent only when the cellular marker was injected within 8 minutes following administration of AII suggesting a transient effect. The

markers were found to be concentrated in the subendothelial space. Robertson and Khairallah (1971) injected ^{3}H-AII and observed that the label was located primarily in the nuclear region of endothelial cells and in the mitochondria of the underlying smooth muscle. Radiolabeled amino acids were distributed randomly throughout the arterial wall. The enhanced permeability induced by AII is not mediated by histamine (Bisset and Lewis, 1962). Thickening of the arteriolar wall and a narrowing of the lumen has been reported following long term AII exposure (Koletsky et al., 1966; Hobbs and Cliff, 1971). Byrom (1964) and Olsen (1968) observed dilation of the main branch of the renal artery with considerable constriction more distally. Giese (1964) observed alternating areas of constriction and dilation of small arteries accompanied by intermittent regions of increased permeability associated with the dilated segments.

Des-Asp^{1}-AII (AIII), a heptapeptide metabolite of AI formed by the sequential activities of ACE and aminopeptidase (Goodfriend et al., 1975), may function as an intracellular mediator of the AII response. Freeman et al. (1976) compared the effect of AII and Des-Asp^{1}-AII on the aldosterone, corticosterone and cortisol responses of receptor sites in the glomerulosa, reticularis and fasciculata regions of adrenal cortiex. In vivo both AII and AIII caused similar increases in steroid synthesis; however, the affinity of the aldosterone-stimulating site was greater for AIII than AII. Bravo and coworkers (1976) found that AII and AIII produced similar steroidogenic activities in vivo and when the peptides were incubated with isolated adrenal glomerulosa cells in vitro. It was concluded that AII could stimulate aldosterone biosynthesis without prior conversion to the

heptapeptide. Angiotensin II stimulates renin substrate synthesis by the liver (Reid, 1977) and inhibits renin secretion from kidneys (Vander and Geelhoed, 1965). Freeman and coworkers (1975, 1976) and Vandongen et al. (1974) reported a similar inhibition of renin secretion by the heptapeptide AIII. A possible intra-renal role of AIII in mediating the AII response is suggested by the demonstration that renal blood flow is decreased following AIII infusion (Freeman et al., 1975, 1976). In summary, the physiological importance of AIII as a mediator of the renin-angiotensin system remains obscure and is a topic of active current investigation.

6. Angiotensinases

The biological half-life of angiotensin II in vivo has been estimated to be approximately one minute (Boyd et al., 1969; Cain et al., 1970). The half-life of the AII when injected into the hepatic portal vessels or renal artery is much shorter than when injected intravenously (Akinkugbe et al., 1966; Chamberlain et al., 1964; Hodge et al., 1967; Kokubu et al., 1967; Methot et al., 1964). Degradation of AII has been examined in vivo, in vitro, and in isolated organs (Bumpus et al., 1964; Hodge et al., 1967; Khairallah et al., 1963; Kokubu et al., 1966; Landesman et al., 1963; Leary and Ledingham, 1969; Osborne et al., 1970, 1971; Regoli et al., 1963). Chromatographic separation of AII peptide fragments has demonstrated that AII is susceptible to hydrolysis by several proteolytic enzymes, including aminopeptidases (Khairallah et al., 1963; Magnan and Regoli, 1978; Moore et al., 1977; Regoli et al., 1963), carboxypeptidases (Johnson and Ryan, 1968) and several endopeptidases (Kokubu et al., 1969; Riniker, 1964). Accordingly, yeast aminopeptidase, pepsin, trypsin,

chymotrypsin and papin have all been shown to degrade angiotensin II (Croxatto and Croxatto, 1942; Plentl and Page, 1943).

Plasma was first suspected as the site of angiotensin degradation, however, subsequent experiments showed the activity of angiotensinases in plasma was insufficient to account for the rapid disappearance of AII *in vivo* (Cain *et al.*, 1970). Several investigators have since demonstrated an important role of systemic vascular beds in the inactivation of AII *in vivo* (Hodge *et al.*, 1967; Ng and Vane, 1968; Bakhle *et al.*, 1969). Tissue distribution studies demonstrated angiotensinase activity to be concentrated in kidney, intestine, pancreas, spleen, liver, salivary gland and thymus (Itskovitz and Miller, 1967; Matsunaga *et al.*, 1969). Tissue fractionation has localized angiotensinase activity in both microsomal and lysosomal subcellular organelles (Glenner *et al.*, 1962; Matsunaga *et al.*, 1968). The high concentration of radioactivity in kidneys following injection of (^{3}H)-AII into rats suggests that a large fraction of the AII is cleared from the circulation by the kidneys (Bumpus *et al.*, 1964). Accordingly, the rate of disappearance of (^{3}H)-AII from plasma was found to be reduced 2-fold in rats nephrectomized 18-24 hrs before AII injection. Only very small amounts of radioactivity appeared in the urine of normal rats suggesting that the major route of elimination of AII by the kidneys was via metabolism to shorter peptide fragments. Oparil and Bailie (1973) reported that 75% of the injected AII was extracted by kidneys and that this metabolism was due primarily to the activities of amino- and endopeptidase enzymes. The fractional removal of AII was not affected by changes in the rate of glomerular filtration but was inversely related to renal blood flow (Bailie and

Oparil, 1977). These investigators therefore concluded that the intrarenal metabolism of AII occurs primarily in the vascular compartment rather than in the tubules. Liver was also found to sequester radioactivity following injection of (^{3}H)-AII (Bumpus et al., 1964), indicating that hepatic metabolism may also contribute to the clearance of circulating AII. The rate of disappearance of AII from plasma incubated in vitro was negligible. In kidney, angiotensinase activity is concentrated in cortical tissue, perhaps associated with proximal tubules and the juxtaglomerular apparatus (Itskovitz and Miller, 1966). Destruction of proximal tubules with sodium potassium tartrate, mercuric chloride or meralluride reduces renal angiotensinase activity in vitro and in vivo.

Angiotensin II survives passage through intact lungs with minimal degradation or extraction (Goffinet and Mulrow, 1965; Biron et al., 1968, 1969; Hodge et al., 1967; Leary and Ledingham, 1969; Osborn et al., 1969). This negligible extraction of AII by lung adds to the efficiency of the pulmonary endothelium as the major site of generation of angiotensin II destined for the systemic circulation (Biron et al., 1968; Ng and Vane, 1967, 1968, 1970) and explains the prolonged half-life of AII when injected into the pulmonary artery compared to that injected into the systemic circulation (Hodge et al., 1967; Biron et al., 1969; Leary and Ledingham, 1969). In contrast, homogenates of lung tissue contain a considerable amount of AII-degrading activity (Itskovitz and Miller, 1967; Bakhle, 1968; Matsunaga et al., 1969), suggesting an intracellular site of these peptidases which are not exposed to circulating AII in the intact lung.

The inactivation of angiotensin II appears to be a rather labile phenomenon. The activity of angiotensinases in plasma is increased by hemolysis (Dexter, 1942), various liver diseases (Kokubu et al., 1965; Biron et al., 1964) and hemorrhage and E. coli endotoxin (Itskovitz et al., 1969). Unilateral renal artery clamping results in decreased angiotensinase activity of the ischemic kidney (Leary and Ledingham, 1970a). Leary and Ledingham (1970a,b) perfused both livers and kidneys of rats previously maintained on low- and high-sodium diets. The rate of hepatic angiotensin II inactivation was unaffected by changes in sodium balance. However, kidneys from sodium-deprived rats were capable of extracting a greater amount of infused AII than controls. Conversely, sodium loading decreased the renal extraction of AII.

7. Concluding Remarks

In conclusion, the renin-angiotensin system is a composite of a series of enzymes and their respective substrates. The synthesis of angiotensinogen by liver is under the influence of several hormonal factors and the direct stimulatory effect of circulating AII. Plasma angiotensinogen is cleared from the circulation by proteolytic digestion to form the biologically inactive decapeptide, AI. Angiotensinogen hydrolysis is catalyzed by renin, an enzyme synthetized and released from juxtaglomerular apparatus of the kidney. Renin secretion is mediated by renal baroreceptors, β-adrenergic receptors and the electrolyte composition of plasma and/or tubular urine. The turnover of plasma renin is regulated by its release from the kidney and its inactivation which occurs primarily via hepatic metaoblism. Angiotensin I is then rapidly metabolized to shorter peptide fragments

by enzymes associated with the vasculature of several tissues. The principal fragment of AI is the octapeptide, AII, which results from the hydrolysis of AI by ACE. The lung is believed to be the major organ contributing to circulating AII whereas extrapulmonary conversion of AI is involved in the local production of AII. Angiotensin II then exerts potent vasopressor effects via vasoconstriction and the release of aldosterone from adrenal cortical tissue. The half-life of circulating AII is very short due to its rapid degradation by nonspecific peptidases located primarily in liver and kidney. Shorter peptide fragments of AII also possess biological activity, however, the physiological significance of this activity remains obscure.

B. Principle Factors Regulating the Steady-State Concentration of AII in Adult Animals

Since all of the major effects of the renin-angiotensin system appear to be mediated through AII or one of its immediate peptide fragments, regulation of circulating AII concentrations is of pivotal importance in understanding the physiological relevance of this important pressor system as a whole. In general, the renin-angiotensin system can be viewed as consisting of 4 enzyme systems acting sequentially in regulating plasma AII concentrations. Being an intermediate in the total reaction sequence, the steady-state concentration of AII in plasma is dependent on both its rate of formation and the rate at which it is degraded. Anabolic processes include the synthesis of angiotensinogen, proteolysis of angiotensinogen by renin to form AI, and the conversion of AI to AII catalyzed by ACE. The catabolism of AII involves multiple nonspecific peptidases of several tissues. Inasmuch as each of the reactions are enzyme-catalyzed processes, the

net rate at which each step proceeds is dependent on both the amount of enzyme present and on the concentration of substrate available to the enzyme.

Since angiotensinogen is not stored to any great extent prior to its release (Nasjletti and Masson, 1971a) the rate at which this protein is secreted by liver is dependent solely upon its rate of de novo synthesis. Several investigators have found that plasma angiotensinogen concentrations appear to be relatively constant under conditions of altered rates of metabolism to AI (Bing, 1972; Nasjletti and Masson, 1971b, 1972, 1973). This may be interpreted to indicate that the steady-state concentration of angiotensinogen in plasma is controlled primarily by its rate of synthesis and not by its rate of hydrolysis to AI by renin. Accordingly, mechanisms must be proposed to account for the regulation of angiotensinogen synthesis which are integrated in some manner with changes in renin activity. Several such mechanisms have been suggested, such as the stimulatory effect of AII on angiotensinogen synthesis (Blair-West et al., 1974; Carratero and Gross, 1967b; Khayyall et al., 1973; Nasjletti and Masson, 1973). Therefore, an increased plasma renin activity would ultimately lead to an elevated plasma AII concentration which would then exert progressively greater positive feedback stimulation of hepatic angiotensinogen synthesis and thereby maintain plasma renin substrate concentrations. Conversely, decreases in renin activity lead to less circulating AII which would then have less stimulatory effect on angiotensinogen synthesis by liver. Alternatively, enhanced rates of synthesis of angiotensinogen may result from stimulation by corticosteroids (Carretero and Gross, 1967b; Haynes et al., 1953; Helmer

and Griffith, 1952; Krakoff et al., 1975; Lazer and Hoobler, 1971; Nasjletti and Masson, 1969; Reid et al., 1973) which commonly are associated with increases in plasma renin activity. Accordingly, under stressful conditions, the resulting increase in plasma corticosteroids acts in a permissive fashion to stimulate the secretion of both renin and angiotensinogen into plasma. If the increase in angiotensinogen synthesis is proportional to the elevated plasma renin activity, the net result will be no change in the plasma renin substrate concentration. Therefore, the overall regulation of plasma angiotensinogen appears to function to provide sufficient substrate for interaction with renin.

The renin-substrate reaction has traditionally been assumed to be the rate-limiting step in the overall regulation of plasma AII concentration. Indeed, changes in plasma renin activity, a measure of the renin-substrate reaction, have been correlated with proportional changes in circulating AII (Barrett et al., 1977; Haber et al., 1969; Lijnen et al., 1978; Sealey et al., 1972; Soveri and Fyhrquist, 1977). This direct correlation of PRA with AII persists under conditions of 10-fold increases in plasma renin activity in normal and hypertensive subjects (Swales and Thurston, 1977). Since plasma renin concentration does not correlate well with plasma AII (Lijnen et al., 1977a,b) the changes in renin activity most likely result from changes in angiotensinogen and/or renin concentration. A kinetic analysis of the renin-substrate reaction in human, dog, and rat plasma suggests that this reaction proceeds via first-order kinetics, the velocity being dependent on both enzyme and substrate concentrations (Gould et al., 1966; Poulsen, 1971a; Reid et al., 1973). In contrast, Rosenthal and

coworkers (1971) have suggested that endogenous angiotensinogen in human plasma is near saturating to renin, yielding reaction velocities 82% of maximum. However, these conclusions were based on substrate determinations by other investigators (Gould et al., 1966; Skinner, 1967). The Michaelis-Menten constant (Km) for the renin-substrate reaction in plasma has been determined in several species including humans. Values for Km in humans range from 0.71×10^{-6}M to 4.0×10^{-6}M angiotensinogen (Gould and Green, 1971; Haas and Goldblatt, 1967; Rosenthal et al., 1971; Skeggs et al., 1967; Skinner et al., 1975b; Weber et al., 1975). This molar concentration represents approximately 500-1000 ng angiotensinogen/ml plasma assuming stoichiometric conversion of angiotensinogen to AII. Inasmuch as serum angiotensinogen concentrations range from 0.78×10^{-6}M in normal plasma to 6.0×10^{-6}M in patients with malignant hypertension (Gould and Green, 1971), it is evident that the endogenous angiotensinogen concentration is near the Km for renin and thus the reaction velocity is most likely dependent at least partially on the plasma substrate concentration. Similarly, endogenous angiotensinogen concentration in plasma from dogs and rats (350 ng/ml - 900 ng/ml) is in a range to influence the velocity of the renin-substrate reaction in these species (Poulsen, 1971a; Reid et al., 1973). This substrate dependence of plasma renin activity has been used to account for the increased rate of AI generation in plasma following bilateral nephrectomy, estrogen administration, or food deprivation, or during human pregnancy or experimentally-induced renal hypertension in rats (Barrett et al., 1976; Blaquier, 1965; Blaquier et al., 1967; Helmer and Judson, 1967; Leenen

et al., 1975; Nasjletti et al., 1971a,b; Nocenti et al., 1975; Sonkodi et al., 1976). In all instances examined, the endogenous angiotensinogen concentration approached but did not exceed the measured Km value for renin and the enhanced plasma renin activity could not be attributed solely to changes in renin concentration.

The correlation of plasma renin activity with plasma AII concentrations suggests that neither ACE nor the rate of degradation of AII exert significant limiting influences on the steady-state concentrations of AII. In this respect, the Km of ACE prepared from a variety of sources has been estimated to be $20\text{-}78 \times 10^{-6}$M AI which is several thousand-fold greater than physiological concentrations of AI in plasma (Das and Soffer, 1975; Das et al., 1977; Dorer et al., 1974; Huggins et al., 1970; Lanzillo and Fanburg, 1976b; Lee et al., 1971b). Furthermore, the conversion of AI by isolated, perfused lungs remains relatively constant (>50%) when doses of 2,000-10,000 times greater than physiological were perfused (Oparil et al., 1970, 1971). Fanburg and Glazier (1973) found that saturation of pulmonary ACE was not approached until doses of 0.5-3.0 mg AI were injected into the pulmonary artery of perfused lungs. This infinitesimally small AI/Km ratio for the conversion of AI by ACE indicates that converting enzyme is present in excess, and thus newly generated AI is converted almost instantaneously and quantitatively to AII during a single circulation. Therefore, from a kinetic perspective, it appears that under most conditions, the concentration of ACE exerts minimal influence on the steady-state concentration of AII in plasma of adult animals.

Although kinetic descriptions of the angiotensinases are lacking, the short half-life of circulating AII suggests that inactivation of this peptide proceeds very rapidly and probably does not influence, to any great extent, the steady-state concentration of AII in plasma (Akinkugbe et al., 1966; Boyd et al., 1969; Cain et al., 1970; Chamberlain et al., 1964; Hodge et al., 1967; Kokubu et al., 1967). The reported Km ($2x10^{-3}$M) of angiotensinase C (prolylcarboxypeptidase) for AII suggests that inactivation of AII by this enzyme is limited by substrate availability (Odya et al., 1978). This is supported by the multitude of enzymes capable of metabolizing AII and the various tissues which possess these enzymes. It seems likely that elimination of a significant proportion of the endogenous angiotensinases would have a negligible affect on the inactivation of AII, and thus changes in plasma AII concentrations are not likely to reflect changes in its rate of inactivation. Similarly, the pressor and steroidogenic responses of AII do not appear to be limited by receptor availability. Estimates of the dissociation constant of smooth muscle receptors for AII in the aorta and uterus and the receptors of the adrenal cortex range from $2\text{-}50x10^{-9}$M (Devynck and Meyer, 1976, 1978; Devynck et al., 1973, 1976; Forget and Heisler, 1976; Glossman et al., 1974; Gurchinoff et al., 1976; Lemorvan and Palaic, 1975; Pernollet et al., 1977; Rouzaire-Dubois et al., 1975). Inasmuch as physiological concentrations of AII are approximately $0.1x10^{-9}$M, it appears, for the most part, that vascular and adrenal receptors for AII are in excess and thus probably do not limit the biological responses to this peptide.

In conclusion, it appears as though the renin-substrate reaction is the rate-limiting step of the renin-angiotensin system of adult

animals in the overall regulation of the steady-state concentration of AII in plasma. Converting enzyme, angiotensinases, and AII receptors all appear to be in excess and thus are substrate-limited processes. Angiotensinogen synthesis, on the other hand, appears complementary to plasma renin activity, functioning primarily to supply renin with sufficient substrate for the changing rates of hydrolysis to AI.

C. Age-related Differences in the Renin-Angiotensin System

1. Feto-placento-maternal unit

a) Fetal

The enhanced activity of the renin-angiotensin system of newborn animals was first proposed by Grossman and Williams in 1938 when they found greater concentrations of a pressor substance in kidneys of young rats and cattle compared to adults. Subsequently, Burlingame and coworkers (1942) and Kaplan and Friedman (1942) described a greater and more prolonged pressor response to the injection of renal extracts from fetal rats and hogs compared to that following injection of extracts from adult kidneys. Indirect evidence suggesting that this renal pressor substance was renin came from the observation of a similar pattern of blood pressure changes following the injection of AII into fetal monkeys and fetal lambs (Assali *et al.*, 1962; Behrman and Kettinger, 1968). Subsequent investigations have confirmed this greater activity of the renal pressor system of immature animals (Mott, 1975).

Direct identification of the renin-angiotensin system as one of the possible renal mechanisms contributing to regulation of

homeostasis in neonates was the finding of greater plasma renin activity in near-term fetuses and newborns compared to adult animals. Plasma renin activity (PRA), is higher in umbilical cord plasma than maternal plasma (Boonshaft et al., 1968; Brown et al., 1966; Geelhoed and Vander, 1968; Gordon et al., 1969). Cord venous AII concentration has also been found to be consistently greater than that in maternal plasma with a significant correlation existing between the two (Lumbers and Reid, 1977; Pipkin et al., 1976, 1977; Pipkin and Symonds, 1977). Mean arterial AII concentration in plasma of infants born to hypertensive mothers is greater than that in normotensive pregnancies (Annat et al., 1978; Pipkin et al., 1976; Pipkin and Symonds, 1977). Babies delivered by lower segment cesarean section have much lower PRA, and AI and AII concentrations than those delivered vaginally (Lammintausta et al., 1977; Lumbers and Reid, 1977; Pipkin et al., 1976). Since both maternal and fetal plasma AII concentrations have been correlated with the duration of labor (Pipkin et al., 1976; Pipkin and Symonds, 1977), it has been suggested that the stress of parturition may be a factor stimulating PRA of newborn infants. The greater AII concentration in cord venous plasma compared to cord arterial plasma during vaginal delivery suggests that the placenta may contribute to the fetal plasma AII concentration (Pipkin and Symonds, 1977).

Lammintausta and coworkers (1977) observed that neonatal PRA and AI following vaginal delivery were greater than those of fetuses delivered by cesarian section. Lumbers and Reid (1977) found higher concentrations of AII in infants delivered vaginally compared to those born by cesarian section (40.3 pg/ml versus 7.5 pg/ml). Anesthetizing

the mother during delivery further stimulated plasma AII of the babies (66.8 pg/ml). Normal, non-pregnant adults had plasma AII concentrations of 7.9±3.4 pg/ml. These investigators suggested that the increase in plasma AII was due to a proportionate increase in PRA.

Since PRC of cord plasma is also greater than that of maternal plasma (Hayduk et al., 1972), it was suggested that the enhanced renin activity may reflect increased release of renin from the feto-placental unit. Renin has been identified in gravid uterine tissues and human amniotic fluid in concentrations greater than that of the maternal or fetal plasma (Brown et al., 1966; Skinner et al., 1968). Therefore, the maturation of placental tissues may contribute to the production of additional renin which may enter both the maternal and fetal circulations. However, the continued transient increase in AII, PRA, and PRC postnatally (Hayduk et al., 1972; Kotchen et al., 1972; Pipkin et al., 1971) suggests that hormonal influences and the placenta are not required for the production of renin in newborns. Pipkin and associates (1974b) found that bilaterial nephrectomy in fetal lambs resulted in the near complete disappearance of renin and AII from fetal plasma. Therefore, it appears that the elevated PRC of fetuses derives from fetal kidney tissue.

In the fetus, plasma angiotensinogen concentration (PAC) is either less or not different from that of the maternal plasma (Carver and Mott, 1978; Godard et al., 1976). Carver and Mott (1978) reported that in fetal lambs, PAC is significantly less than that of the paired ewe. Fetal nephrectomy increased PAC of both maternal and fetal plasma. In view of the substrate-dependence of PRA, it was concluded that the elevated fetal PRA compared to that of the mother

understated the difference in PRC due to the greatly reduced PAC in fetal plasma. This suggests that the PRC of fetal plasma is most likely several-fold greater than that reflected by the elevated PRA and that substrate is a predominant limiting factor to PRA in fetal plasma.

The concentration of renin in plasma of fetal lambs 111-144 days gestational age (term = 147 days) is approximately 7-fold greater than that of maternal plasma (Pipkin et al., 1974b). Similarly, PRA of fetal lambs is 8-fold greater than their respective mothers. Fetal PRC increased from 9.2 ng/ml/hr to 12.6 ng/ml/hr during the final 20% of gestation and was accompanied by increases in fetal plasma Na^+ and K^+ concentrations. Plasma pH, P_{O_2} and P_{CO_2} remained constant throughout the last quarter of gestation (7.36, 22.5 mmHg, and 50.8 mmHg, respectively). Hemorrhage (<3% blood volume) increased fetal PRA reflecting the extreme sensitivity of this system in utero to physiological stimuli (Pipkin et al., 1974b). Administration of low O_2 mixtures decreased fetal P_{CO_2} to 14.6 mmHg and significantly reduced the fetal PRA response to hemorrhage. It was therefore concluded that fetal hypoxia during birth was not responsible for the elevated PRC and plasma AII concentration in newborns. The responsiveness of fetal PRA to hypovolemia suggests that loss of blood volume during the birth process may be a significant contributory factor to the elevated PRC immediately after birth.

The increased PRC of fetal plasma is easily explained on the basis of the mechanisms controlling renin secretion. The presence of juxtaglomerular granules containing renin indicates that the fetal kidney is a potential source of renin (Molteni et al.,

1974a). Ischemia and maternal nephrectomy both increased renal renin concentration of fetal dogs (Hodari and Hodgkinson, 1968; Hodgkinson et al., 1967). Increased rates of renin release from fetal kidneys may be the result of the relatively low arterial blood pressure (Dweck et al., 1974) or the decreased plasma sodium concentration (Mellor and Slater, 1971; Pipkin et al., 1974b). The responsiveness of the macula densa of immature kidneys to sodium has been demonstrated by the increased PRA of fetuses born to ewes maintained on sodium-restricted diets during pregnancy (Joppich and Weber, 1976). The relative ischemia of the renal cortex of fetal kidneys may potentiate the enhanced secretion of renin from outer cortical juxtaglomerular apparatus (Kotchen et al., 1972). It is not likely that the increased fetal PRC is due to lesser rates of inactivation of renin since no difference was observed in the half-life of injected renin between nephrectomized fetal and adult sheep (Mott, 1973). Alternatively, the enhanced fetal PRA may reflect an undeveloped negative feedback mechanism of AII on fetal kidneys (Bunag et al., 1967; DeChamplain et al., 1966; Vander and Geelhoed, 1965). Similar relative insensitivities to injected AII have been observed under other conditions of elevated plasma AII concentrations in adults (Chinn and Dusterdieck, 1972; Pipkin, 1971).

The autnomic nervous system of rabbits has been reported to be functionally incomplete at birth (Friedman et al., 1968). Infusion of norepinephrine (NE) into fetal lambs did not affect fetal PRA whereas maternal administration of one-half that dose of NE resulted in a tripling of fetal PRA (Pipkin et al., 1974b). Therefore, stimulation of the fetal PRA may be due to the effects of

increased concentrations of circulating humoral factors in maternal plasma. The lack of direct effect of NE on fetal PRA may be due to a relative insensitivity of fetal kidneys to adrenergic transmitters and other hormones. The effect of maternal NE must, therefore, be mediated by other secondary factors not yet identified. Since fetal blood loss, plasma Na^+ and K^+, hypoxia, and arterial pressure were not correlated to the duration of labor (Pipkin et al., 1976; Pipkin and Symonds, 1977), the increase in fetal PRA is best explained by the transfer of maternal hormones across the placenta.

The elevated plasma AII concentrations in newborns immediately following birth suggests that angiotensin-converting enzyme is present at birth. However, the observation of normal plasma AII concentrations in the presence of a greatly elevated PRA in fetuses suggests that fetal animals may be deficient in ACE (Pipkin et al., 1974b). Since the lungs are the major site of production of AII in plasma (Ng and Vane, 1967, 1968) the amount of converting enzyme in lung must be sufficiently large to compensate for the limited pulmonary blood flow in the fetus (Dawes et al., 1953; Rudolph, 1970; Rudolph and Heymann, 1970, 1974). Indeed, ACE has been identified in fetal human plasma (Oparil et al., 1978) and in fetal rat and rabbit lungs (Wallace et al., 1978; Wigger and Stalcup, 1978). Converting enzyme activity of cell-free preparations of rabbit lung is first detectable at day 24 of gestation and increases 5-fold to term (Kokubu et al., 1977). In rats, pulmonary ACE activity in near-term fetuses is approximately 1/8 that of adults (Wallace et al., 1978). Wigger and Stalcup (1978) identified ACE in the luminal plasma membrane of pulmonary endothelial cells during the second trimester in fetal

rabbits. Using a fluorescein-conjugated antibody to ACE, these investigators described an age-related increase in the density of converting enzyme *in utero*. Perfusion of lungs isolated from fetal rabbits demonstrated an increased ability of intact lungs to remove AI and bradykinin with gestational age (Stalcup *et al.*, 1978). These investigators attributed the increased clearance to both an age-related increase in ACE content along with an increase in the vascular suface area available for the interaction with the peptide substrates. It was concluded that under conditions of equal plasma AI concentrations, the rate of liberation of AII from adult lungs is greater than that from newborn lungs due to the larger ACE content and the greater perfusion of mature lungs. This decreased rate of generation of AII may limit the steady-state concentration of AII in systemic arterial plasma of fetal animals.

b) Maternal

The increased concentration of AII in maternal plasma compared to non-pregnant adults is accompanied by a greater plasma aldosterone concentration (Watanabe *et al.*, 1963). Aldosterone concentration in maternal plasma following cesarean section was 0.4 ng/ml whereas women delivering by the vaginal route had 1.5 ng aldosterone/ml (Beitins *et al.*, 1972). Sodium restriction further increased plasma aldosterone concentration compared to non-pregnant females. Cord plasma aldosterone was significantly greater than maternal aldosterone and restriction of maternal sodium resulted in an increased aldosterone concentration in cord plasma. The increase in maternal plasma aldosterone is most likely due to enhanced rates of secretion during pregnancy (Martin and Mills, 1956; Rinsler and

Rigby, 1957; Venning and Dyrenfurth, 1956; Venning et al., 1956, 1957), and is reflected by greater urinary excretion of aldosterone in gravid women (Genest, 1964; Gross, 1968; Jones et al., 1959; Watanabe et al., 1963). Giry and Delost (1977) found that in the fetus, both plasma and adrenal aldosterone concentrations increased gradually to term. Birth greatly stimulated aldosterone synthesis and release in both the fetus and mother. In contrast to renin and AII, however, maternal plasma aldosterone concentration was consistently greater than that of fetal plasma. Lammintausta and Erkkola (1977) demonstrated that the daily excretion of aldosterone increases throughout pregnancy in humans, being approximately 12-times greater at 34 weeks of gestation compared to non-pregnant adults.

Although the increased maternal plasma AII and aldosterone concentrations most likely represent a greater rate of AII formation, decreased degradation could also account for elevated concentrations of AII. However, just the converse occurs during pregnancy. Lubash and coworkers (1969) found that angiotensinase activity was elevated in pregnant women and in women receiving contraceptive steroids. The electrophoretic profile of angiotensin peptide fragments was not different for any of the groups studied. Since changes in angiotensinase activity did not correlate with changes in PRA or blood pressure it was assumed that the differences in AII half-life represented non-specific hydrolytic enzyme activities.

The similar pattern of changes in plasma AI, AII, and aldosterone in maternal plasma suggests that the predominant factor

mediating these changes is the rate of formation of AI. Skinner and associates (1975b) described the renin-substrate reaction in plasma of sheep as a first-order process, being dependent on both PRC and the concentration of circulating angiotensinogen. Since AI is the direct product of the renin-substrate reaction, the increased rate of generation of AI in vivo (PRA) may reflect either increases in PRC or elevated plasma angiotensinogen (renin substrate) concentrations. Helmer and Judson (1967) attributed the elevated plasma angiotensinogen concentration (PAC) during pregnancy to the stimulatory effect of estrogens on angiotensinogen synthesis. The increased plasma renin substrate concentration resulted in an increased PRA of maternal plasma whereas PRC was unchanged. Nasjletti and coworkers (1971a) treated non-pregnant rats with estrogens and found that these animals had an increased plasma renin substrate concentration compared to non-treated rats. This increase in PAC resulted in a decrease in PRC which together maintained PRA at normal values. Plasma renin substrate was increased during estrus and suppressed by ovariectomy. Castration of male rats did not change PAC. Stibestrol treatment increased PAC of female rats which was accompanied by a decrease in PRC and renal renin content but no change in PRA. Administration of progesterone or testosterone had no effect on PAC. Therefore, it is possible that the elevated PRA of maternal plasma may be due to increases in PAC rather than PRC. However, several investigators have reported PRC to be increased in plasma of pregnant women (Godard et al., 1976; Hayduk et al., 1972; Pipkin et al., 1974b, 1977; Trimper and Lumbers, 1972). Thus, in the mother, the elevated PRA may be due to increases in both PRC and PAC.

The increased PRC of maternal plasma may result from either increased rates of renin secretion or from a slower rate of destruction. Although the inactivation of renin in maternal plasma has not been examined, many investigations have dealt with factors concerning renin production during pregnancy. Renal blood flow is increased in pregnant women (Sims and Krantz, 1958) which may be the cause for the increased glomerular filtration rate (Gordon et al., 1969). However, despite the 2-fold increase in filtration of sodium, enhanced tubular sodium reabsorption leads to a positive sodium balance during pregnancy (Chesley, 1972). The increased blood flow and net sodium retention would inhibit renin secretion through mechanisms involving the baroreceptor and the macula densa. Thus, the increased PRC of the mother most likely does not result from mechanisms involving the macula densa and juxtaglomerular baroreceptor cells. Alternatively, increased rates of renin secretion may be due to the permissive effects of adrenal and pituitary hormones in maternal plasma. Although it is well accepted that pregnancy produces changes in the hypophyseal-pituitary-adrenal axis, the mechanisms by which these changes may enhance renin secretion have not been evaluated. Finally, the increased PRC of pregnancy may reflect a reduced sensitivity of the kidney to the negative feedback effect of AII. Gant and coworkers (1974) found that the vascular sensitivity to AII was reduced during pregnancy which may account for the decreased peripheral vascular resistance (Pyorala, 1966). A similar loss of sensitivity of the renal receptors for AII may lead to increased renin secretion and thus an elevated PRC of maternal plasma.

The specific mechanisms responsible for the increased PRC of pregnancy remain to be determined. However, at present it appears that hormonal or neural mechanisms are the primary factors leading to the increased maternal PRC. In contrast, the elevated PRC of fetal plasma may result from a variety of factors which most likely are not neurogenic in nature and are thus different from those mechanisms controlling PRC of the mother. It appears that in the fetus, plasma AII concentration is limited primarily by the relatively reduced plasma angiotensinogen concentration and a slower rate of AI conversion by ACE. Perhaps age-related differences in AII degradation may also affect plasma AII concentrations of the fetus, however, this possibility remains to be investigated.

2. Postnatal maturation

a) Maternal

Hyperactivity of the renin-angiotensin-aldosterone system during pregnancy extends into the postpartum period in both neonates and mother. Maternal PRA, PRC and plasma AII concentrations decrease rapidly following birth (Pipkin et al., 1977). Lammintausta and Erkkola (1977) found that maternal PRA and AI decreased progressively reaching normal adult values by 7 days postpartum. These investigators found a similar decrease in urinary aldosterone excretion returning to non-pregnant values by the seventh puerperal day. Postnatal changes in the concentration of aldosterone in maternal plasma are similar to that reported for urinary aldosterone (Giry and Delost, 1977). The return of PRA, AII and aldosterone to normal values shortly following parturition suggests that either the feto-placental

unit or hormonal responses due to the stress of labor are responsible for stimulating renin secretion in the mother.

b) Fetal

In contrast to the rapid return of the renin-angiotensin-aldosterone system to normal values in the mother, neonatal renin, angiotensin, and aldosterone remain elevated for several days following birth. Plasma renin activity is elevated shortly following birth and decreases progressively with age (Dillon and Ryness, 1975; Granger et al., 1971; Mott, 1975; Sassard et al., 1975). Pohlova and associates (1973) found that PRA of infants 1-10 days of age was 35 ng/ml/hr and decreased to 9 ng/ml/hr at 6 months. Non-pregnant adult PRA was approximately 5 ng/ml/hr. A similar postnatal decrease in PRA was observed in infants born prematurely (Richer et al., 1977). No difference in PRA was observed between preterm infants and normal children of the same postmentrual age. PRA was negatively correlated to postmentrual age up to approximately 9 weeks postpartum. Pipkin and Smales (1977) found that systolic blood pressure of newborn infants was inversely correlated to postmentrual age and that plasma AII concentration was inversely related to blood pressure, suggesting a relationship between the renin-angiotensin system and homeostasis in newborns.

The postnatal changes in plasma and urine aldosterone concentrations parallel that of PRA (Siegler et al., 1977; Vagnucci et al., 1974). Urinary aldosterone excretion is low immediately following birth but increases to maximum rates at 10-14 days of age (745 ng/day). Daily excretion of aldosterone decreases to adult values (190 ng/24 hr) by 4 weeks of age (Hubl et al., 1976). Siegler and

coworkers (1977) found that in humans, plasma potassium and aldosterone and PRA decrease with age during the first year of life. Kowarski and associates (1974) have attributed the increased plasma aldosterone in newborns to result primarily from increased rates of secretion rather than decreased metabolic clearance (Beitins et al., 1972, 1974; Kowarski et al., 1974). Inasmuch as the aldosterone in both urine and plasma correlate well with PRA during the first 12 months postpartum (Siegler et al., 1977) it was proposed that aldosterone secretion during development is regulated primarily by the activity of the renin-angiotensin system.

The greater PRA of newborns is due primarily to an increased PRC rather than angiotensinogen concentration. Hayduk and coworkers (1972) found that maternal PRC returned to normal values within 4 days following birth, whereas the PRC in 1-week old infants was approximately 3-fold greater than that of adults. It was not until 3-24 months postpartum that newborn PRC returned to that of adults. In humans, PRA increases to maximum values at 24-72 hours postnatally (Godard et al., 1976; Hayduk et al., 1972; Kotchen et al., 1972). Similarly, PRC in humans increases immediately following birth to maximum concentrations at 2 days following birth (Hayduk et al., 1972). In contrast to changes in PRC, Kotchen and coworkers (1972) attributed the greater PRA of infants less than one week of age to the elevated plasma angiotensinogen in newborns compared to adults. However, angiotensinogen did not change between birth and 6 weeks of age whereas PRA increased during the first week then declined to adult values by 6 weeks of age (Kotchen et al., 1972). The changes in PRA

in the face of a constant angiotensinogen concentration suggest that renin concentration is the primary factor mediating the postnatal changes in renin activity.

Pipkin and coworkers (1974a) found that plasma AII concentration in newborn lambs increases to maximum values (0.9 ng/ml) at 8 hours postpartum and attributed these changes to enhanced rates of renin secretion induced by the stress of parturition. In contrast, PRA (Godard et al., 1976; Hayduk et al., 1972; Kotchen et al., 1972) and plasma AII concentration (Pipkin et al., 1971, 1974a) increase gradually following birth suggesting that factors associated with delivery are not responsible for the postnatal changes. In rabbits (Pipkin et al., 1971) maximum plasma AII concentrations are not attained until 10-14 days after birth suggesting that the increase in renin results from processes associated with the maturation of renal tissue. Maximum plasma AII concentrations occurred at the same time during development as when the relative kidney weight (g/kg body wt) was maximum.

Several investigators have demonstrated that despite the elevated PRA, the renin-angiotensin system of newborns is sensitive to stimuli which enhance renin secretion. Removal of 25% of the calculated blood volume of rabbits induced a 5-fold greater increment in plasma AII concentration in immature rabbits than in adults (Pipkin et al., 1971), however, the percent change in AII was similar for the two ages. These investigators were not able to demonstrate slower rates of AII inactivation in newborn rabbits. The incremental increase in plasma AII concentration produced by standardized

hypovolemia was also greater in newborn lambs compared to fetuses and adult ewes (Pipkin et al., 1974a). Similarly, bilateral carotid occlusion resulted in larger increases in plasma AII of newborn lambs compared to adult ewes. Hypovolemia produced by furosemide diuresis also stimulates PRA of newborn lambs (Siegel and Fisher, 1977; Trimper and Lumbers, 1972). The increase in PRA and plasma aldosterone produced by furosemide administration was greater in fetal and newborn lambs than adult ewes. This greater responsiveness of the renin-angiotensin-aldosterone system to hypovolemia may suggest a greater role of this system in homeostasis of bodily fluids in newborn animals compared to that of adults. Furthermore, the postnatal decrease in PRA paralleled the age-dependent decrease in sodium-retention, extracellular fluid volume and urinary excretion of aldosterone metabolites (Pohlova et al., 1973). This relationship of PRA and body fluid composition suggests an important role for the renin-angiotensin system in regulation of homeostasis early during development.

Pohlova and Jelinek (1974) observed an age-dependent decrease in the half-life of AII when incubated with homogenates of kidney and suggested that the elevated plasma AII concentration in newborns may be partially due to lesser rates of inactivation. Pipkin (1972) observed a similar age-related increase in angiotensinase activity of livers perfused in situ. Although a two-fold difference in the rate of AII inactivation existed between rabbits 5-29 days old and adults, the difference was not sufficient to account for the 5-fold difference in arterial AII concentrations between these two ages. It was, therefore, concluded that the predominant factor

responsible for the postnatal decrease in plasma AII is the progressively decreasing rates of secretion of renin from the developing kidneys.

Factors which may contribute to the enhanced renin secretion in newborns may be related to the functional immaturity of these kidneys (Edelmann and Spitzer, 1969; Kleinmann and Lubbe, 1972). Glomerular filtration rate (GFR) of newborn infants is approximately one-third that of adults (Loggie et al., 1975) which may reflect the lower arterial blood pressure and renal blood flow. In newborn puppies, acute changes in blood pressure produced proportionate changes in glomerular filtration rate (GFR) (Kleinmann and Lubbe, 1972) indicating a direct dependence of GFR on perfusion pressure. Consequently, the delivery of sodium to the macula densa is probably less in the immature kidney (Loggie et al., 1975). This combination of low perfusion pressure and decreased sodium load to the macula densa may act concurrently to stimulate renin release in newborns (Davis and Freeman, 1976). The existence of an inverse relationship between mean arterial blood pressure during development and circulating plasma AII concentrations supports this concept (Pipkin and Smales, 1977; Pipkin et al., 1971, 1974b).

A third mechanism controlling renin release involves the sympathetic nervous system (Davis and Freeman, 1976). However, it is unlikely that stimulation of β-adrenergic receptors in the immature kidney is responsible for the elevated PRC of newborns since it has been demonstrated that the autonomic nervous system of rabbits is incompletely developed at birth (Friedman et al., 1968). Alternatively, the increased neonatal PRC may reflect a relative lack of the

negative feedback of AII on renin secretion (Vander and Geelhoed, 1965). Similar relative insensitivities of AII receptors have been identified under various conditions of elevated plasma AII concentrations in adults (Chinn and Dusterdieck, 1972; Pipkin, 1971).

Aoi and Weinberger (1976) demonstrated that the basal rate of secretion of renin from kidney slices decreased from 25 ng/mg at 6 weeks of age to 10 ng/mg in rats 5 months old. This decrease may be reflected by the age-related increase in renin content of the juxtaglomerular apparatus of developing rats (Bruhl et al., 1974). In newborn rats, PRA remained elevated for 3 weeks postpartum then decreased to adult values by 4 weeks of age (Solomon et al., 1977). The changes in PRA of newborns were not correlated to changes in total renal renin content, which increased following birth to reach adult values by 6 weeks of age. Solomon and coworkers (1977) found that the rate of inactivation of renin in nephrectomized newborn rats (less than 2 weeks old) was much slower than that of nephrectomized adult rats. The estimated half-life of endogenous renin in newborns was 25 min compared to 18 min in adult rats. It was concluded that the elevated PRA of immature rats may result from a slower rate of inactivation of renin in these young animals.

More recent investigations suggest that the maturation of ACE may limit the production of AII in developing animals. Pipkin and Smales (1977) found that children less than 1 week of age suffering from respiratory distress syndrome possessed plasma AII concentrations twice that of normal infants. The observation of Mattioli and coworkers (1975) that serum ACE activity was enhanced in children

with respiratory distress suggests that converting enzyme may be a limiting factor in regulating plasma AII concentration in newborns.

Hebert and coworkers (1972) first demonstrated an age-related increase in pulmonary ACE activity by comparing the systemic blood pressure response to AI or bradykinin following injection into the pulmonary artery and injections into the ascending aorta. The pressor response of fetal, newborn and adult ewes to AI was greatest following pulmonary arterial injections indicating pulmonary conversion of AI to AII. The fraction of injected AI which was converted in adult lungs was significantly greater than that converted by newborn lungs (44% versus 22%). Using similar techniques, Friedli and coworkers (1973) found that only 68% of the injected bradykinin was inactivated by the pulmonary circulation of newborn lambs, whereas adult lungs inactivated 93% of the injected bradykinin. Furthermore, these investigators were unable to detect pulmonary inactivation of bradykinin in preterm fetal lambs, suggesting that pulmonary ACE activity appears late during development.

Using more sensitive assays for ACE, Kokubu and coworkers (1977) detected converting enzyme activity in homogenates of fetal rabbit lungs as early as day 24 of gestation (term = 32 days). The postnatal development of pulmonary ACE consisted of a rapid 2-fold increase, reaching adult values by 10 days postpartum. A similar age-dependent increase in ACE activity has been demonstrated for lungs and kidneys of both mice and rats (Wallace et al., 1978). Pulmonary ACE activity of near-term fetal rats was one-seventh that of adults. The postnatal increase in ACE activity was biphasic with the first

increase occurring between birth and 10-days of age and the second equally large increase occurring between 4- and 5-weeks. The postnatal increase in ACE activity in all tissues examined was attributed to a greater enzyme content rather than differences in the affinity of the enzyme for substrate (Wallace et al., 1978). The increased PRA of newborns indicates a more rapid production of AI available for conversion to AII. However, the lower ACE content of immature lungs may limit quantitative conversion of AI to AII thereby dissociating the relationship between PRA and plasma AII concentration. Accordingly, converting enzyme activity may limit the production of AII in newborn animals.

In conclusion, the postnatal decrease in maternal plasma AII concentration appears to be due to the depletion of hormonal influences stimulatory to renin secretion. In contrast, the elevated plasma AII of newborns has been attributed to increased secretion of renin and a reduced rate of clearance of renin from plasma. Decreased activity of angiotensinases in newborns may contribute to the elevated plasma AII concentrations, however, the limited availability of ACE may function to restrict plasma AII concentrations relative to the elevated PRA.

D. Objectives

The unifying purpose of this investigation was to assemble a basic description of the stoichiometry of the renin-angiotensin system in developing rats. The specific objectives were: 1) to define age-related changes in the concentrations of angiotensinogen, angiotensin I, and angiotensin II; 2) to determine the maturational changes in renin and angiotensin-converting enzyme; 3) to integrate changes in

enzyme activities and angiotensin concentrations to formulate an understanding of the factors regulating the steady-state concentration of AII in plasma during development; 4) to establish basic evidence suggesting age-related transitions in the rate-limiting step responsible for the turnover of AII. Rats were used in these investigations because the relative immaturity of this species at birth facilitated the monitoring of gradual changes occurring early during postnatal development. Males were used to avoid influences of estrogens on angiotensinogen secretion.

METHODS

A. General

1. Experimental animals

Male Sprague-Dawley rats of various ages were purchased from Spartan Research Animals, Inc., Haslett, Michigan and allowed to acclimate to the laboratory environment for at least one day prior to their use. Suckling animals were housed 6-8 pups per lactating female and weaned at 28-days of age. Near-term pregnant rats were housed separately and allowed to deliver spontaneously. Pregnant rats of known gestational age were purchased and the fetuses used for prenatal determinations. Fetal and neonatal littermates were pooled to obtain sufficient sample for individual measurements.

2. Plasma samples

Rats were killed by decapitation and blood collected directly from the severed neck vessels into chilled vials containing 0.5 M ethylenediaminetetraacetic acid (EDTA, Mallinckrodt, Inc., St. Louis, MO) to prevent coagulation and inhibit angiotensinases. Plasma was separated by centrifugation (1,000 x g at 4°C for 10 min) and stored at -20°C until assayed.

3. Preparation of plasma angiotensinogen

Adult male Sprague-Dawley rats were anesthetized with anhydrous ethyl ether then bilaterally nephrectomized. Incisions were

closed and the animals allowed to recover from anesthesia. At 24-36 hours post-nephrectomy, the rats were anesthetized with sodium-pentobarbital (50 mg/kg) and exsanguinated. The blood was pooled in a large flask containing 0.5 M EDTA, then the plasma separated and kept at 4°C.

Plasma from male mongrel dogs (15-25 kg) and crossbred hogs (30-40 kg) was used as the source of homologous angiotensinogen in the kinetic experiments comparing the renin-substrate reaction of various species. Adult dogs and hogs were anesthetized with 10 mg/kg sodium thiamylal, intravenously (Parke-Davis, Detroit, MI) and maintained on methoxyflurane. Bilateral nephrectomies were performed and the animals allowed to recover. After 48 hr, the hogs and dogs were anesthetized with sodium-pentobarbital (35 mg/kg, iv) and exsanguinated into chilled vessels containing 0.5% EDTA. Plasma was pooled from 2 animals of each species and used as homologous angiotensinogen.

Angiotensinogen was extracted from plasma by the method described by Green and Bumpus (1954) as modified by Skeggs and coworkers (1963) and Nasjletti and Masson (1971b). Plasma pH was adjusted to 6.0 with 2.5 N H_2SO_4 (Mallinckrodt, Inc) and the angiotensinogen concentrated by differential precipitation with ammonium sulfate. The 2.3 M $(NH_4)_2SO_4$ precipitate was collected, washed, then redissolved in a sufficient volume of distilled water to reach the limit of solubility then stored at -20°C. Each concentrated preparation of angiotensinogen was analyzed for contaminating AI and renin activity.

B. Angiotensin Radioimmunoassay

Angiotensin I and II were quantified by radioimmunoassay employing a specific antibody to pure [Asp^1, Ile^5]-AI or AII (CIBA Pharmaceutical Co., Summit, NJ) as previously described (Bailie et al., 1971; Haber et al., 1969). The sample was incubated at 4°C for 18-24 hrs in the presence of antibody and ^{125}I-[Asp^1, Ile^5]-angiotensin I or II (New England Nuclear, Boston, MA). Free and antibody-bound angiotensin were separated using a 10% dextran-coated charcoal suspension and centrifugation (1,000 x g for 20 min). The concentration of AI and AII was estimated from the relative amount of free and bound radiolabel as determined from gamma scintillation counting (Searle Analytic, Inc., Model 1185) and comparison to commercial standards (E.R. Squibb & Sons, Princeton, NJ)).

C. Angiotensin-Converting Enzyme (ACE) of Cell-Free Preparations

Adult, newborn, and fetal rats were killed by decapitation and the lungs immediately removed and washed in cold NaCl (0.9%). Lungs were then gently blotted, weighed and homogenized (Polytron, Brinkman Instruments, Westbury, NY) in 4 volumes of cold 100 mM potassium phosphate-300 mM sodium chloride buffer (pH 8.3). The homogenate was then centrifuged (International Equipment Company, Needham, Hts, MA) and the supernate fraction assayed the same day for converting enzyme activity.

Activity of ACE was determined as described previously (Wallace et al., 1978) by a modification of the method described by Cushman and Cheung (1971b). The supernatant fraction of lung homogenates was incubated at 37°C for 30 min (Dubnoff metabolic shaking incubator,

Precision Scientific, Chicago, IL) with 5.0 mM hippuryl-L-histidyl-L-leucine (HHL; Vega Fox Biochemicals, Tucson, AZ) in 100 mM potassium phosphate-300 mM sodium chloride buffer (pH 8.3). The final volume of the incubation mixture was 0.25 ml. The reaction was stopped by addition of 0.25 ml 1 N HCl and vortex mixing (Vortex-Genie, Scientific Products, Evanston, IL).

The hippuric acid formed by the enzymic hydrolysis of HHL was extracted into 1.5 ml ethyl acetate by vortex mixing and centrifugation at 2,000 x g for 10 min. A 1.0 ml aliquot of the ethyl acetate phase was then evaporated under nitrogen at 40°C. The residual hippuric acid was then dissolved in 1 M NaCl and the optical density recorded at 228 nm (Beckman DB-GT spectrophotometer, Fullerton, CA). The concentration of hippuric acid was calculated from the absorbance at 228 nm using a molar extinction coefficient of 9.8 mM^{-1} cm^{-1}. ACE activity was expressed as nmole hippuric acid formed/min·mg lung protein or on the basis of lung wt. Protein was determined by the method of Lowry et al. (1951).

D. Prenatal Development of ACE

1. Estimation of fetal lung ACE

Pregnant female Sprague-Dawley rats of known gestational age were killed and the fetuses decapitated in utero. Lungs from littermates were pooled to provide sufficient tissue for a single determination. Fetal lungs were immediately removed and homogenized in 4 volumes of cold 100 mM potassium phosphate-300 mM sodium chloride buffer (pH 8.3). The homogenate was centrifuged at 20,000 x g for 20 min and the supernatant fraction assayed for converting enzyme activity.

Because of the relatively low activity of fetal ACE, the following modifications of the enzyme assay were made. The 0.25 ml incubation mixture contained 0.5-1.0 mg fetal lung protein and 5.0 mM HHL. Following the extraction of the hydrolytic product in ethyl acetate and evaporation of the acetate solvent, the residual hippuric acid was dissolved in 1.0 ml 1 N NaCl and the optical density at 228 nm measured using 1.5 ml quartz cuvettes. ACE activity was expressed as nmole hippuric acid formed/min·mg protein.

2. Substrate-dependence of fetal ACE

The substrate-dependence of fetal lung ACE was determined by incubating 100 µl aliquots of the 20,000 x g supernatant fraction of fetal lung homogenates with increasing concentrations of HHL. The substrate-dependence of each enzyme preparation was determined separately. The rate of production of hippuric acid was used as an index of the reaction velocity.

3. Estimation of kinetic parameters of fetal ACE

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) of the ACE catalyzed hydrolysis of HHL was estimated from the substrate-dependence of the reaction. Assuming first-order reaction kinetics as reflected by a hyperbolic substrate-dependence, the reaction velocity can be predicted from the classical Michaelis-Menten equation:

$$v = \frac{Vmax\ [S]}{Km + [S]}$$

dividing the numerator and denominator by [S] gives:

$$v = \frac{Vmax}{\frac{Km}{[S]} + 1}$$

Therefore: $$Vmax = Km \frac{v}{[S]} + v$$

Thus: $$v = Vmax - Km \frac{v}{[S]}, \quad v = -Km \frac{v}{[S]} + Vmax$$

Therefore, a plot of v versus $\frac{v}{[S]}$ will result in a straight line having a slope of -Km and intercepting of the ordinate at Vmax. In this calculation, [S] represents the concentration of HHL (mM) in the incubation medium and v reflects the velocity of HHL hydrolysis expressed as nmoles hippuric acid formed/min·mg protein.

4. Effect of ventilation on fetal lung ACE

Fetuses from near-term pregnant female rats were delivered by caesarian section. Four litters of fetuses were immediately decapitated whereas 4 other litters were delivered and allowed to breathe room air for 30 minutes prior to decapitation. The fetuses which were allowed to breathe were kept warm under a heat lamp. Lungs were then immediately removed and homogenized in 100 mM potassium phosphate-300 mM sodium chloride buffer and the 20,000 x g supernatant fraction assayed for ACE.

E. Postnatal Changes in Pulmonary ACE Content

1. Determination of ACE content of cell-free preparations

Newborn and adult rats were killed by decapitation and the lungs homogenized in 4 volumes of 100 mM potassium phosphate-300 mM

sodium chloride buffer (pH 8.3). Lungs from rats 3 weeks of age or younger were pooled such that each litter represented a single determination. The 600 x g supernatant of lung homogenates was then assayed for ACE activity.

2. Determination of ACE fractionation in homogenates of developing lungs

In order to ascertain whether age-related differences in ACE activity of 600 x g supernatant of lung homogenates was due to differences in fractionation of enzyme-membrane complex, the activity of ACE was determined in the 600 x g supernate and in the whole homogenate of lungs from newborn and adult rats. Lungs were homogenized in the phosphate-chloride buffer as described above. A portion of the homogenate was kept on ice while the remainder was centrifuged at 600 x g for 10 min. ACE activity was then determined in the 600 x g supernatant and compared to that of the whole homogenate. The fraction of the total enzyme activity appearing in the 600 x g supernatant (expressed per mg protein) from lungs at differents ages was compared to insure equal solubilization of the enzyme.

F. Measurement of Angiotensins in Plasma from Developing Rats

Male rats of various ages were killed by decapitation and blood collected directly from the severed neck vessels into chilled vials containing 0.5 M EDTA. Blood from rats less than 4-weeks of age was pooled such that each litter represented a single determination. Plasma was separated by centrifugation and plasma AI and AII concentrations determined by radioimmunoassay.

G. Measurement of Renin

1. Plasma renin activity (PRA)

The activity of renin in plasma from developing rats was estimated from the amount of AI generated during incubation of the plasma sample with inhibitors of AI degrading enzymes (Oparil, 1976; Skinner, 1967). A 50 µl aliquot of the plasma sample was incubated at 37°C in a total of 1.0 ml 0.2 M maleate buffer (pH 6.0) containing 8.1 mM dimercaprol (BAL; Hynson, Westcott and Dunning, Inc., Baltimore, MD) and 1.7 mM 8-hydroxyquinoline (8-HQ; Aldrich Chemical Co., Milwaukee, WI) to prevent the degradation of newly generated AI. The reaction was stopped after 60 minutes of incubation by placing the samples on ice. Microliter aliquots of the incubation mixture were then transferred to radioimmunoassay tubes for quantitation of AI as described above. The concentration of AI in the original sample was determined prior to incubation and this value subtracted from the total AI present at the end of incubation to give the amount of enzymatically generated AI representative of renin activity. PRA was expressed as ng AI formed/hr·ml plasma.

2. Plasma renin concentration (PRC)

The concentration of renin in plasma of developing rats was estimated by assuming that the concentration of substrate in the incubation was saturating to renin. Under these conditions, the velocity of AI generation would theoretically be dependent solely upon the amount of enzyme present. Saturation with substrate was accomplished by adding 300 µl of a concentrated angiotensinogen preparation to the incubation mixture containing renin. With exception of the

addition of exogenous substrate, PRC was estimated under conditions identical to the determination of PRA (Skinner, 1967). The data were corrected for contaminating AI and renin present in the concentrated angiotensinogen preparation. PRC was expressed as ng AI generated per ml plasma during the 60 minute incubation.

3. Time-dependence of AI generation during incubation of plasma renin

Incubation mixtures were prepared with maleate buffer containing 300 µl concentrated angiotensinogen preparation. The mixtures were preincubated at 37°C for 10 min and the reaction initiated by addition of 100 µl plasma renin. Aliquots of the reaction mixture were withdrawn at various times during the incubation and analyzed for AI by radioimmunoassay.

4. Comparison of PRA and PRC during development

Renin activity and concentration were compared in plasma of developing rats by simultaneous incubations of the plasma sample in the presence (PRC) and absence (PRA) of additional exogenous angiotensinogen. Two incubation tubes were prepared for each plasma sample tested. One tube (PRA) contained 0.2 M maleate buffer (pH 6.0) with BAL and 8-HQ. The other incubation mixture (PRC) was prepared identically except that 300 µl of a concentrated angiotensinogen preparation was substituted for an equal volume of maleate buffer. Both incubation mixtures were handled identically thereafter. The amount of radioimmunoassayable AI generated during incubation was used to compare PRA and PRC of the given sample. Statistical comparison of these two values was by the Student's *t*-test for paired comparisons (Sokal and Rohlf, 1969).

5. Renal renin concentration (RRC/g)

The concentration of renin in kidneys of developing rats was estimated from the rate of AI generation during incubation of cell-free preparations of kidney tissue with exogenous angiotensinogen. Kidneys from rats of various ages were decapsultated and homogenized (Polytron, Brinkman Instruments) in 4 volumes of 0.1 M Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 4°C (500 x g for 10 min) and the supernatant fraction assayed for renin.

Renal renin content was determined by incubating a 50 µl aliquot of the 500 x g supernate at 37°C in maleate buffer (pH 6.0) containing BAL, 8-HQ, and 300 µl of a concentrated angiotensinogen preparation. In order to determine renin concentration as opposed to renin activity, it was necessary to establish that the added angiotensinogen was saturating to renin and therefore the reaction process was zero-order with respect to substrate. Renal renin content was estimated from the amount of AI generated during the 1 hour incubation period and expressed on the basis of kidney wt (RRC/g) and per whole organ (RRC). The data were collected for contaminating AI and renin present in the concentrated angiotensinogen preparation.

H. Estimation of the Kinetics of the Renin-Substrate Reaction

1. Collection of plasma renin samples

Plasma containing elevated renin activity was used in the experiments describing the kinetics of the renin-substrate reaction in various species. High renin rat plasma was obtained from sodium-pentobarbital anesthetized, adult male rats which had previously been maintained on a sodium-deficient diet (Ralston Purina Co., St. Louis,

MI) and 0.05% NaCl drinking water for 3 weeks. High renin dog plasma was obtained 10 min following furosemide injection (5 mg/kg) into adult dogs anesthetized with sodium-pentobarbital (35 mg/kg). Hog renin was obtained from 1- to 5-day old piglets anesthetized with ketamine (5-10 mg/kg, im) and supplemented with 80% nitrous oxide -20% oxygen. Following a 30 minute stabilization period, saralasin (5-10 μg/kg/min) was infused into the femoral vein and blood collected from the femoral artery 30 min later.

Plasma collected from 4 unanesthetized, normal adult male rats was pooled and served as the source of adult renin for experiments comparing the kinetics of the renin-substrate reaction of adult and newborn plasma. Blood samples were collected from unanesthetized 7-day old rats using the vacuum-assisted cardiac puncture method described by Gupta (1973). Blood from 4 litters of 7-day old rats was pooled and the plasma separated by centrifugation. This plasma served as the source of newborn renin for the kinetic determinations.

2. Preparation of angiotensinogen for kinetic determinations

Plasma from bilaterally nephrectomized animals served as the source of homologous angiotensinogen. Two preparations of angiotensinogen with different degrees of purity were used in the kinetic investigations. Plasma from bilaterally nephrectomized rats or dogs was pooled and divided into two portions. One portion was frozen without further purification and employed as the unextracted substrate preparation. The second portion of plasma was concentrated for angiotensinogen by the ammonium sulfate precipitation method described above and served as the source of high renin substrate for the kinetic experiments.

3. Test of zero-order kinetics for PRC determinations

Measurement of PRC requires that substrate is saturating to renin and therefore the rate at which the reaction proceeds is dependent solely on the amount of enzyme present. Substrate-saturation was tested by incubating the high renin plasma with exogenous substrate for various periods of time and monitoring the rate of generation of AI. Incubation tubes were prepared identically to those used in estimating PRC as described above. At various times during incubation, an aliquot of the mixture was withdrawn and analyzed for AI.

Zero-order reaction kinetics reflect conditions of saturating substrate ([S]>>Km). Therefore, the classical Michaelis-Menten equation,

$$v = \frac{Vmax \cdot S}{Km + S}$$

reduces to $$v = \frac{Vmax \cdot S}{S} = Vmax = \frac{d[P]}{dt}$$

Since $d[P] = Vmax \cdot dt$, a plot of product formation versus time will result in a straight line with a slope of Vmax under zero-order conditions.

4. Prediction of PRC at various concentrations of renin

The validity of PRC measurements was further tested by incubating a given amount of exogenous angiotensinogen with several volumes of renin and monitoring the rate of AI generation. Each incubation mixture contained 300 µl of the concentrated angiotensinogen preparation in maleate buffer (pH 6.0) containing BAL and 8-HQ. The reaction was initiated by the addition of 25, or 50 µl of a given

plasma renin sample from adult rats and the rate of AI generation determined by sequential sampling of the different incubation mixtures.

Assuming substrate saturation, increasing the amount of renin in the reaction mixture should result in a proportionate increase in the amount of AI generated. This can be tested by measuring porportionate increases in the rate of AI formation for the respective renin concentrations as shown above, or by demonstrating a uniform rate of AI generation when velocity is normalized to the product of enzyme volume and time of incubation.

If indeed substrate is saturating to all enzyme concentrations tested, $\frac{d[P]}{dt}$ should increase in direct porportion to the amount of enzyme present in the reaction mixture. Thus, the slope of a line describing the rate of AI generation should be constant if the rate is normalized for enzyme concentration.

Inasmuch as it was technically difficult to vary enzyme volume over a sufficiently large range to obtain rates of AI generation for several enzyme concentrations, the data were plotted as [AI] formed versus volume plasma renin times time, which allowed for a greater number of points to describe a single line. A linear plot of such data verifies this method of data analysis.

5. Effect of doubling substrate concentration on the rate of AI generation

A linear time course for the production of AI may reflect substrate saturation and thus a zero-order reaction process. Alternatively, during the initial stage of a first-order reaction process the rate of product formation appears to be linear. In contrast to a

true zero-order process, however, the initial rate of product formation is dependent upon the concentration of substrate available to the first-order reaction process. Therefore, in order to differentiate between a true zero-order process and the initial stages of a first-order process, the rate of AI generation was determined under conditions of varying substrate concentration.

Samples of high renin plasma from the different species were incubated with various volumes of an angiotensinogen preparation and the rate of AI production determined by measuring the amount of AI present in the reaction mixture at various times during the incubation. Plasma from bilaterally nephrectomized animals served as the source of homologous substrate for these experiments. The data were analyzed by the velocity equation derived in Appendix A and plotted by the method described in Appendix B. Contaminating AI and renin present in the substrate preparations were subtracted when calculating the reaction velocity. The endogenous rate of formation of AI (k_{app}) was calculated from the respective PRA using the same renin sample as employed in the kinetic determinations.

6. Determination of substrate-dependence of renin-angiotensinogen reaction in plasma from adult and newborn rats

Plasma from adult and 7-day old unanesthetized rats was incubated for various times with differing amounts of angiotensinogen. Unextracted plasma from bilaterally nephrectomized adult rats served as the source of substrate for both newborn and adult plasma renin. Inasmuch as angiotensinogen was not quantifiable, the substrate concentration was expressed as the volume of a standard substrate preparation added to the reaction mixture. The same pool of substrate

plasma was used for all enzyme assays to avoid artifacts due to non-uniform preparations of angiotensinogen. Since it is not possible to express renin activities on the basis of molar equivalents of enzyme, the rate of generation of AI was used as an index of renin activity.

The reaction mixture (1.0 ml) consisted of 0.2 M maleate buffer (pH 6.0) containing BAL and 8-HQ and varying amounts of angiotensinogen and plasma renin. The volume of the angiotensinogen added to the reaction mixture ranged from 25-300 µl. Renin was added in volumes of 0, 10, 25 and 50 µl. The reaction was initiated upon placing the incubation tubes into a waterbath at 37°C. At various times following initation of the reaction, tubes were withdrawn and placed in a water bath at 4°C. Aliquots of the respective incubation mixtures were then analyzed for AI by radioimmunoassay.

The data were analyzed by the first-order velocity equation derived in Appendix A and plotted by the double-reciprocal method described in Appendix B. Contaminating AI and renin present in the substrate preparation were subtracted when calculating the reaction velocity.

The apparent rates of AI generation were calculated from the slope of the line describing the time-dependence of the reaction as determined by linear regression, the least squares method. The equation describing the reciprocal plot of k_{app} at various substrate volumes is derived in Appendix B:

$$\frac{1}{d[P]/[R_t](dt)} = \frac{K_S}{kp}\ \frac{1}{[S]} + \frac{1}{kp}$$

where P = product, R = renin, dt = time elapsed, K_S = Michaelis-Menten constant, kp = rate constant for the reaction, and S = substrate. The data of the double-reciprocal plot were fitted to the best line determined by the method of weighted least squares described by Wilkinson (1961).

7. Estimation of plasma angiotensinogen concentration from the kinetics of renin-substrate reaction

The endogenous rate of generation of AI in plasma from adult and 7-day old rats was determined under conditions identical to those described for the measurement of plasma renin activity (PRA). The apparent velocity (k_{app}) was calculated from the respective PRA by correcting for time and the dilution of plasma in the incubation mixture.

Considering the equation: $v = \frac{Vmax\ [S]}{K_S + [S]}$, if we assume PRA to represent the velocity of the reaction, we can predict [S] using the K_S and kp (Vmax) obtained from the kinetic analysis of the reaction. Because of the differences in enzyme concentration of the different plasma renin samples, this method for quantifying substrate concentration is valid only for the same plasma sample as that used in the kinetic experiments. The velocity equation can be rewritten as:

$$k_{app} = \frac{Vmax\ [S]}{Km + [S]} = \text{ng/ml·min}$$

where [S] is a measure of the plasma angiotensinogen concentration. Therefore,

$$\frac{k_{app}}{Vmax} = \frac{[S]}{Km + [S]}$$

and

$$\frac{Vmax}{k_{app}} = \frac{Km}{[S]} + 1$$

rearranging

$$(\frac{Vmax}{k_{app}} - 1)[S] = Km$$

and thus

$$[S] = \frac{Km}{(\frac{Vmax}{k_{app}} - 1)}$$

The expression $\frac{[S]}{Km}$ reflects the substrate concentration relative to that required for half maximum velocity of the reaction.

I. Isolated, Perfused Lung

1. Surgical procedures and ventilation of lungs

Adult (200-250 g) and newborn (7- and 21-day old) male, Sprague-Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Heparin (sodium salt, 2 KU/kg; Sigma Chemical Co., St. Louis, MO) was then injected into the inferior vena cava to prevent clotting and 3-5 minutes allowed for complete distribution of the heparin throughout the body. The thorax was then opened and the diaphragm cut from the lower rib cage. The pulmonary artery was separated from the aorta and a suture placed around the artery (00 silk, adult; 000 silk, 21-day old; 6-0 silk, 7-day old). A small incision was made in the right ventricle and a polyethylene cannula passed through the ventricle to the pulmonary artery (PE-160, adult; PE-90, 21-day old; PE-50, 7-day old). During cannulation, medium was perfused through the tubing to prevent the formation of air spaces

in the cannula. The cannula was secured in place by tightening the suture around the pulmonary artery. Caution was taken to prevent passing the cannula beyond the bifurcation of the left and right pulmonary arteries. Lungs were then continuously perfused with Krebs-bicarbonate perfusion medium (pH 7.4) supplemented with 4% bovine serum albumin (BSA; Sigma Chemical Co.). The left atrium was cut and perfusion medium allowed to flow freely from the severed atrium. The trachea was then cannulated (PE-200, adult; PE-160, 21-day old; PE-90, 7-day old) and the cannula secured in place with silk suture. Care was taken not to pass the cannula beyond the bifurcation of the trachea to the right and left lungs. The lungs were carefully removed from the thorax and suspended in a perfusion chamber maintained at 37°C by a heat lamp. The chamber was closed to form an air tight space and the lungs ventilated at 30 strokes/min (Harvard Apparatus Respiration Pump; Millis, MA) with a 95% O_2-5% Co_2 gas mixture. The chamber was designed such that the respirator displaced 100 cc of air from the completely enclosed chamber thereby creating a negative pressure which tended to expand the lungs (Figure 2). Expansion of the lungs tended to draw the 95% O_2 mixture passing over the tracheal cannula into the lungs. Reversal of the pressure within the chamber led to deflation of the lungs, the expired air leaving via the tracheal cannula. The flow of the gas mixture was adjusted to maximize the tidal volume. Under these conditions, end expiratory volume was estimated to be approximately 20% of the total lung capacity. Tidal volume was estimated from the displacement of water in a graduate cylinder into which was placed the tubing which passed across the tracheal cannula.

Figure 2. Diagram of the isolated, perfused lung model. The lungs were inflated by negative pressure ventilation with a mixture of 95% O_2-5% CO_2. Lungs were perfused by pulsatile flow with Krebs-bicarbonate buffer (pH 7.4) maintained at 37°C. Effluent medium dripped freely from the severed left atrium and was collected from a funnel located directly beneath the suspended lungs. Inflow pressure, tidal volume and the temperature in the chamber were monitored throughout the perfusion.

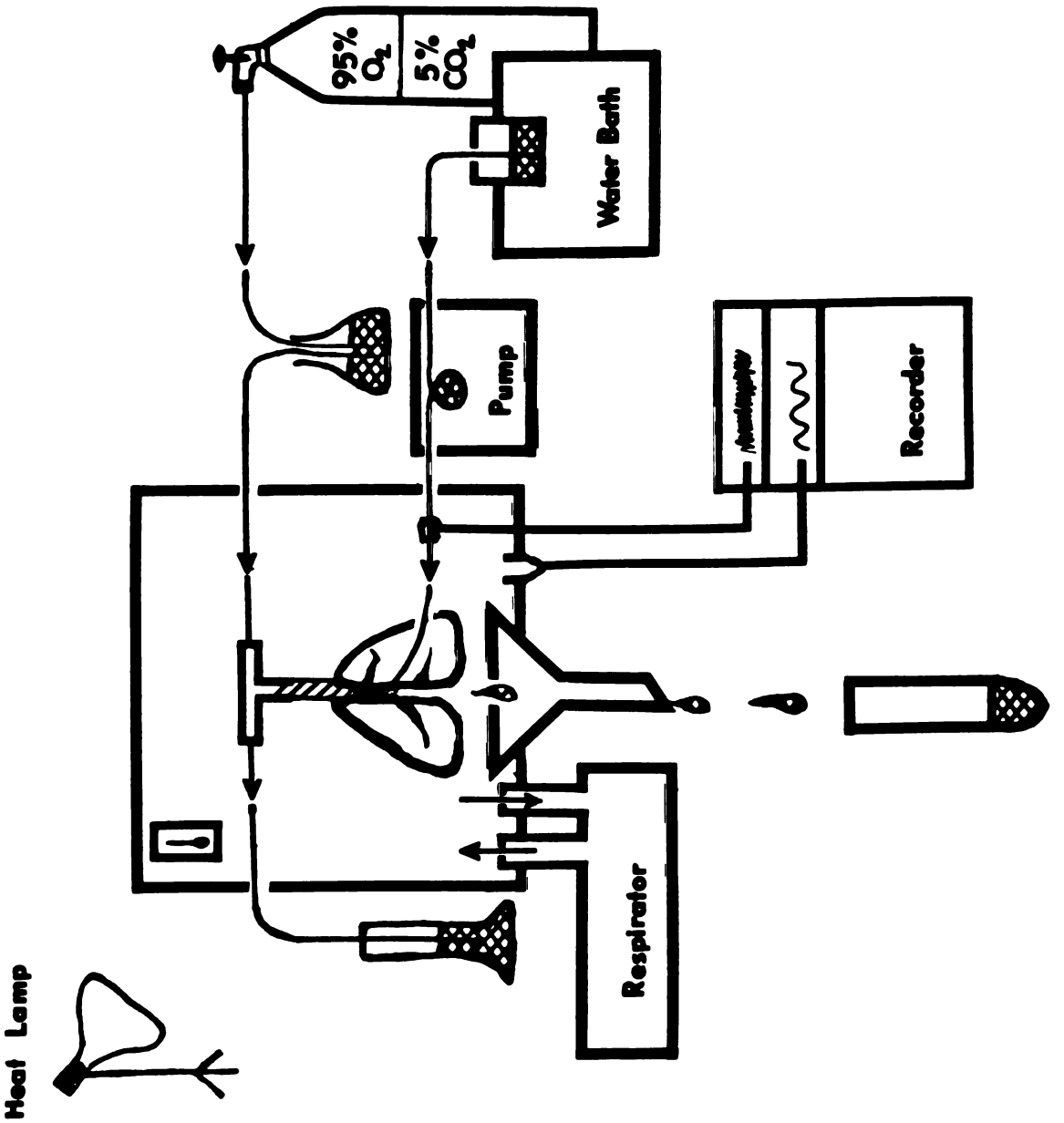


Figure 2

2. Perfusion of lungs

Lungs were perfused with Krebs-bicarbonate perfusion medium (pH 7.4) supplemented with 4% bovine serum albumin (Polystaltic pump, Buchler Instruments; Fort Lee, NJ) at 37°C in a single-pass system. The rate of perfusion was adjusted to approximately 4.5 ml/min·g wet lung wt. The perfusion medium was kept in a water bath maintained at 37°C (Thelco, Precision Scientific, Chicago, IL). Temperature within the chamber was kept at 37°C with a heat lamp.

Perfusion pressure was continuously monitored on a Grass Model 7 polygraph (Grass Instr. Co., Quincy, MA) using a P23Ac Statham pressure transducer (Statham, Hato Rey, Puerto Rico). The most common problem encountered when using the isolated, perfused lung model is the development of pulmonary edema. Since edema is invariably associated with an increased lung wt and greater perfusion pressure at constant flow, an increase in the inflow pressure was used as an early indicator of edema formation. Lungs which became edematous during the perfusion were immediately discarded and the data not included in the experimental data analysis.

Lungs were initially perfused for 5-10 minutes with drug-free perfusion medium and the inflow pressure allowed to stabilize. Following this stabilization period, the perfusion medium was changed to one containing the test substance. Agents which were perfused through the different lungs include angiotensin I (AI; E.R. Squibb & Sons, Inc., Princeton, NJ), ^{125}I-AI (New England Nuclear), and blue dextran (MW 2,000,000; Sigma Chemical Co.). Under standard conditions, AI (1 ng/ml) was perfused through the lungs and samples of the

effluent medium (0.5-1.0 ml) collected into chilled test tubes. The midpoint of the collection period was 2 min after beginning perfusion with the test substance. The time required to fill the arterial cannula was subtracted when calculating collection times. At the end of the experiment, lungs were removed from the chamber, gently blotted and weighed, then dried at 200°C to constant weight (Mettler, A.H. Thomas Co., Philadelphia, PA).

3. Determination of transpulmonary metabolism

The concentrations of AI in the perfusion medium and the effluent sample were determined by radioimmunoassay. The percent removal of AI was calculated from the fraction of perfused AI appearing in the effluent medium.

$$\%R = \frac{AI_{perfused} - AI_{effluent}}{AI_{perfused}} 100$$

4. Estimation of distribution of angiotensin within perfused lungs

Lungs from adult rats were coperfused with AI (1 ng/ml), ^{125}I-angiotensin I (1 pg/ml, 1 μCi/ng) and dextran blue (2 mg/ml, MW = 2,000,000). The rate of appearance of the test substances was determined by collecting samples of the effluent medium every 10 sec after beginning perfusion. Following 2 min of perfusion, the pump was stopped and the inflow medium changed to one containing no test substance, then perfusion restarted. Effluent samples were again collected every 10 sec for one-half minute.

The concentration of AI was determined by radioimmunoassay. ^{125}I was quantified by gamma scintillation counting and dextran was

measured by spectrophotometry at 630 nm. The effluent concentration of all agents was expressed as the percent of perfused substance.

5. Effect of SQ20,881 on AI removal by perfused lungs

The effect of inhibiting converting enzyme on AI removal by perfused lungs was examined by coperfusing lungs with AI and a specific competitive inhibitor of ACE, SQ20,881 (E.R. Squibb & Sons). Lungs from adult rats were initially perfused with 1 ng/ml AI and the percent removal determined. The perfusion medium was then switched to one containing AI (1 ng/ml) plus 1 nM, 1 μM or 1 mM SQ20,881. The order in which the various concentrations of inhibitor were perfused was randomized for each lung. Following 2 min of perfusing with AI and SQ20,881, a sample of the effluent was collected and the fractional removal of AI determined.

6. Measurement of vascular volume of isolated, perfused lungs

The perfused vascular volume was estimated from the dilution of blue dextran (MW 2,000,000) in the pulmonary circulation. Following perfusion of lungs with AI, the perfusion medium was changed to one containing approximately 2 mg/ml blue dextran. Lungs were perfused with the dye for 2 min at which time a sample of the effluent medium was collected. The arterial cannula was then immediately clamped, the lungs rapidly removed, rinsed with 0.9% NaCl, gently blotted then homogenized in 3% trichloroacetic acid (TCA). The homogenate was then centrifuged at 1000 x g for 10 min and the supernatant fraction analyzed for the blue dye. The amount of blue dextran in the supernatant was quantified by subtracting the absorbance at 700 nm from the maximum absorbance at 630 nm (Beckman DB-GT spectrophotometer).

Acidification of the sample did not affect the optical density as measured by this difference in absorbance.

Vascular volume was then determined from the following equation.

$$\text{Vascular volume} = \frac{(OD_{supernatant})(Vol_{supernatant})}{(OD_{effluent})}$$

RESULTS

A. Postnatal Changes in Plasma Angiotensin II Concentration and Renin Activity

1. Angiotensin II (AII)

The concentration of AII in plasma decreased between birth and 3-weeks of age, then increased approximately 20-fold to maximum values (0.8-1.0 ng/ml) in rats 5- to 6-weeks of age (Figure 3). Plasma AII concentration subsequently declined to adult values by 60 days (approximately 0.5 ng/ml). The concentration of AII in plasma of rats 80-days old was 2-fold greater than that in rats less than 3-weeks old.

2. Plasma renin activity (PRA)

Plasma renin activity increased following birth to maximum values 1- to 2-weeks postpartum then declined to adult values by 3-weeks of age (Figure 4). PRA of 2-week old rats was approximately twice that of adult rats (11.8 ng/ml·hr versus 4.8 ng/ml·hr, respectively). Renin activity did not change appreciably between 21- and 80-days postpartum. PRA was greater in rats less than 24-hr old than that in adult animals.

B. Evaluation of Methods Used for Estimating Plasma Renin Concentration

1. Comparison of rates of generation of AI in the presence and absence of exogenous angiotensinogen

Incubation of high renin rat plasma with 300 µl exogenous angiotensinogen resulted in a greater rate of generation of AI

Figure 3. Plasma angiotensin II (AII) concentration in developing rats. AII was quantified by radioimmunoassay. Littermates less than 3 weeks of age were pooled to obtain sufficient plasma for a single determination. Each point represents mean ± S.E. of at least 3 determinations. Points without error bars indicate that the variability fell within the radius of the circle.

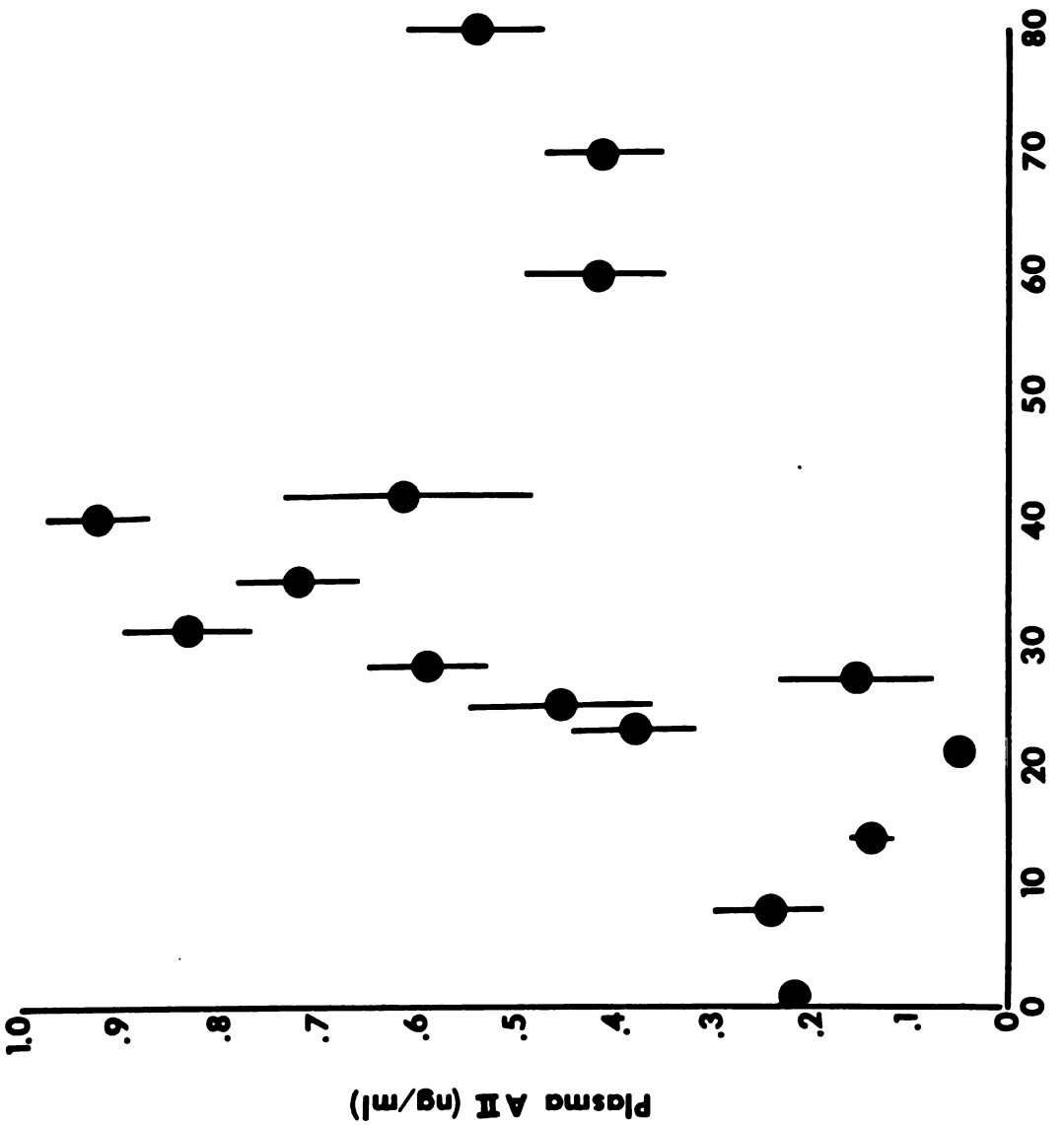


Figure 3

Figure 4. Age-related changes in plasma renin activity (PRA). Each point represents mean ± S.E. of 3-6 determinations. Blood from littermates less than 3-weeks old was pooled to obtain sufficient plasma for a single determination.

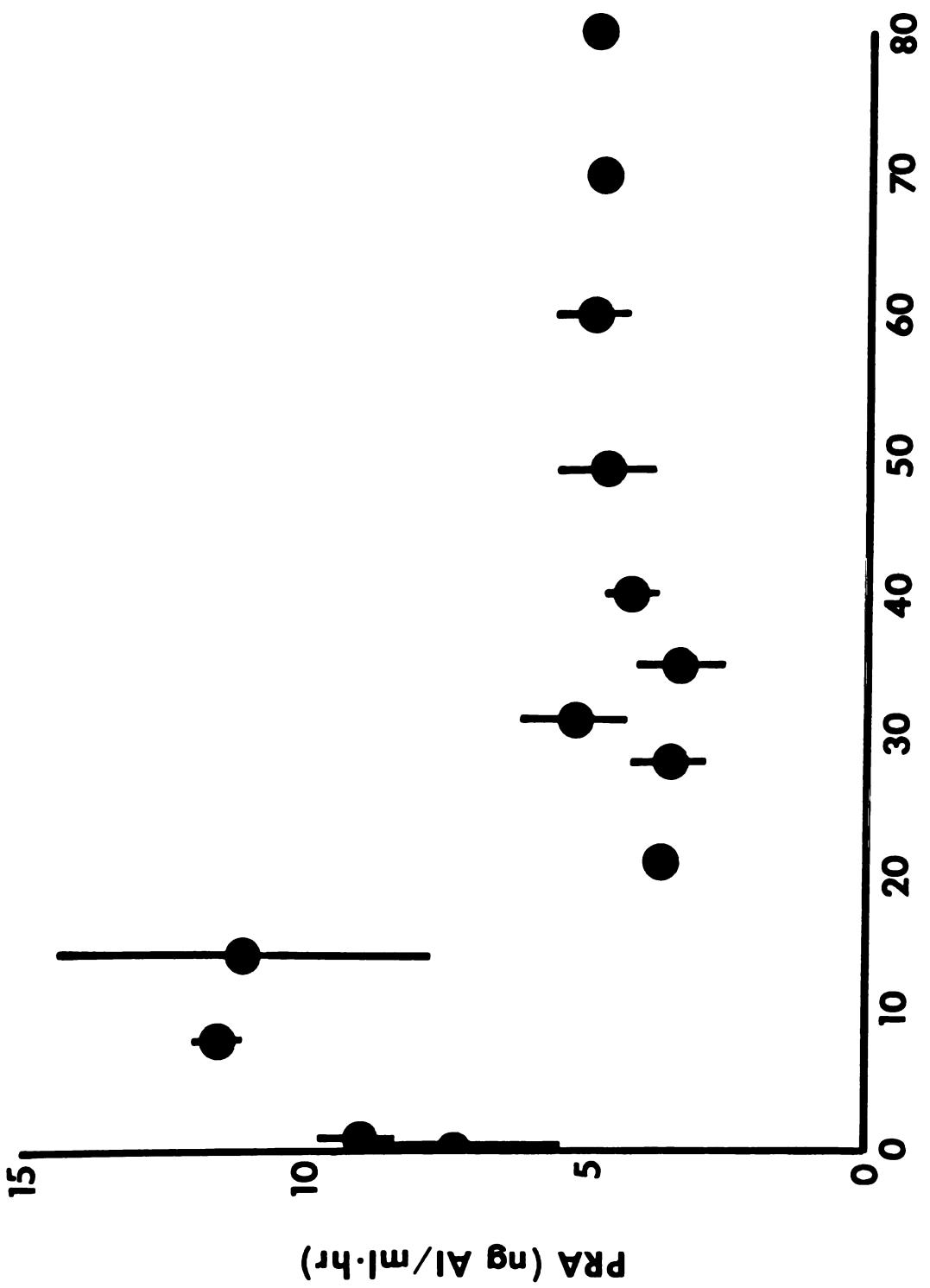


Figure 4

compared to that in the absence of additional substrate (Figure 5). In both cases, the concentration of AI in the incubation medium was linear with time up to 2 hrs. The rate of generation of AI from endogenous angiotensinogen was approximately 1.2 ng/min whereas the velocity of the renin-substrate reaction was doubled when 300 µl of exogenous angiotensinogen was present in the incubation mixture.

2. Effect of altering substrate concentration on the rate of generation of AI

The rate of the renin-substrate reaction was 0.009 ng AI/ml·min when high renin rat plasma was incubated in the presence of 100 µl plasma from nephrectomized rats (Figure 6). Doubling the volume of high substrate plasma resulted in a 180% increase in the rate of formation of AI. Further increases in the volume of substrate did not, however, change the slope of the line appreciably, suggesting that the maximum rate was approximately 0.017 ng AI/ml·min. In contrast, addition of 100 µl of a partially purified preparation of angiotensinogen increased the rate of generation of AI to 0.024 ng/ml·min (Figure 6). The generation of AI was linear regardless of the substrate used.

3. Effect of altering renin concentration on the rate of generation of AI

Doubling the volume of renin added to the incubation medium resulted in a two-fold increase in the rate of generation of AI (Figure 7). When 300 µl plasma angiotensinogen was incubated with 25 µl high renin rat plasma, the rate of generation of AI was 0.042 ng/ml·min. When renin was increased to 50 µl, the slope of the line increased proportionately to 0.082 ng/ml·min. The rate of formation of AI was constant for each renin concentration examined. The data

Figure 5. Comparison of the rate of generation of angiotensin I in the absence (PRA) and in the presence (PRC) of exogenous angiotensinogen. Both PRA and PRC were determined for the same samples. Angiotensin I was quantified by radioimmunoassay. Each point represents the mean ± S.E. of individual measurements. The data were fitted to the best line calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

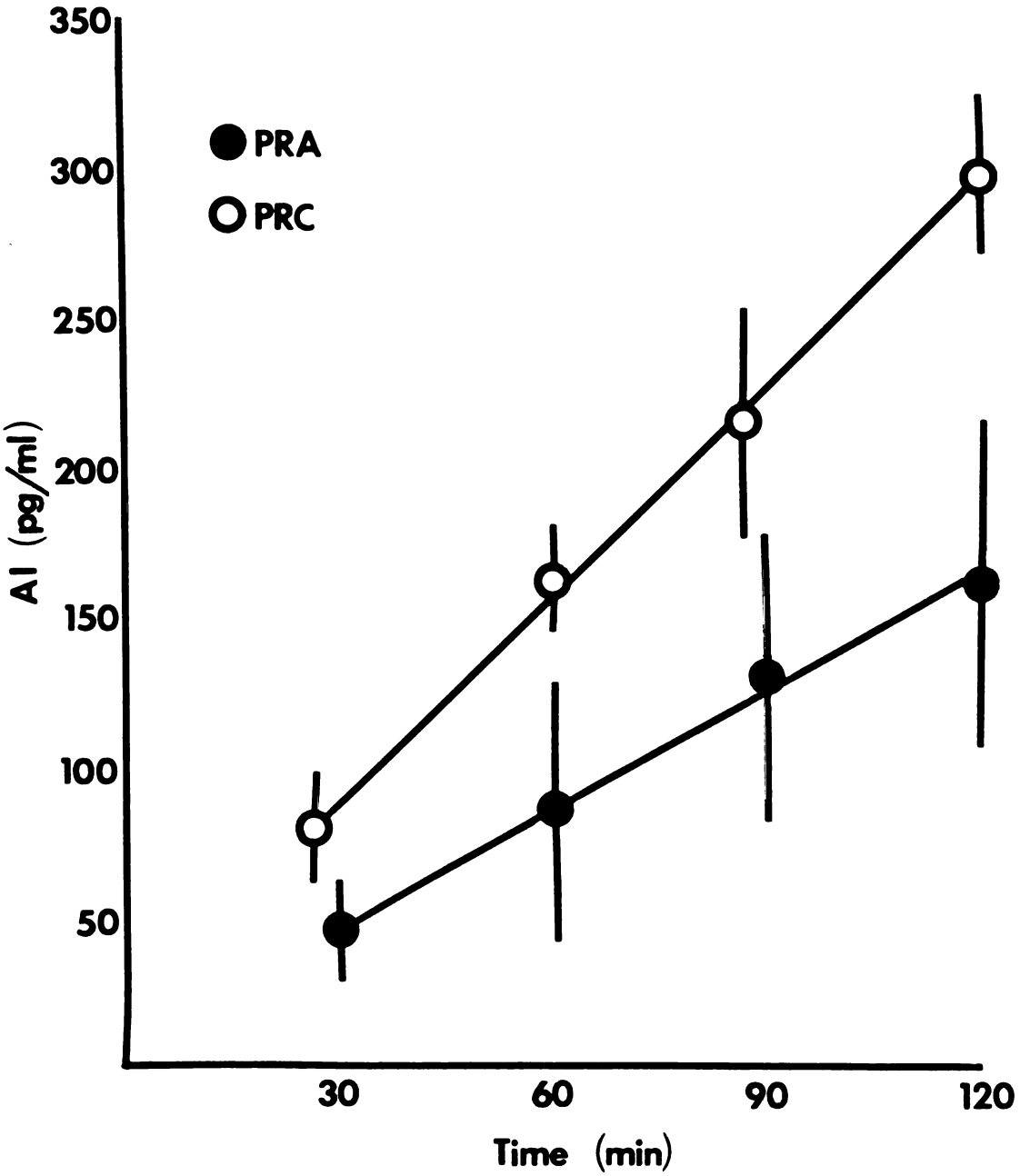


Figure 5

Figure 6. Effect of increasing volumes of angiotensinogen on the rate of generation of angiotensin I in rat plasma. Angiotensin I was quantified by radioimmunoassay. Each point represents the mean of 2 determinations. Open circles represent AI formation when the same renin sample was incubated with a preparation of angiotensinogen partially purified from the pooled plasma from nephrectomized rats. Data were fitted to the best line calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

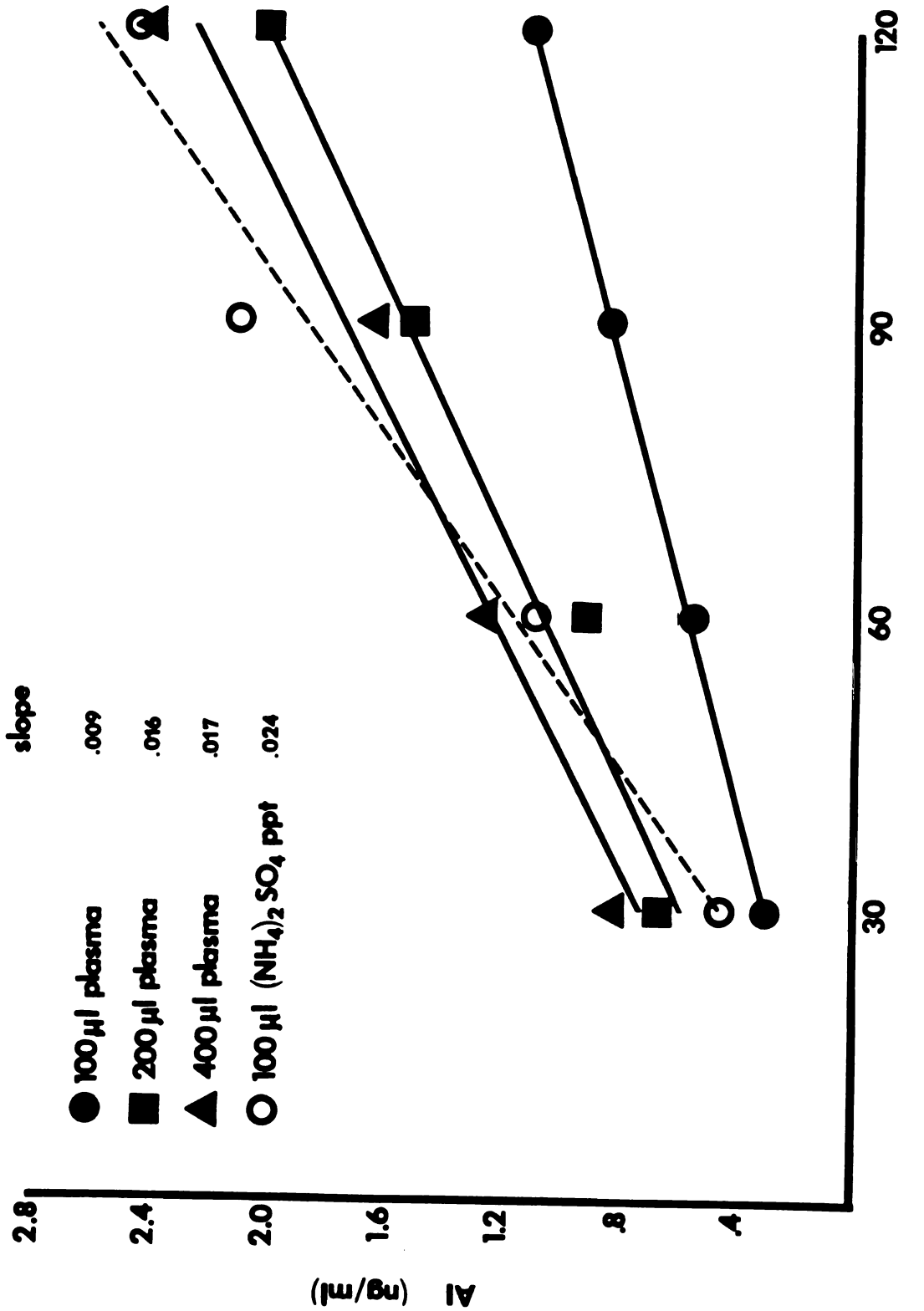

slope
100 µl plasma .009
200 µl plasma .016
400 µl plasma .017
100 µl $(NH_4)_2SO_4$ ppt .024
2.8
2.4
2.0
1.6
1.2
.8
.4
Al (ng/ml)
30
60
90
120
Time (min)

Figure 6

Figure 7. Effect of altering renin concentration on the rate of AI generation in rat plasma. Angiotensin I was quantified by radioimmunoassay. Each point represents a single determination. The data were fitted to the best line determined by linear regression, the method of least squares (Sokal and Rohlf, 1969).

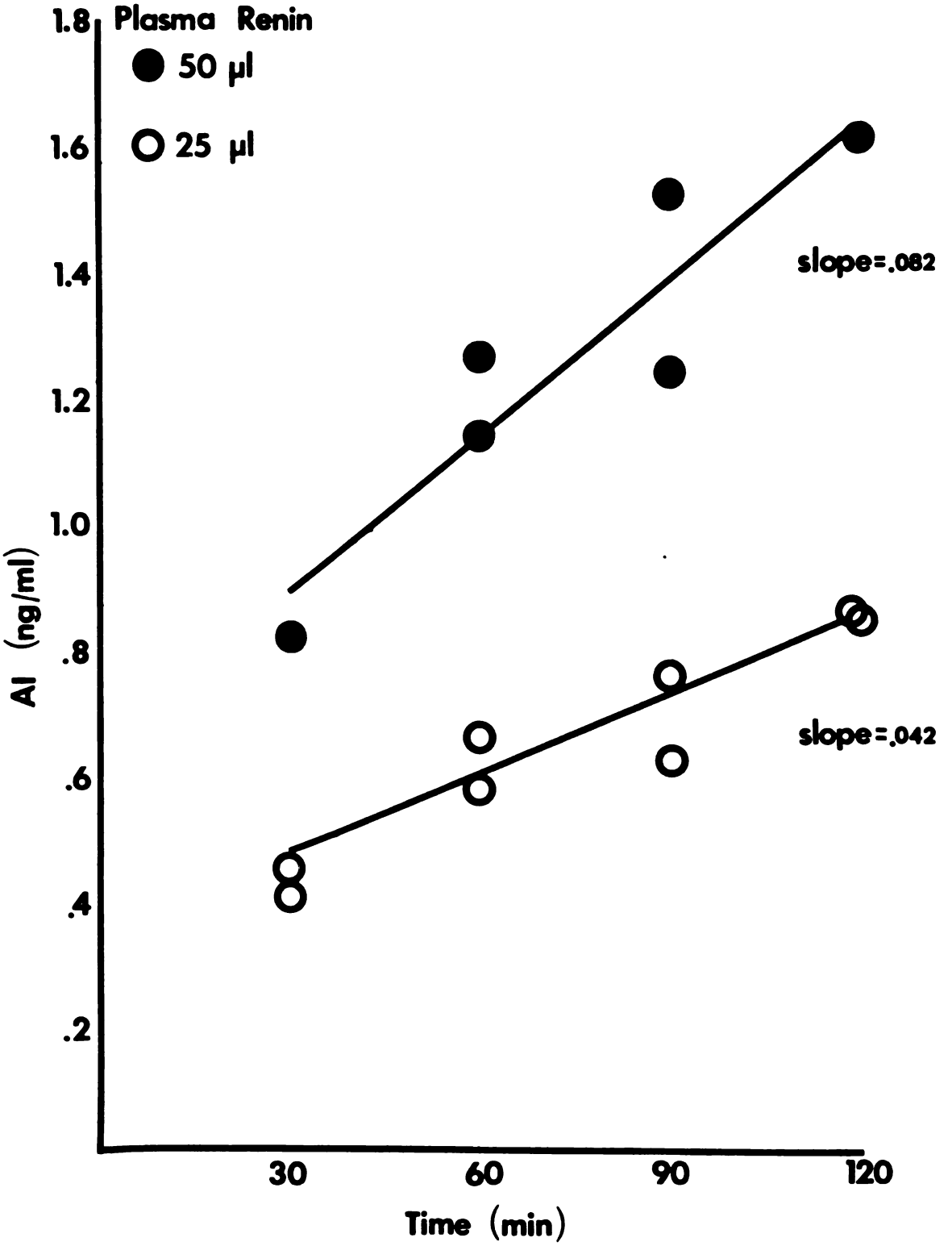

Plasma Renin
50 µl
25 µl
slope=.082
slope=.042
AI (ng/ml)
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
30
60
90
120
Time (min)

Figure 7

suggest that under these conditions, the rate of formation of AI is an accurate reflection of the concentration of renin present in the sample.

4. Correlation of AI generation to the zero-order velocity equation

The velocity of a zero-order process is described by the equation; $v = k[E] = \frac{d[AI]}{dt} = k[renin]$. Rearranging this equation to $d[AI] = k([renin] \cdot dt)$ suggests that a linear plot of [AI] versus [renin]·time is indicative of a true zero-order reaction process. Indeed the velocity of the renin-substrate reaction in the presence of 300 µl of plasma angiotensinogen conformed closely with that predicted by the zero-order velocity equation (Figure 8). The concentration of AI in the incubation medium was directly proportional to both the volume of renin added and the time of incubation. The rate constant (k) describing this zero-order process was estimated to be 0.41 ng AI/min.

C. Comparison of the Kinetics of the Renin-Substrate Reaction in Plasma from Various Species

1. Rat

Addition of increasing volumes of unextracted, nephrectomized rat plasma to the incubation medium containing homologous renin resulted in progressively greater rates of generation of AI (Figure 9). Normalizing the rates of AI formation to the volume of renin added generated significantly straight lines for each substrate volume examined. The slopes of the lines increased from 0.114 ng/min at 50 µl substrate to 0.383 ng/min when 400 µl plasma angiotensinogen was added to the incubation medium. The incremental changes in the slopes

Figure 8. Correlation of angiotensin I formation with the zero-order velocity equation. Plasma (300 µl) from nephrectomized adult male rats was incubated for various times with increasing volumes of high renin rat plasma. Each point represents a single determination. The data were fitted to the best line calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

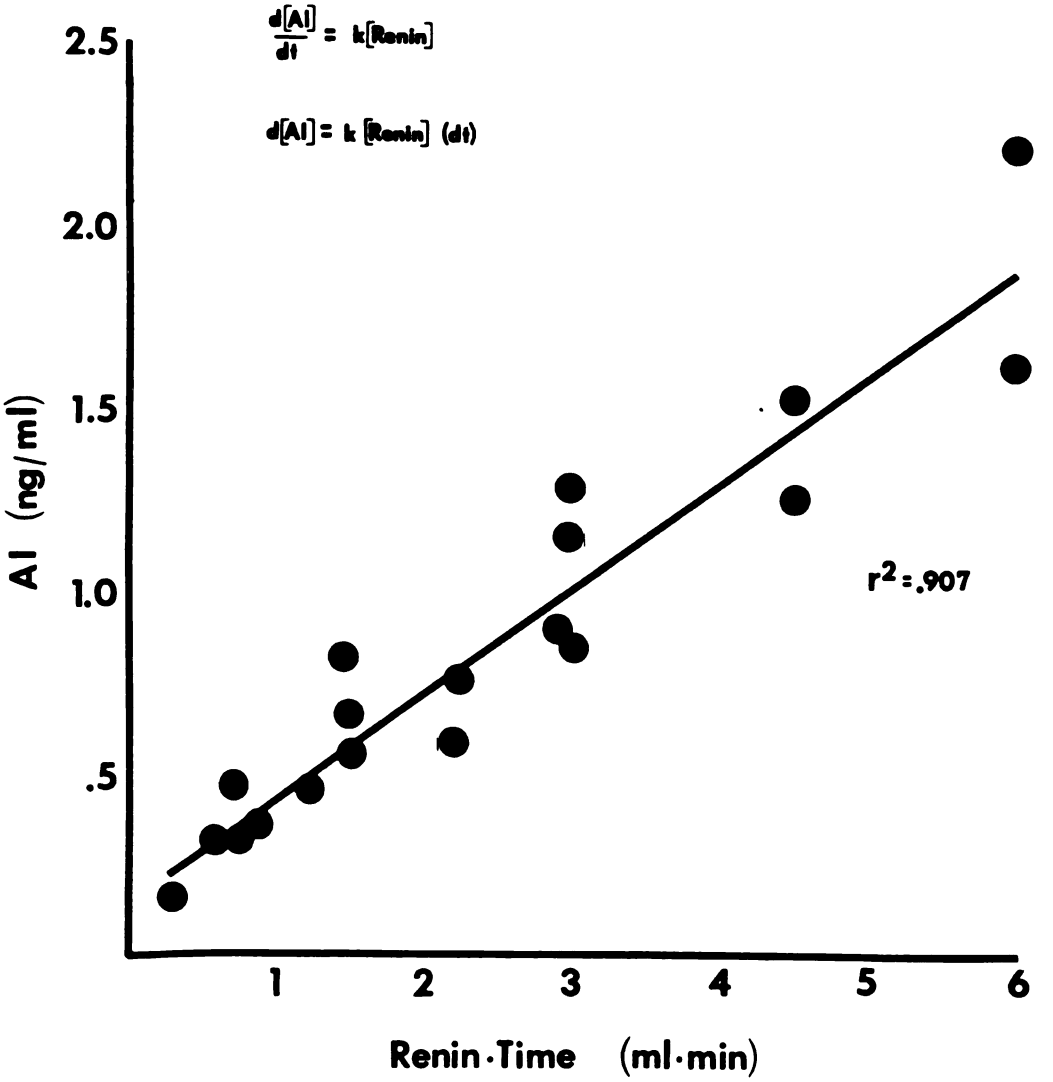

$\frac{d[AI]}{dt} = k[Renin]$
$d[AI] = k[Renin]\ (dt)$
2.5
2.0
1.5
1.0
.5
AI (ng/ml)
$r^2 = .907$
1
2
3
4
5
6
Renin·Time (ml·min)

Figure 8

Figure 9. Effect of varying plasma angiotensinogen on rate of generation of angiotensin I in rat plasma. Angiotensin I was quantified by radioimmunoassay. Final incubation volume was 1.0 ml. Each point represents a single determination. All lines were calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

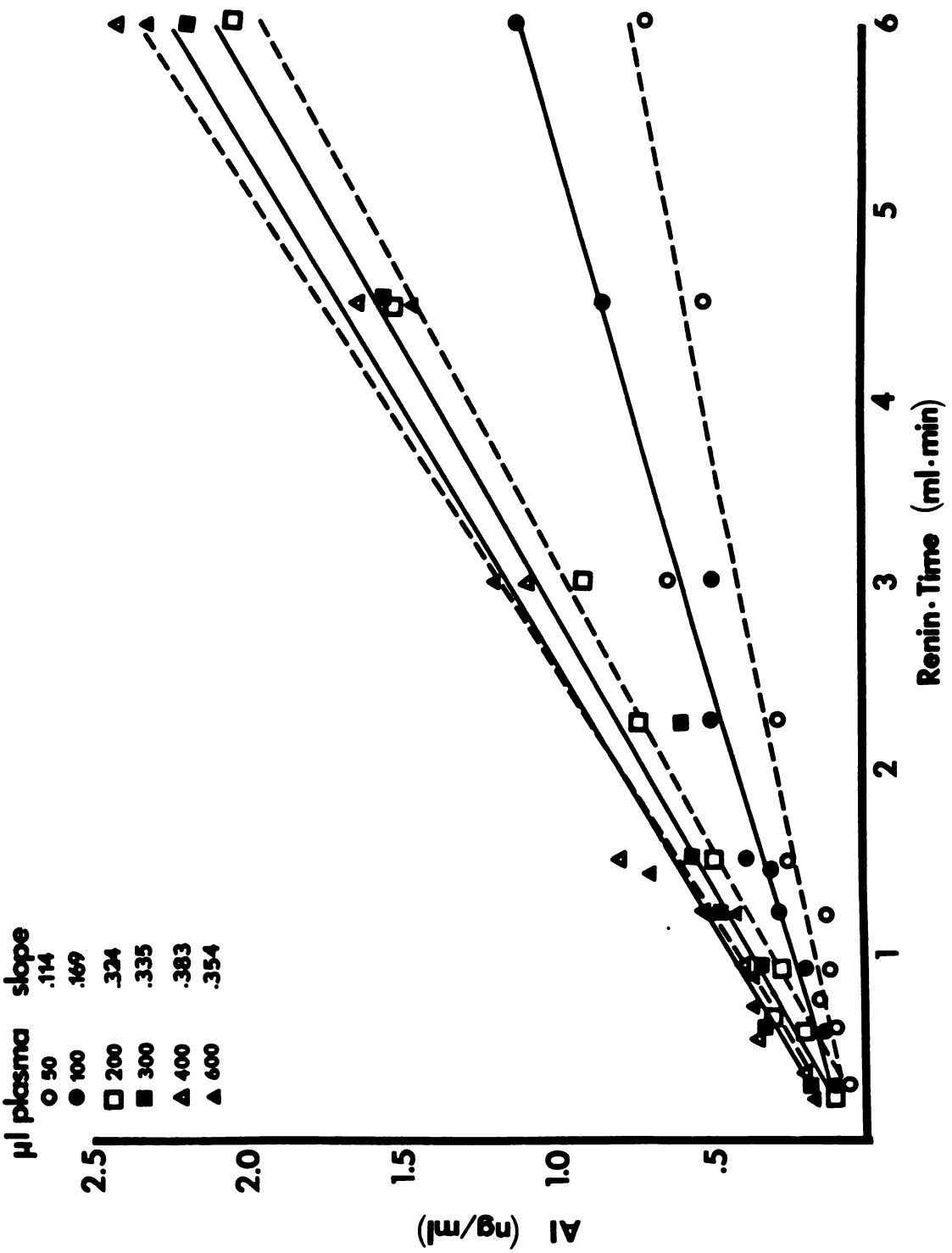

µl plasma slope
50 .114
100 .169
200 .324
300 .335
400 .383
600 .354
2.5
2.0
1.5
1.0
.5
Δ1 (ng/ml)
1
2
3
4
5
6
Renin·Time (ml·min)

Figure 9

of the lines, however, tended to decrease progressively with increasing volumes of angiotensinogen and did not change appreciably when the volume of substrate exceeded 300 µl.

Similarly, the rate of AI formation increased with the addition of progressively greater volumes of a preparation of partially purified angiotensinogen (Figure 10). The production of AI was linear at each substrate concentration examined. The rate of formation of AI when rat renin was incubated with 50 µl purified angiotensinogen was 0.302 ng/min whereas the rate increased to 0.588 ng/min in the presence of 300 µl angiotensinogen. There was no apparent difference in the rate of generation of AI when the volume of concentrated angiotensinogen was increased above 300 µl, suggesting that substrate was approaching saturating concentrations to renin.

Rearranging the data concerning the rates of generation of AI on a double-reciprocal plot with respect to the volume of substrate added generated a single straight line for each substrate examined (Figure 11). The line describing the data using unextracted, nephrectomized rat plasma had a slope of 0.3 ml·min/ng and intercepted the ordinate at 2.1 min/ng, whereas the line representing the purified substrate had a slope of 0.1 ml·min/ng and intercepted the ordinate at 1.3 min/ng. The differences in the slopes and intercepts of the lines indicated differences in the maximum velocity and affinity of renin for the two substrates. The maximum velocity (Vmax) increased from 0.49 ng/min in unextracted plasma to 0.75 ng/min using the concentrated preparation of angiotensinogen. Furthermore, renin had a greater affinity for the purified angiotensinogen (Km = 71 µl) than for the unextrated plasma substrate (Km = 141 µl).

Figure 10. Effect of increasing volumes of purified angiotensinogen on the rate of generation of angiotensin I in rat plasma. Each point represents a single measurement. All lines were calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

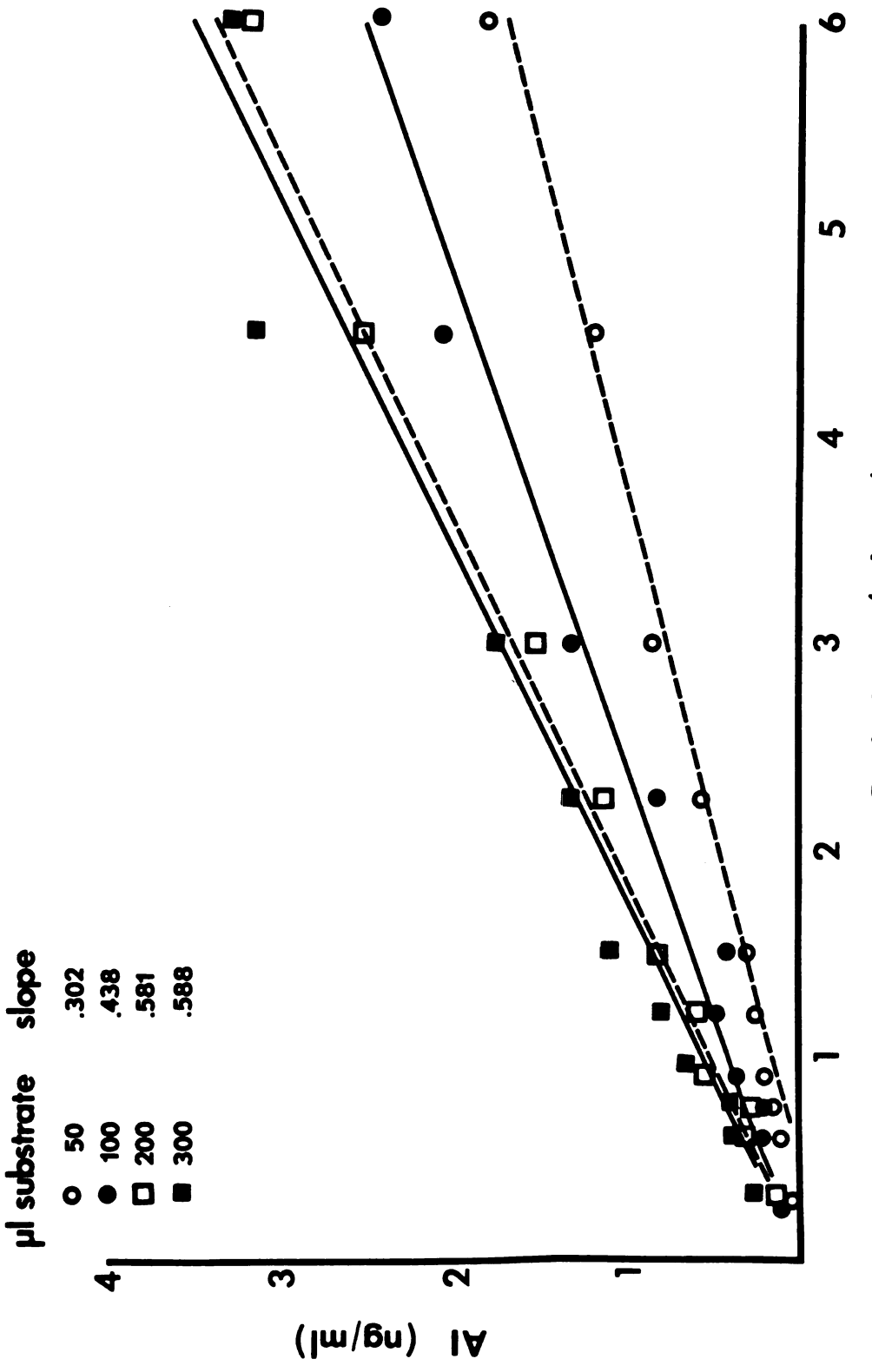

µl substrate slope
50 .302
100 .438
200 .581
300 .588
AI (ng/ml)
Renin·Time (ml·min)
1
2
3
4
5
6

Figure 10

Figure 11. Double-reciprocal plot of the substrate-dependence of renin from sodium-depleted, anesthetized, adult male rats. Closed circles represent determinations using unextracted plasma as substrate. Open circles represent determinations using a preparation of angiotensinogen partially purified from the pooled plasma. Each point represents a single determination. The data were fitted to the respective line calculated by the method of Wilkinson (1961).

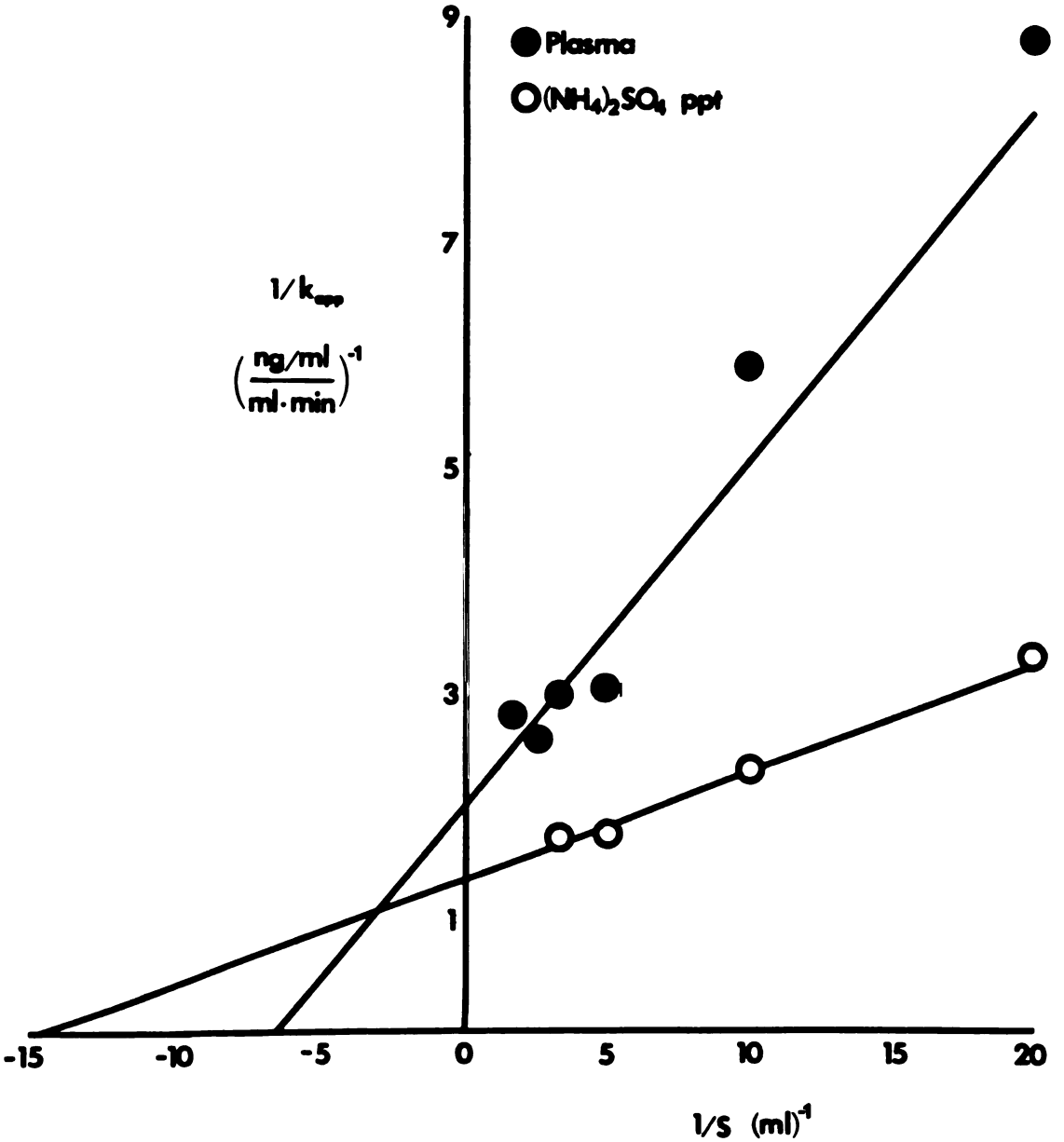

Plasma
$O(NH_4)_2SO_4$ ppt
$1/k_{app}$
$\left(\frac{ng/ml}{ml \cdot min}\right)^{-1}$
9
7
5
3
1
-15
-10
-5
0
5
10
15
20
1/S $(ml)^{-1}$

Figure 11

2) Hog

The rate of generation of AI by hog renin also increased with increasing volumes of plasma angiotensinogen (Figure 12). The data were linear and the rates increased progressively when the volume of unextracted angiotensinogen was raised from 50 µl to 600 µl. Replotting these data on a double-reciprocal plot resulted in the generation of a straight line intercepting the ordinate at 7.1 min/ng and having a slope of 0.1 ml·min/ng (Figure 13).

3. Dog

Similar to that observed for both rat and hog renins, the rate of the renin-substrate reaction in dog plasma was increased by increasing the volume of unextracted plasma angiotensinogen added to the incubation medium (Figure 14). The rate of AI production increased from 0.045 ng/min to 0.370 ng/min with increasing volumes of angiotensinogen between 50- and 600 µl. The incremental increases in the reaction rate, however, decreased at higher concentrations of substrate.

A similar substrate-dependence of dog renin was observed using a partially purified preparation of angiotensinogen (Figure 15). The data at each substrate concentration generated straight lines with increasing slopes at greater substrate concentrations. The slope of the line using 50 µl exogenous angiotensinogen was 0.237 ng/min whereas that with 600 µl substrate was 0.666 ng/min. Increasing the volume of angiotensinogen above 400 µl did not alter the rate of AI formation suggesting that substrate saturation was approached.

Figure 12. Substrate-dependence of renin from unanesthetized, saralasin-treated piglets. Angiotensin I was quantified by radioimmunoassay. Each point represents a single determination. All lines were calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

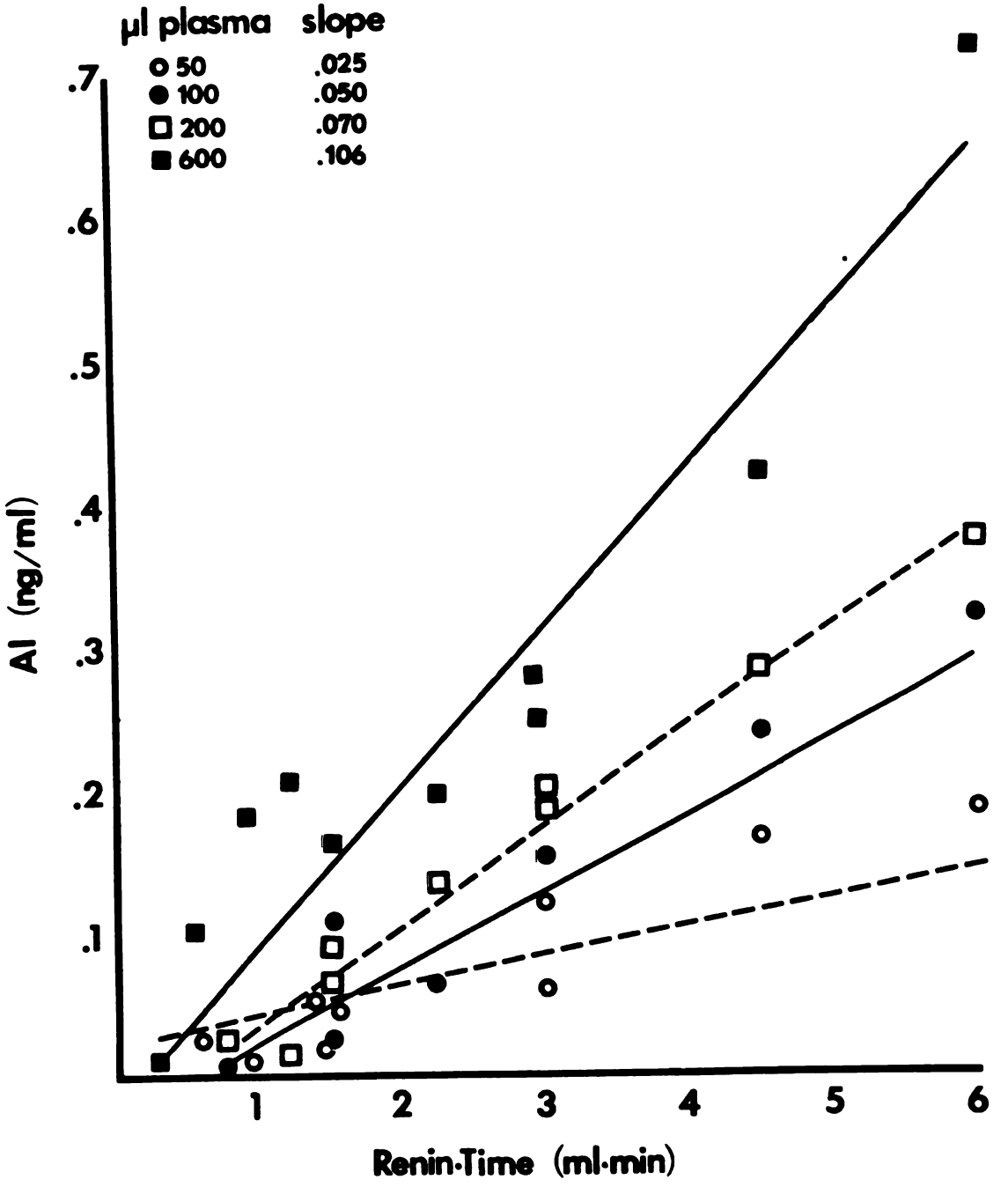

µl plasma
slope
50
.025
100
.050
200
.070
600
.106
.7
.6
.5
.4
.3
.2
.1
AI (ng/ml)
1
2
3
4
5
6
Renin·Time (ml·min)

Figure 12

Figure 13. Double-reciprocal plot of the substrate-dependence of hog renin. Each point represents a single determination of the rate (k_{app}) of generation of AI at each concentration of angiotensinogen. The data were fitted to the best line calculated by the method of Wilkinson (1961).

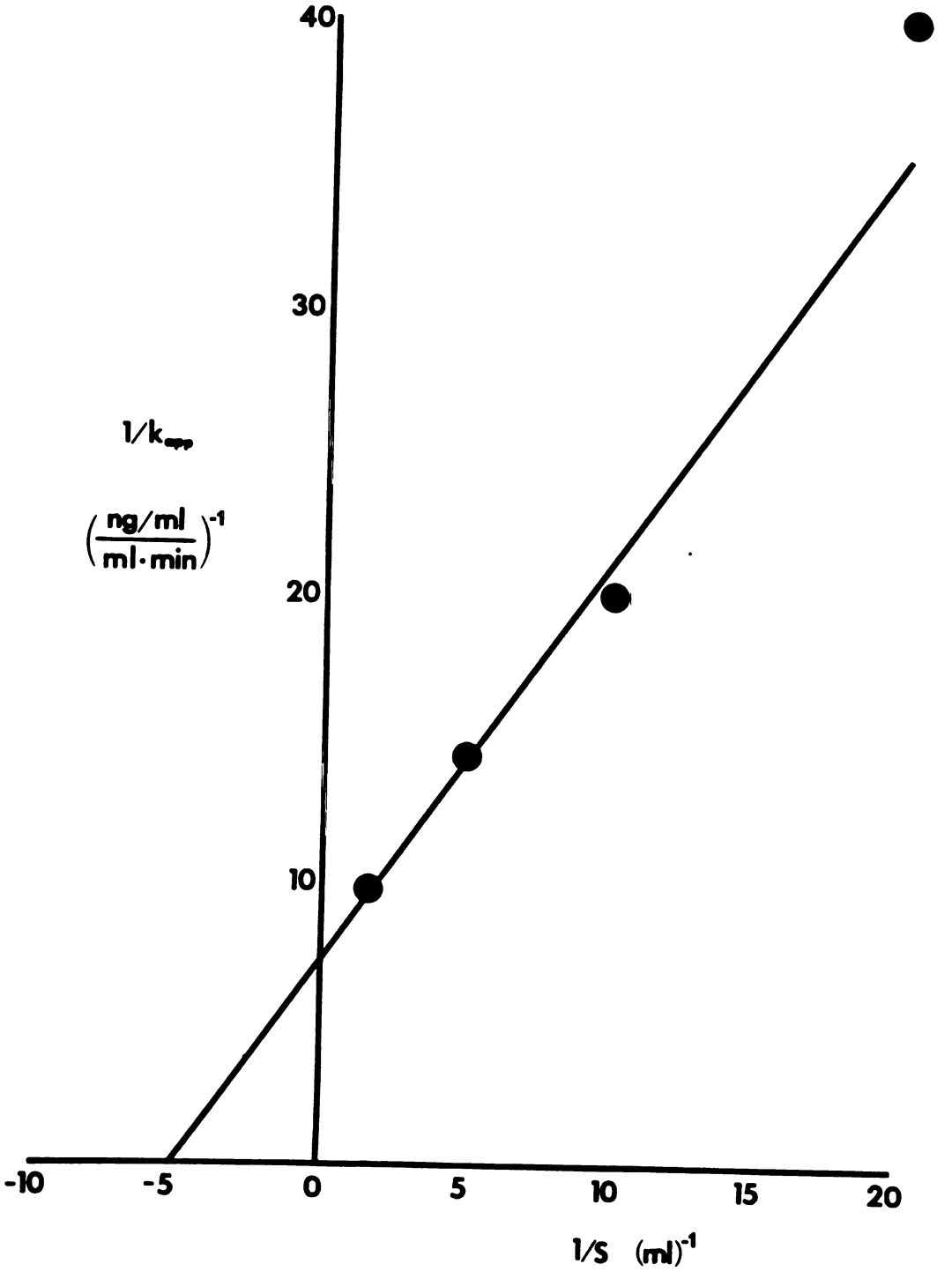

40
30
20
10
$1/k_{app}$
$\left(\frac{ng/ml}{ml \cdot min}\right)^{-1}$
-10
-5
0
5
10
15
20
$1/S \quad (ml)^{-1}$

Figure 13

Figure 14. Effect of varying plasma angiotensinogen on the rate of AI formation by dog renin. Each point represents a single determination. All lines were calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

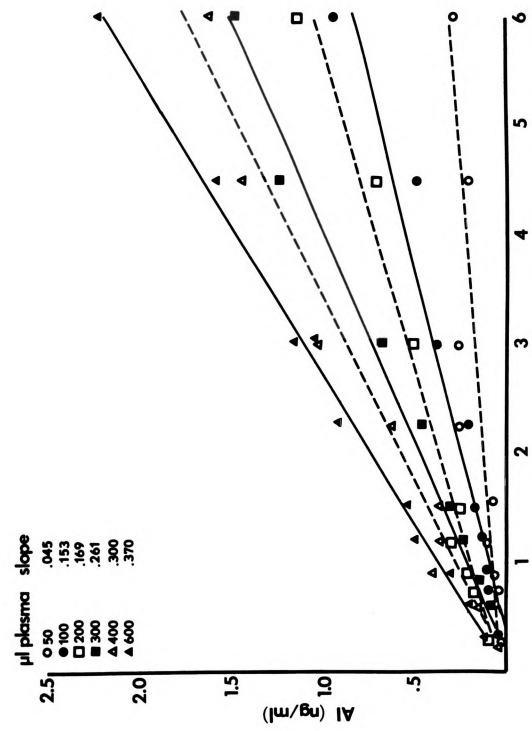

µl plasma slope
○ 50 .045
● 100 .153
□ 200 .169
■ 300 .261
△ 400 .300
▲ 600 .370
AI (ng/ml)
2.5
2.0
1.5
1.0
.5
1
2
3
4
5
6
Renin·Time (ml·min)

Figure 14

Figure 15. Effect of increasing volumes of purified angiotensinogen on the rate of the renin-substrate reaction in dog plasma. Each point represents a single determination. Data were fitted to the best line as calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

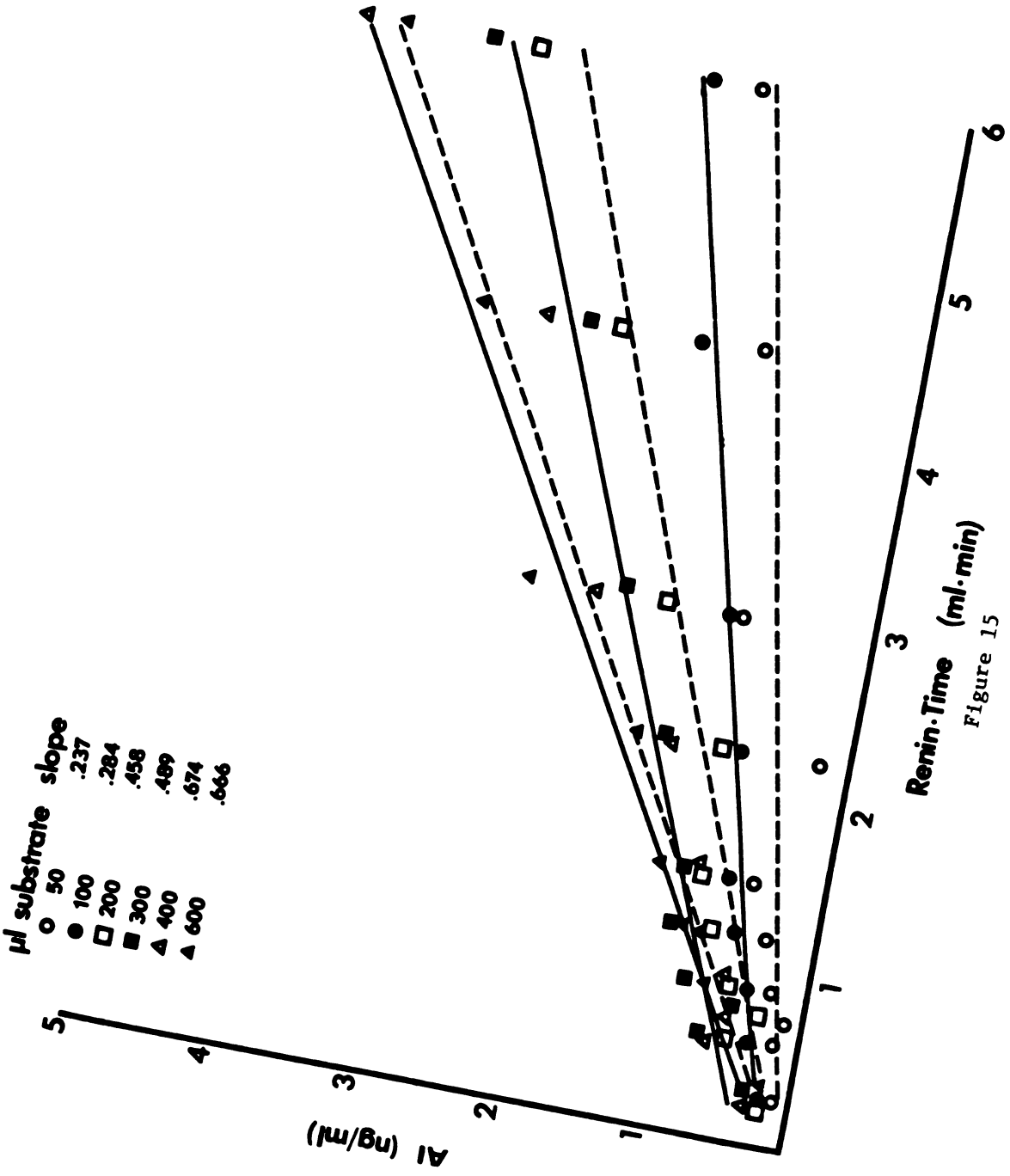

µl substrate slope
50 .237
100 .284
200 .458
300 .489
400 .674
600 .666
AI (ng/ml)
Renin Time (ml·min)
1
2
3
4
5
6

Figure 15

Plotted in double-reciprocal fashion, the data describing the substrate-dependence of dog renin generated a straight line for each of the substrates examined (Figure 16). The line generated from the data using the partially purified substrate had a smaller slope and intercept on the ordinate than did the line describing the reaction using unextracted dog plasma as substrate. The values of Vmax calculated from the intercept on the ordinate were 0.63 ng/min for unextracted plasma and 0.88 ng/min for the partially purified preparation of angiotensinogen. Similarly, purification of angiotensinogen altered the affinity with which dog renin interacted with the substrate (Km = 418 µl plasma versus Km = 183 µl ammonium sulfate precipitate).

Kinetic analysis of the data concerning the renin-substrate reaction in the different species was performed by the method described in Appendix B. The equation describing the double-reciprocal plots derives from the classical Michaelis-Menten first-order reaction velocity,

$$\frac{1}{d[AI]/[R_t](dt)} = \frac{K_m}{V_{max}} \left(\frac{1}{[S]}\right) + \frac{1}{V_{max}}$$

Therefore, a double-reciprocal plot of $\frac{1}{d[AI]/[R_t](dt)}$ versus $\frac{1}{[S]}$ results in a straight line intercepting the ordinate at $\frac{1}{V_{max}}$ and having a slope of K_m/V_{max}. The intercept of the abscissa is then equal to $-1/K_m$. Accordingly, the values of K_m and V_{max} for each species was estimated from the line best describing the double-reciprocal plots of the respective data.

Figure 16. Double-reciprocal plot of the substrate-dependence of renin from furosemide-treated dogs. Closed circles depict the data obtained using unextracted plasma as substrate. Open circles represent similar determinations using a preparation of angiotensinogen partially purified from the pooled plasma. Each point represents a single determination of the rate of generation of AI. The data were fitted to the respective line calculated by the method of Wilkinson (1961).

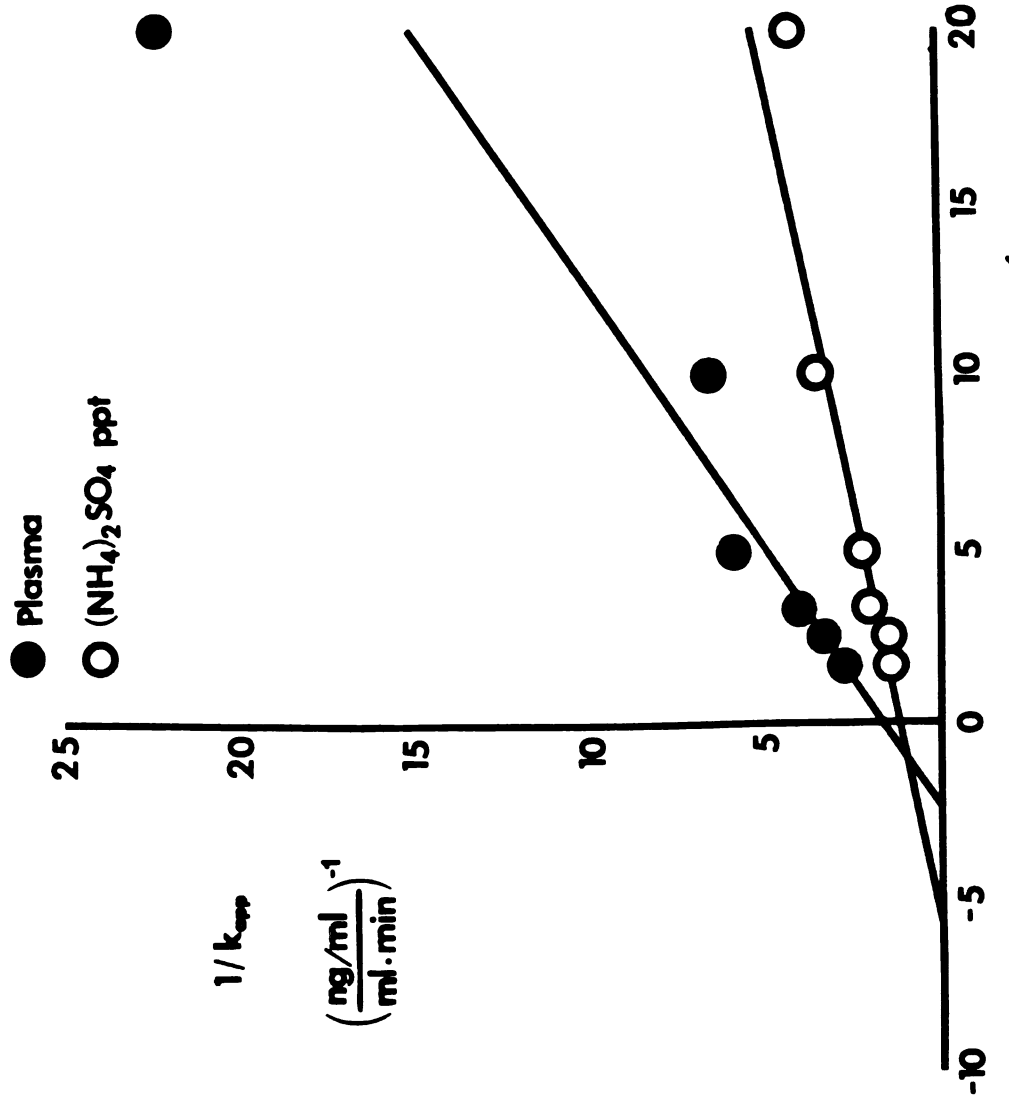

Plasma
$(NH_4)_2SO_4$ ppt
$1/k_{ap}$
$\left(\frac{ng/ml}{ml \cdot min}\right)^{-1}$
25
20
15
10
5
0
-5
-10
1/S $(ml)^{-1}$
5
10
15
20

Figure 16

The kinetic parameters obtained from the double-reciprocal plots using unextracted angiotensinogen as homologous substrate are summarized in Table 1. The Vmax of dog renin was greater than that for the other two species examined. The Michaelis-Menten constants (Km) for hog and rat plasma were similar (0.198 versus 0.141 ml) whereas the Km for dog plasma was markedly greater than that for the other two species (0.418 ml).

The observed velocities of the renin-substrate reaction for all 3 species fell on or very near the theoretical line calculated from the Michaelis-Menten equation (Figure 17), suggesting that the reactions conformed very closely with that predicted by a true first-order process. The curve describing hog renin plateaued at a much lower velocity than the other two species. Hog renin appeared to be saturated at approximately 400 µl angiotensinogen. The rate of generation of AI by rat renin also exhibited a hyperbolic substrate-dependence, becoming saturated with approximately 400 µl plasma angiotensinogen. The velocity of the rat renin reaction was approximately 3-fold greater than that of hog renin. The velocity of the renin-substrate reaction in dog plasma was similar to that of rat at 600 µl of homologous angiotensinogen (0.370 ng/min and 0.354 ng/min, respectively). In contrast to the rat and hog, however, the renin reaction of dog plasma was not saturable with unextracted angiotensinogen up to 600 µl.

The relative concentration of angiotensinogen in plasma was calculated from the Michaelis-Menten equation given the Km, Vmax, and the rate of generation of AI from endogenous substrate as described in

TABLE 1

Kinetic Parameters of the Renin-Substrate Reaction in Plasma from Adult Hogs, Dogs and Rats[a]

	V_{max} ($\frac{\text{ng AI/ml medium}}{\text{ml renin}\cdot\text{min}}$)	K_m (ml plasma angiotensinogen)
Hog	0.141±0.008	0.198±0.025
Dog	0.628±0.092	0.418±0.098
Rat	0.485±0.054	0.141±0.043

[a]Values represent mean ± S.E. as determined by the method of Wilkinson (1961).

Figure 17. Correlation of the substrate-dependence of renin from different species with that predicted by Michaelis-Menten reaction kinetics. Each point represents the rate of generation of AI at each substrate concentration. The theoretical lines were calculated by substituting the respective values for V_{max} and K_m into the Michaelis-Menten equation.

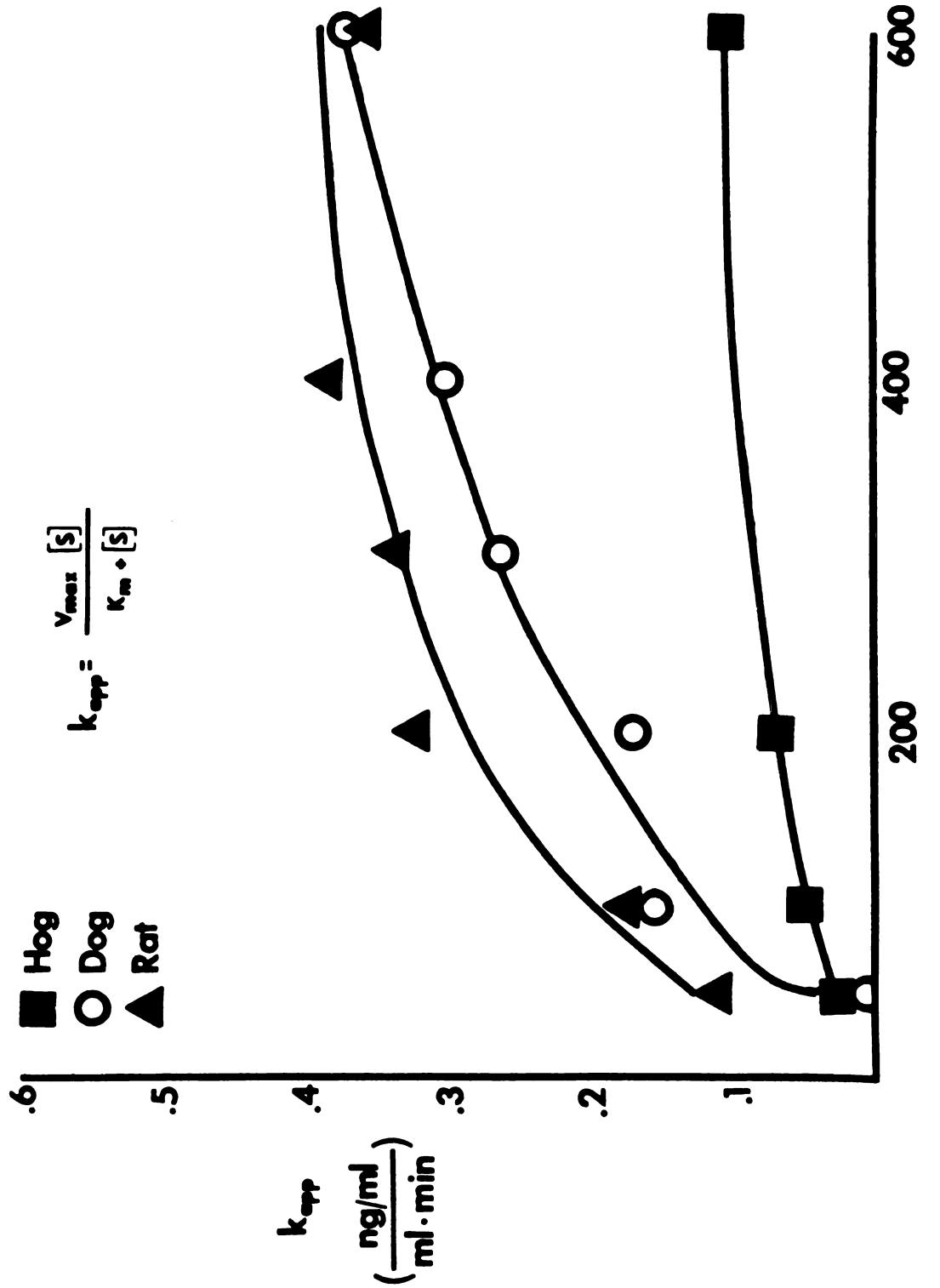

Hog
Dog
Rat
$k_{app} = \frac{V_{max}[S]}{K_m + [S]}$
k_{app} $\left(\frac{ng/ml}{ml \cdot min}\right)$
.6
.5
.4
.3
.2
.1
200
400
600
Plasma Angiotensinogen (µl)

Figure 17

the methods section. Endogenous velocities (k_{app}) were calculated from the PRA of the same renin sample as that used to estimate the respective Km and Vmax.

The rate of generation of AI from endogenous angiotensinogen was greatest in dog plasma compared to that of hog and rat (Table 2). Calculation of the apparent velocity (k_{app}) of the renin-substrate reaction from PRA provided an estimation of the fractional maximum velocity (k_{app}/V_{max}) of the reaction in vivo. Values less than 100 indicate limitation of PRA by substrate. The renin-substrate reaction of hog plasma was found to proceed at 91% of the maximum velocity in vivo. In contrast, the rate of generation of AI from endogenous angiotensinogen in rat plasma was only 16% that of the maximum velocity. The fractional maximum velocity of dog renin in vivo was intermediate between that of hog and rat (42% maximum).

Rearranging the Michaelis-Menten equation and substituting the ratio of k_{app}/V_{max} provides an estimation of the concentration of endogenous angiotensinogen in ml equivalents of the high substrate plasma. The relative concentration of angiotensinogen in hog plasma was 7-fold greater than that required for half maximum velocity (Table 2). The relative concentration of angiotensinogen in dog plasma was 74% of the Km value. In contrast, the concentration of endogenous angiotensinogen in rat plasma was only 19% that required for half maximum velocity of the renin-substrate reaction.

TABLE 2

Relative Angiotensinogen Concentration and Velocity of the Renin-Substrate Reaction in Plasma from Different Species[a]

	Hog	Dog	Rat
PRA (ng AI/ml plasma·hr)	7.66 ±0.31	15.99±0.34	4.61±0.06
k_{app} ($\frac{\text{ng AI/ml medium}}{\text{ml renin·min}}$)[b]	0.128±0.005	0.266±0.006	0.077±0.001
$\frac{k_{app}}{V_{max}}100$[b]	90.65 ±3.63	42.46 ±0.91	15.85±0.20
Angiotensinogen (ml plasma)[c]	2.725±1.578	0.309±0.011	0.027±0.001
$\frac{\text{Angiotensinogen}}{K_m}100$[c]	704.9±70.8	73.8±2.7	18.8±0.3

[a]Values represent mean ± S.E. of 4 determinations.

[b]The apparent rate of generation of AI (k_{app}) was calculated from PRA. V_{max} was derived from the kinetic analysis.

[c]The concentration of angiotensinogen was calculated by substitution of k_{app}/V_{max} into the Michaelis-Menten equation. K_m was obtained from the kinetic analysis.

D. Age-Related Differences in the Kinetics of the Renin-Substrate Reaction in Rat Plasma

Similar kinetic experiments were performed to provide an indication of the stoichiometry of the renin-substrate reaction of newborn rat plasma compared to that of adults. In order to make this comparison, it was necessary to employ the same pooled plasma sample as the source of angiotensinogen for both enzymes. Unextracted plasma from bilaterally nephrectomized, adult male rats was used as substrate for both adult and newborn renin.

The rate of generation of AI during incubation of plasma from unanesthetized, normal adult male rats was found to be dependent on the volume of exogenous angiotensinogen added to the incubation mixture (Figure 18). The concentration of AI in the incubation medium was directly proportional to the product of renin and time for all volumes of angiotensinogen examined. The slope of the line increased with increasing substrate concentrations, however, the incremental change in slope decreased as larger volumes of angiotensinogen were added. The maximum slope of the line was estimated to be approximately 0.177 ng/min.

The rate of the renin-substrate reaction of newborn (7 days) rat plasma also increased progressively with increasing angiotensinogen (Figure 19). The reaction appeared to be zero-order in nature at each substrate concentration as reflected by the linear relationship between the concentration of AI and the product of renin and time. The increment of increase in the slopes was less at larger substrate volumes. The maximum rate of generation of AI (approximately 0.874

Figure 18. Substrate-dependence of renin from unanesthetized adult male rats. Unextracted plasma from 24 hr bilaterally nephrectomized, adult male rats served as the source of angiotensinogen. Each point represents a single determination. All lines were calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

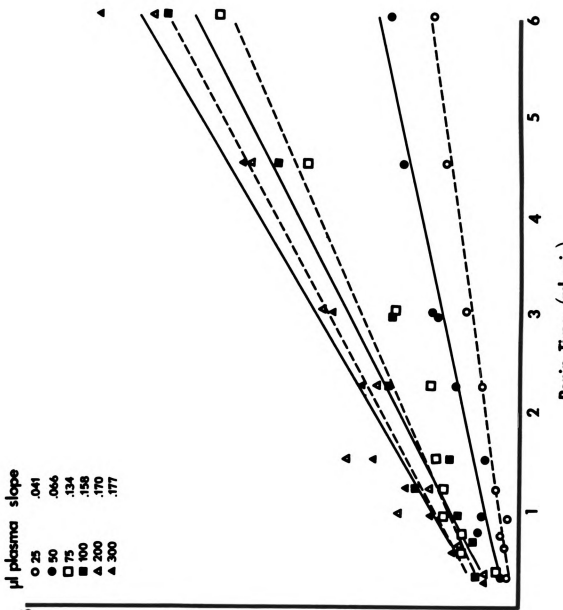

µl plasma slope
○ 25 .041
● 50 .066
□ 75 .134
■ 100 .158
△ 200 .170
▲ 300 .177
1.5
1.0
.5
AI (ng/ml)
1
2
3
4
5
6
Renin · Time (ml · min)

Figure 18

Figure 19. Substrate-dependence of renin from unanesthetized newborn (7 days) rats. Unextracted plasma from 24 hr bilaterally nephrectomized, adult male rats served as the source of angiotensinogen. Each point represents a single determination. All lines were calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

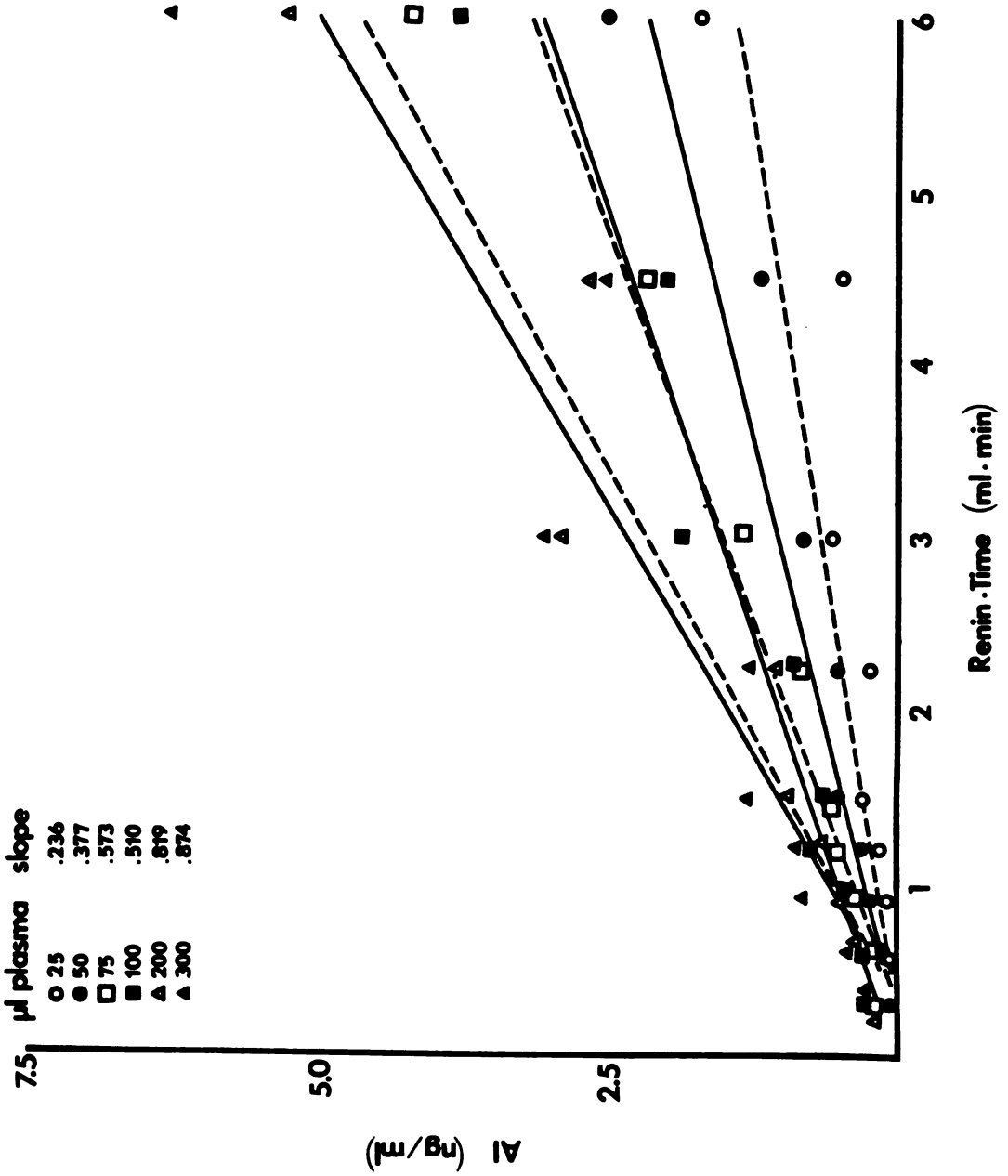

μl plasma slope
○ 25 .236
● 50 .377
□ 75 .573
■ 100 .510
▲ 200 .819
▲ 300 .874
7.5
5.0
2.5
AI (ng/ml)
1
2
3
4
5
6
Renin·Time (ml·min)

Figure 19

ng/min) was observed when newborn renin was incubated in the presence of 100-300 µl angiotensinogen.

Replotting the data in double-reciprocal fashion generated a straight line for each of the enzymes examined (Figure 20). The line describing the adult reaction had a greater slope and intercept on the ordinate than that for newborn renin using the same pooled sample of nephrectomized rat plasma as substrate for both enzymes. On the other hand, both lines intersected the abscissa at approimately the same location (-10 to -15 ml^{-1}).

The values for Vmax and Km obtained from the double-reciprocal plots of the data are tabulated in Table 3. The maximum velocity of the renin-substrate reaction in newborn rat plasma was 5-fold greater than that of adult plasma (1.215 ng/min versus 0.239 ng/min, respectively), however, the volume of nephrectomized rat plasma required for half-maximum velocity was similar for both enzymes (109 and 74 µl, respectively).

Substitution of the calculated values for Km and Vmax into the Michaelis-Menten equation resulted in a line which closely described the observed reaction velocities for both adult and newborn rat plasma (Figure 21). The maximum rate of generation of AI in adult plasma was approximately 1/4 that of newborn plasma. Adult renin appeared to be saturated with 100-300 µl plasma angiotensinogen. The newborn enzyme was capable of generating approximately 1.0 ng AI/min·ml when incubated with large volumes of angiotensinogen. The fact that the data were superimposed on the theoretical curve is indicative of true first-order kinetics for the renin-substrate reaction of both adult and newborn plasma.

Figure 20. Double-reciprocal plot of the substrate-dependence of adult and newborn (7 days) rat renin. Plasma from unanesthetized adult and newborn rats served as the respective source of renin. Plasma from 24 hr bilaterally nephrectomized adult rats was used as substrate for both enzymes. Each point represents a single determination of the rate of generation of AI. Both lines were calculated by the method of Wilkinson (1961).

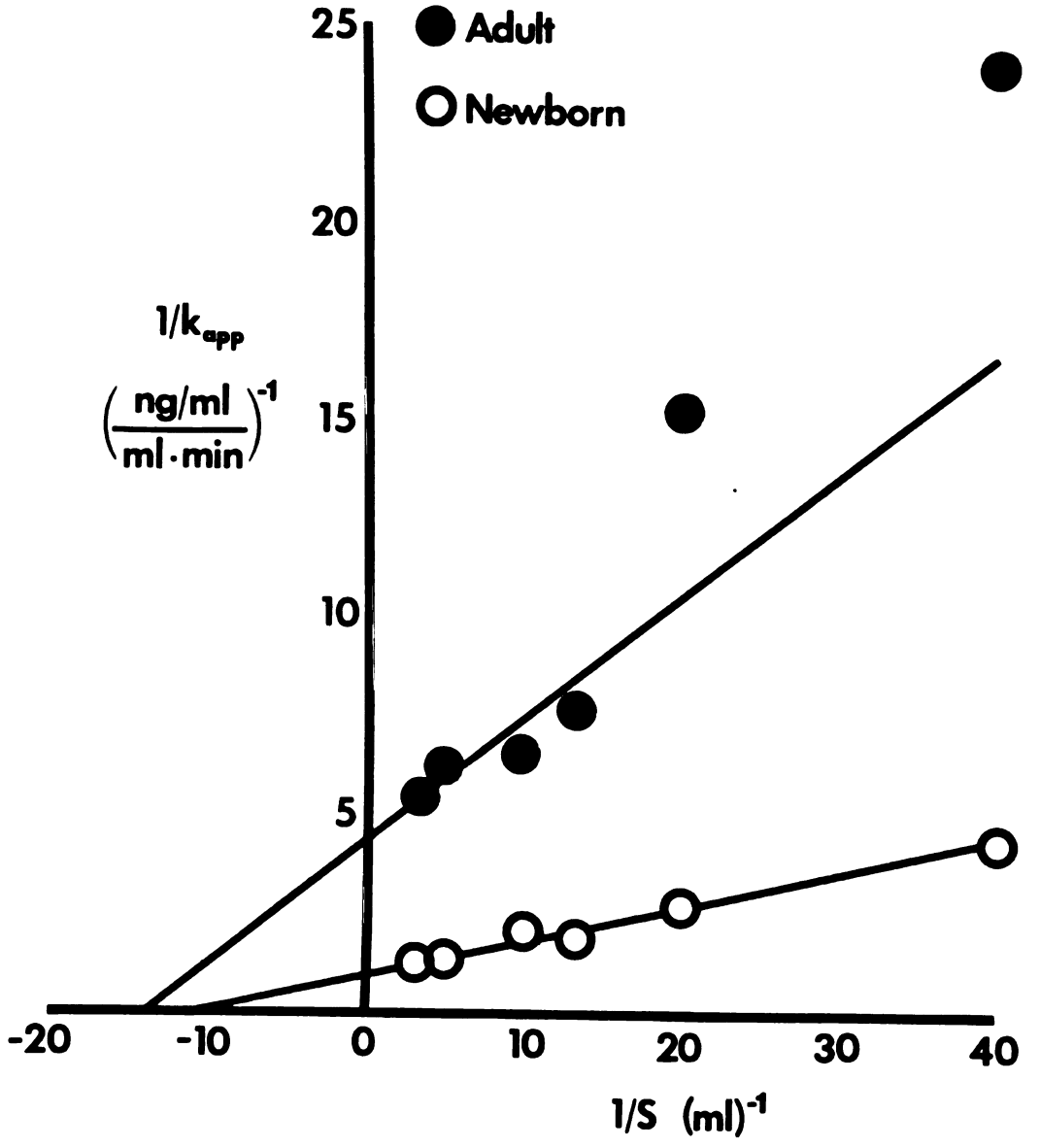

Adult
Newborn
25
20
15
10
5
$1/k_{app}$
$\left(\frac{ng/ml}{ml \cdot min}\right)^{-1}$
-20
-10
0
10
20
30
40
1/S $(ml)^{-1}$

Figure 20

TABLE 3

Kinetic Parameters of the Renin-Substrate Reaction in Plasma from Newborn and Adult Rats[a]

	7-Day	Adult
V_{max} ($\frac{\text{ng AI/ml medium}}{\text{ml renin}\cdot\text{min}}$)	1.215±0.129	0.239±0.029
K_m (ml plasma substrate)	0.109±0.025	0.074±0.019

[a]Values represent mean ± S.E. as determined by the method of Wilkinson (1961).

Figure 21. Correlation of the substrate-dependence of adult and newborn (7 days) rat renin with that predicted by the equation describing a first-order reaction process. Each point represents the rate (k_{app}) of generation of AI at each substrate concentration. The lines were calculated by substituting the values for V_{max} and K_m into the respective Michaelis-Menten equation.

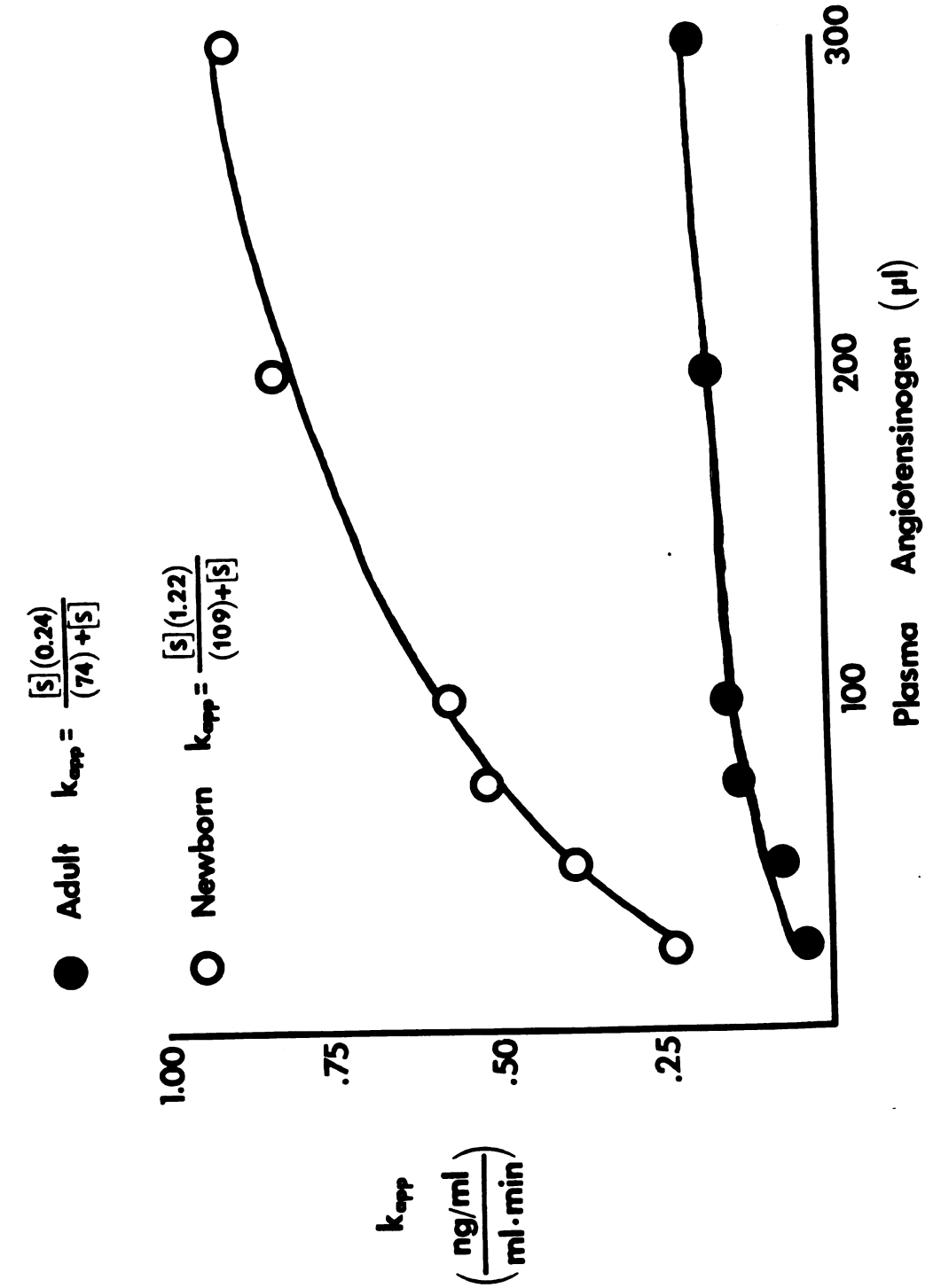

● Adult $k_{app} = \frac{[S](0.24)}{(74)+[S]}$
○ Newborn $k_{app} = \frac{[S](1.22)}{(109)+[S]}$
1.00
.75
.50
.25
k_{app} $\left(\frac{ng/ml}{ml \cdot min}\right)$
100
200
300
Plasma Angiotensinogen (μl)

Figure 21

The velocity of the renin-substrate reaction in the absence of exogenous angiotensinogen (PRA) was greater in newborn plasma (11.5 ng/ml·hr) compared to adults (4.8 ng/ml·hr). Comparison of this endogenous rate of generation of AI (k_{app}) to the maximum rate indicates that the relative rate of generation of AI *in vivo* (k_{app}/V_{max}) was 2-fold greater in adult plasma than that in newborns (Table 4). Values less than 100 reflect limitation of PRA by angiotensinogen. Rearranging the Michaelis-Menten equation and substituting the ratio k_{app}/V_{max} provides an estimation of the relative concentration of endogenous angiotensinogen expressed as ml equivalents of the high angiotensinogen plasma. The relative concentration of angiotensinogen in adult plasma was 50% that required for half maximum velocity (Table 4). In contrast, the concentration of angiotensinogen in newborn plasma was only 11% that of the Km for the reaction.

E. Evaluation of Using Partially Purified Angiotensinogen as Substrate for Renin Measurements

Because of the dissimilarities between the manner in which unextracted plasma and purified angiotensinogen interact with homologous renin, it was of interest to compare the results of renin measurements using the two substrates. Plasma from bilaterally nephrectomized rats was divided into two portions. One portion was kept frozen while the angiotensinogen in the remaining plasma was concentrated by ammonium sulfate precipitation. The rates of generation of AI from the two preparations of angiotensinogen were then compared.

The velocity of the renin-substrate reaction in newborn rat plasma was 0.87 ng/min when incubated with 300 µl unextracted plasma

TABLE 4

Estimated Angiotensinogen Concentration in Plasma from Newborn and Adult Rats[a]

	7-Day	Adult
PRA (ng AI/ml plasma·hr)	11.49 ±0.42[d]	4.79 ±0.28
k_{app} ($\frac{\text{ng AI/ml medium}}{\text{ml renin·min}}$)[b]	0.192±0.007[d]	0.080±0.005
$\frac{k_{app}}{V_{max}}100$[b]	15.76 ±0.58[d]	33.37 ±1.98
Angiotensinogen[c] (ml plasma)	0.020±0.001[d]	0.037±0.003
$\frac{\text{Angiotensinogen}}{K_m}100$[c]	11.2 ±3.0[d]	50.4 ±4.4

[a] Values represent mean ± S.E. of 4 determinations.

[b] The apparent rate of AI generation (k_{app}) was calculated from PRA. V_{max} was obtained from the kinetic analysis of the data.

[c] The concentration of angiotensinogen was estimated by substitution of k_{app}/V_{max} into the Michaelis-Menten equation. K_m was determined from the kinetic analysis of the reaction.

[d] Significantly different from adult ($p<.05$).

angiotensinogen (Table 5). This value is 4-fold greater than that of adult renin (0.18 ng/min) using the same substrate preparation. However, incubation of the same volume of either adult or newborn renin with as little as 25 µl of the concentrated angiotensinogen resulted in rates of generation of AI several fold greater than the respective estimated maximum velocities (Vmax) using the unextracted angiotensinogen. Increasing the volume of the partially purified preparation of substrate to 200 µl did not further increase the rate of generation of AI by either adult or newborn renin. These data suggest that angiotensinogen purified by the ammonium-sulfate precipitation technique may yield rates of AI generation greater than the calculated maximum.

F. Postnatal Changes in Plasma and Renal Renin

1. Comparison of renin activity and concentration in plasma during development

The concentration of renin in plasma increased following birth reaching maximum values (20-35 ng/ml·hr) at 2-3 weeks of age (Figure 22). PRA was maximum earlier during development (1- to 2-weeks postpartum). PRA and PRC of 2-week old rats were respectively 2- and 5-fold greater than that of adults. Both PRA and PRC decreased progressively after 3-weeks of age to minimum values observed in adult rats. The largest decrease in PRA and PRC occurred between the second and fourth postnatal week. In newborn rats, PRC was consistently greater than PRA suggesting that the relative substrate concentration was limiting PRA in newborn plasma. However, PRA and PRC tended to converge in older animals until no significant difference existed in

TABLE 5

Saturation of the Renin-Substrate Reaction with Angiotensinogen[a]

Substrate	7-Day Plasma Renin ($\frac{\text{ng AI/ml medium}}{\text{ml renin·min}}$)	Adult Plasma Renin ($\frac{\text{ng AI/ml medium}}{\text{ml renin·min}}$)
300 µl plasma[b]	0.87	0.18
V_{max} Plasma[c]	1.22±0.13	0.24±0.03
25 µl $(NH_4)_2SO_4$ ppt[d]	3.43	1.30
300 µl $(NH_4)_2SO_4$ ppt[d]	3.63	1.25

[a]Values represent mean of 3 determinations.

[b]Unextracted plasma from bilaterally nephrectomized adult rats was used as substrate.

[c]V_{max} was calculated from the kinetic analysis of the data.

[d]Angiotensinogen partially purified from the nephrectomized rat plasma by ammonium sulfate precipitation was used as substrate for renin.

Figure 22. Comparison of plasma renin activity (PRA) and plasma renin concentration (PRC) in developing rats. Blood from littermates less than 3 weeks of age was pooled to obtain sufficient plasma for a single determination. Each point represents mean ± S.E. of at least 3 determinations. At ages where both renin activity and concentration were determined in the same plasma sample, significant differences between the means of PRA and PRC were determined by the Student's *t*-test for paired comparisons ($p<.05$).

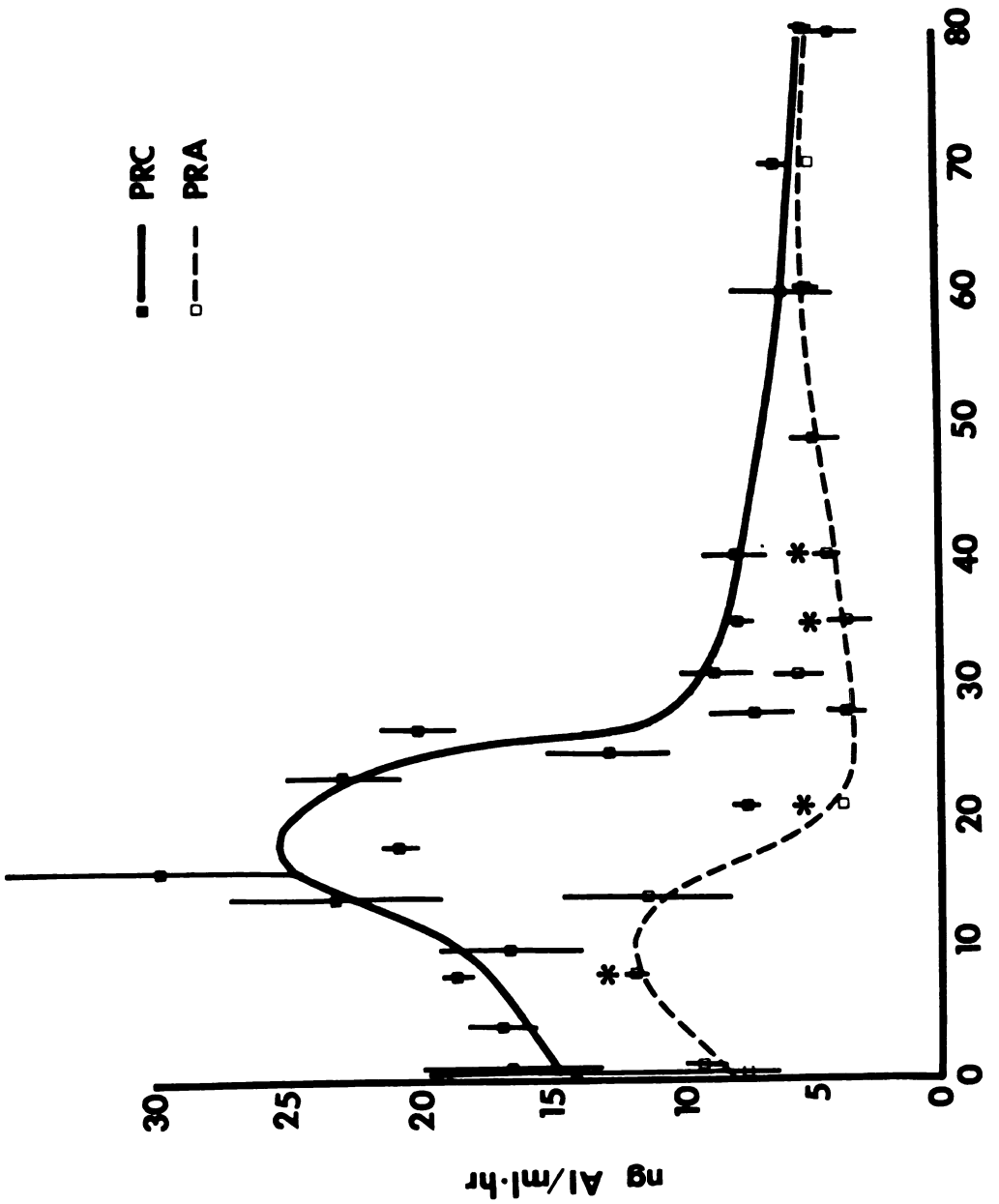

PRC
PRA
ng Al/ml·hr
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
Age (days)
*
*
*

Figure 22

adult rats (approximately 5 ng AI/ml·hr). Both plasma renin activity (PRA) and concentration (PRC) were greater in rats 2-days old than in adults.

The specific activity of renin (PRA/PRC) increased progressively throughout development (Figure 23). Ratios less than 1.00 indicate subsaturating angiotensinogen. The ratio of PRA to PRC was 0.60±.17 in 4-day old rats and did not change appreciably until the fourth week postpartum. The specific activity increased gradually from 0.58 at 3 weeks to 0.98 in rats 60-days old. The ratio of PRA/PRC of 60- and 80-day old rats was not significantly different from 1.00.

2. Renal renin

Renal renin concentration (RRC/g) was lower in 1-day old rats (31±11 μg/hr·g) compared to adults (Figure 24). RRC/g increased in a nonlinear fashion between 2- and 6-weeks of age. Renin concentration did not change appreciably between birth and 2-weeks postpartum. The concentration of renin in kidneys at 6-weeks of age was not different from that of adults (136±22 μg AI/hr·g).

Renal renin content (RRC), expressed per whole kidney, also increased with age (Figure 25). Kidneys from 1-day old rats contained 6.3 μg AI/hr renin whereas that of adults was 300 μg AI/hr. This 50-fold increase in RRC proceeded in a somewhat linear fashion between 2- and 6-weeks of age. Renin content did not change appreciably during the first 2 weeks postpartum.

Figure 23. Specific activity of renin in plasma from developing rats. PRA and PRC were determined for the same plasma samples. Value represents the mean ± S.E. of 4 comparisons.

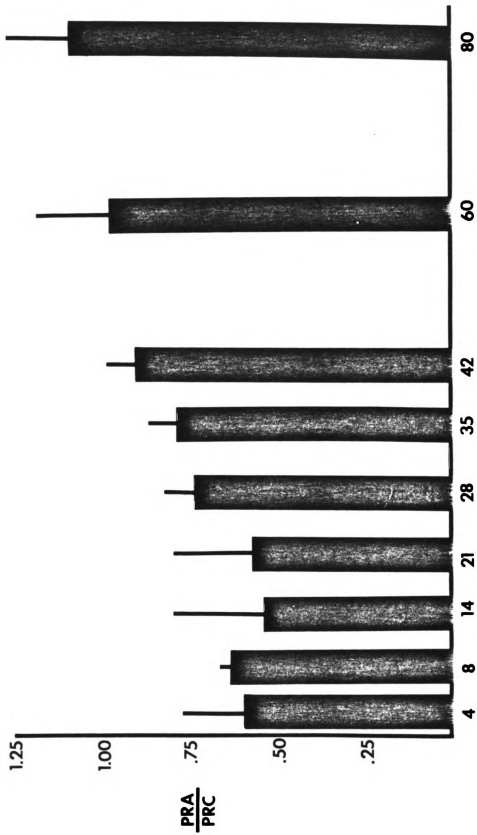


Figure 23

Figure 24. Renal renin concentration (RRC/g) in developing rats. Renin concentration is expressed as the rate of formation of AI per gram kidney. Each point represents the mean ± S.E. of 3-5 determinations.

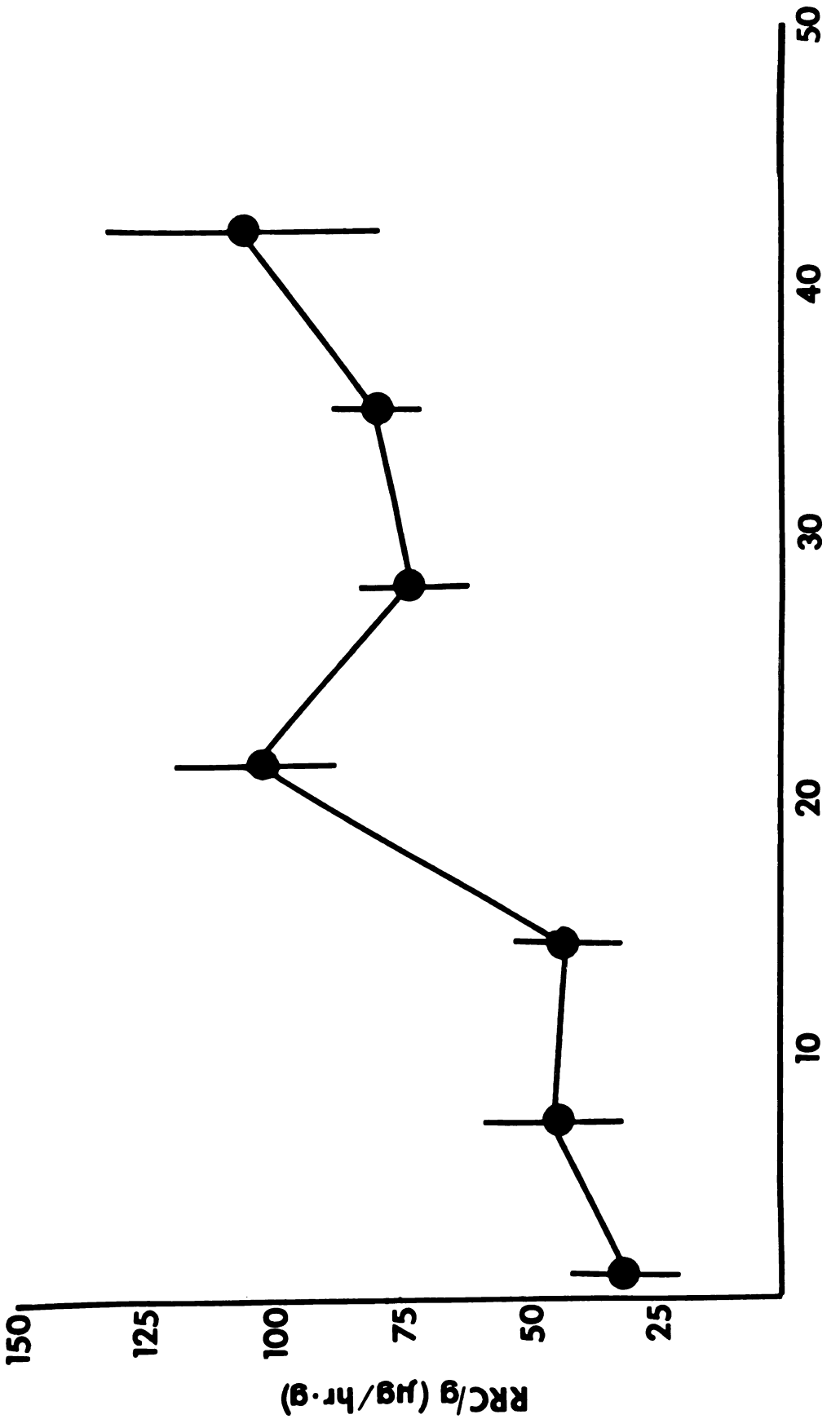


Figure 24

Figure 25. Renal renin content (RRC) in rats at various ages. Each point represents a single determination. Adult animals were defined as having body weights exceeding 200 g.

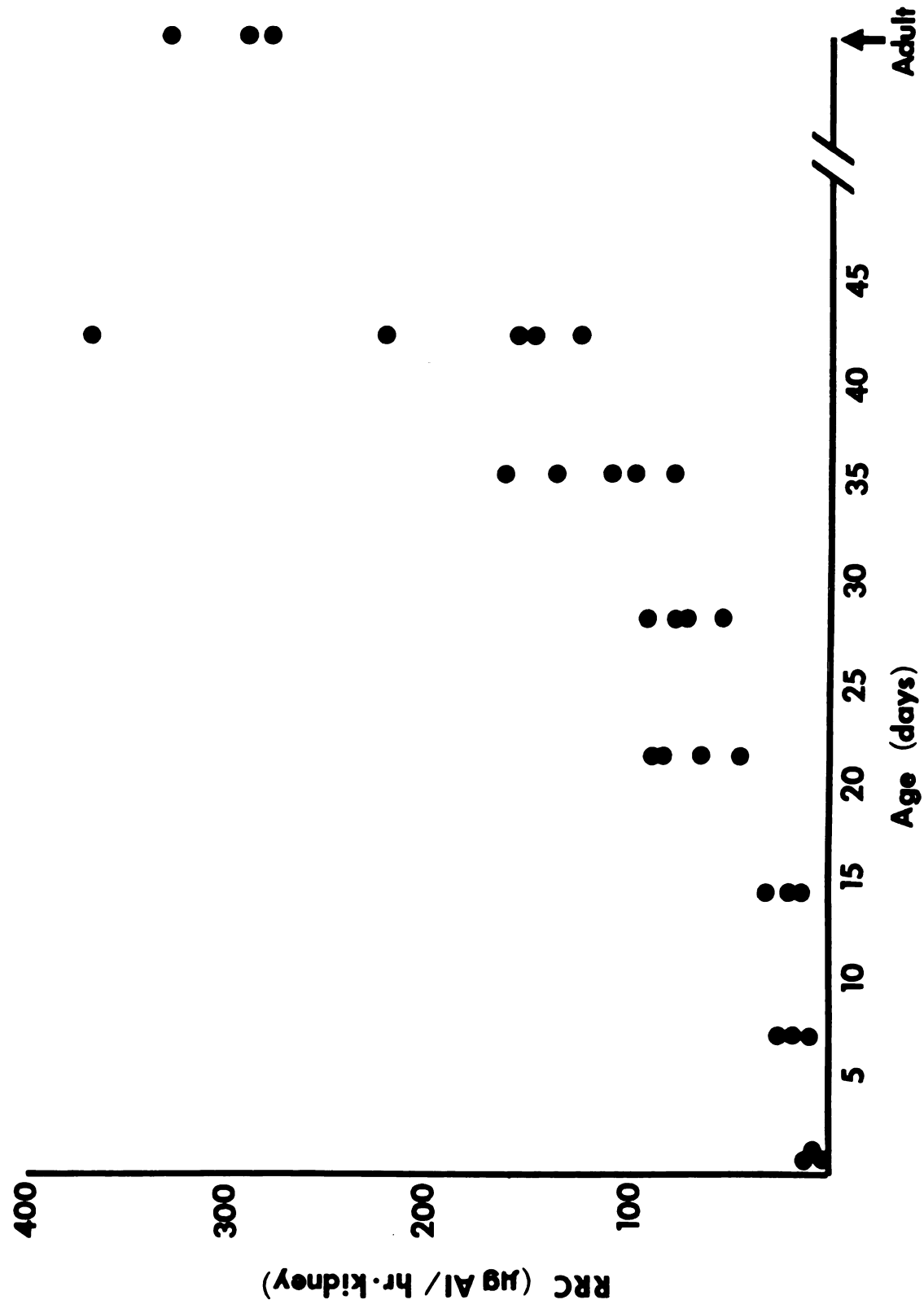

RRC (µg Al/ hr·kidney)
400
300
200
100
5
10
15
20
25
30
35
40
45
Age (days)
Adult

Figure 25

G. Changes in Plasma Angiotensin I Concentration During Development

The concentration of angiotensin I in plasma of newborn rats was highly variable (Figure 26). Plasma AI concentration in rats less than 3-weeks old tended to be slightly greater than in older rats. Although no profound changes were evident, AI tended to increase only slightly from 3- to 6-weeks and decrease from 60- to 80-days. These changes, however, were not statistically significant. The concentration of AI in adult rat plasma was approximately 0.5 ng/ml.

H. Maturation of Pulmonary Angiotensin-Converting Enzyme (ACE) in Utero

1. Fetal ACE

Fetal lung weight increased rapidly from day 18 of gestation to term (Figure 27). The rate of lung growth approached 0.05 g/day during the last 3 days in utero. Lung weight of newborn rats less than 24 hours old was not significantly different from that of near-term fetuses (approximately 0.12 g).

Pulmonary ACE activity also increased rapidly during the last 10% of gestation (Figure 28). ACE activity was first detectable in fetal rat lungs on day 18 of gestation (term = 21 days). It was not possible to measure converting enzyme activity in younger fetuses using the spectrophotometric assay employed in these experiments. Converting enzyme activity of the 20,000 x g supernatant fraction of fetal lung homogenates doubled between day 18 and day 20 of gestation. Pulmonary ACE activity 1-day after birth was approximately 2-fold that of near-term fetuses (2.9 nmol/min·mg versus 1.2 nmol/min·mg protein, respectively). This increase in ACE activity could not be attributed

Figure 26. Concentration of angiotensin I (AI) in plasma of developing rats. Littermates less than 3 weeks of age were pooled to obtain sufficient plasma for a single determination. Each point represents mean ± S.E. of at least 3 determinations.

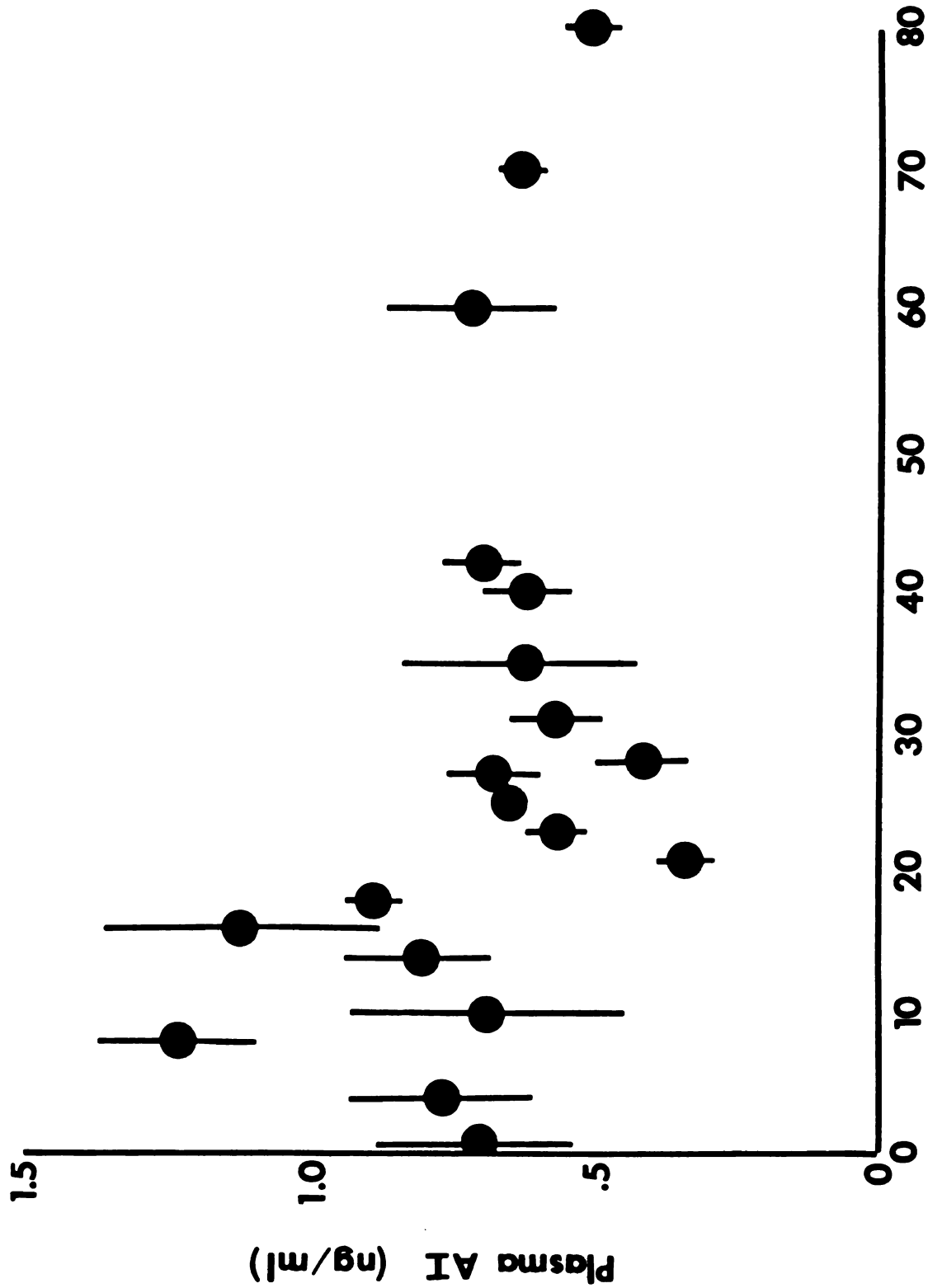


Figure 26

Figure 27. Fetal lung wet weight at various gestational ages. Littermates were pooled for each measurement. Each point represents mean ± S.E. of 4 determinations. Lung weight of one-day-old rats is plotted to the right of the vertical dashed line representing the day of birth.

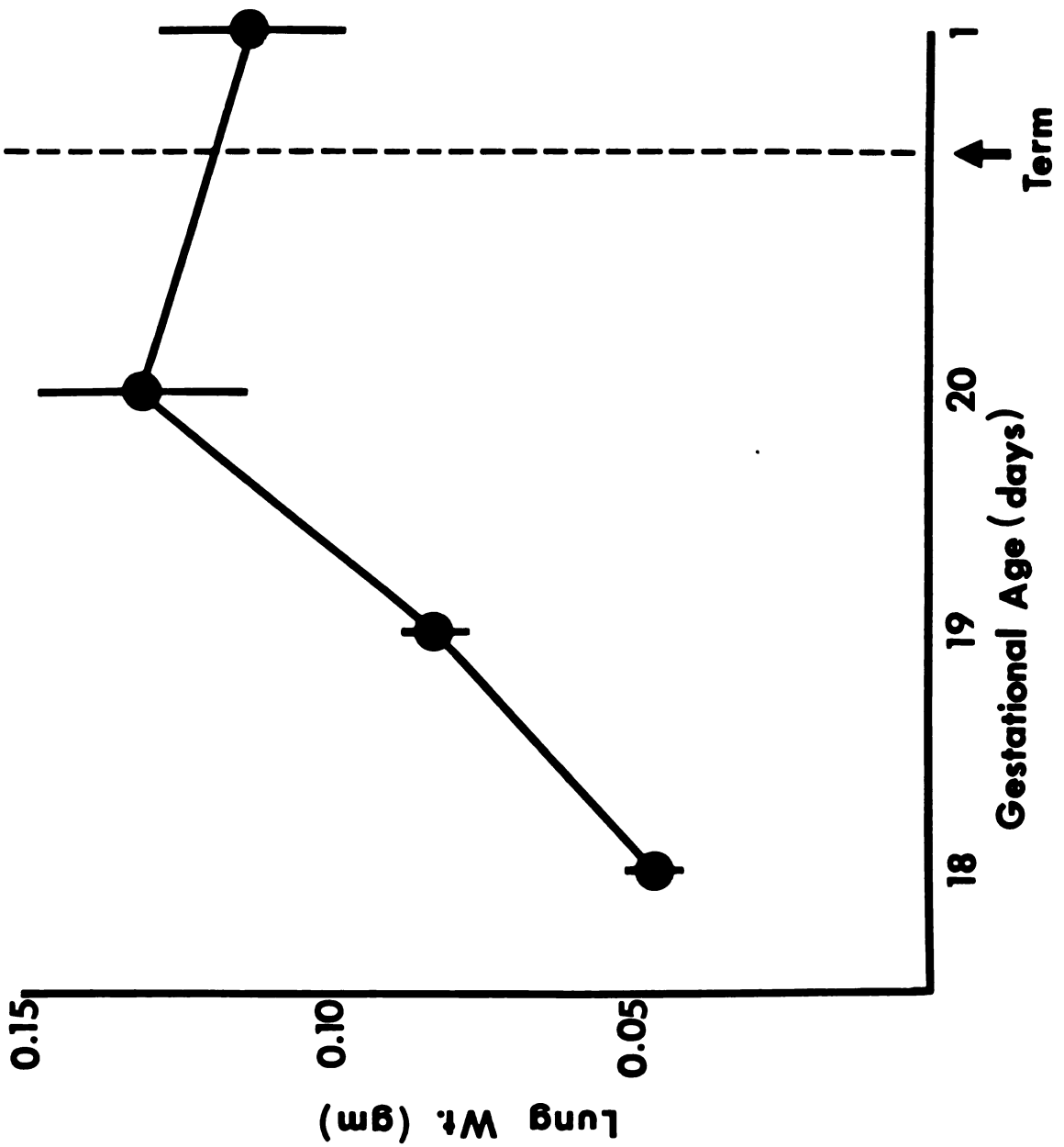


Figure 27

Figure 28. Angiotensin-converting enzyme (ACE) activity in fetal rat lungs at various gestational ages. Littermates were pooled in order to obtain sufficient enzyme for each determination. Data points represent mean ± S.E. of 4 enzyme preparations. Points without error bars indicate the S.E. fell within the radius of the circle. Pulmonary converting enzyme activity of one-day-old rats is plotted to the right of the vertical dashed line representing the day of birth (day 21).

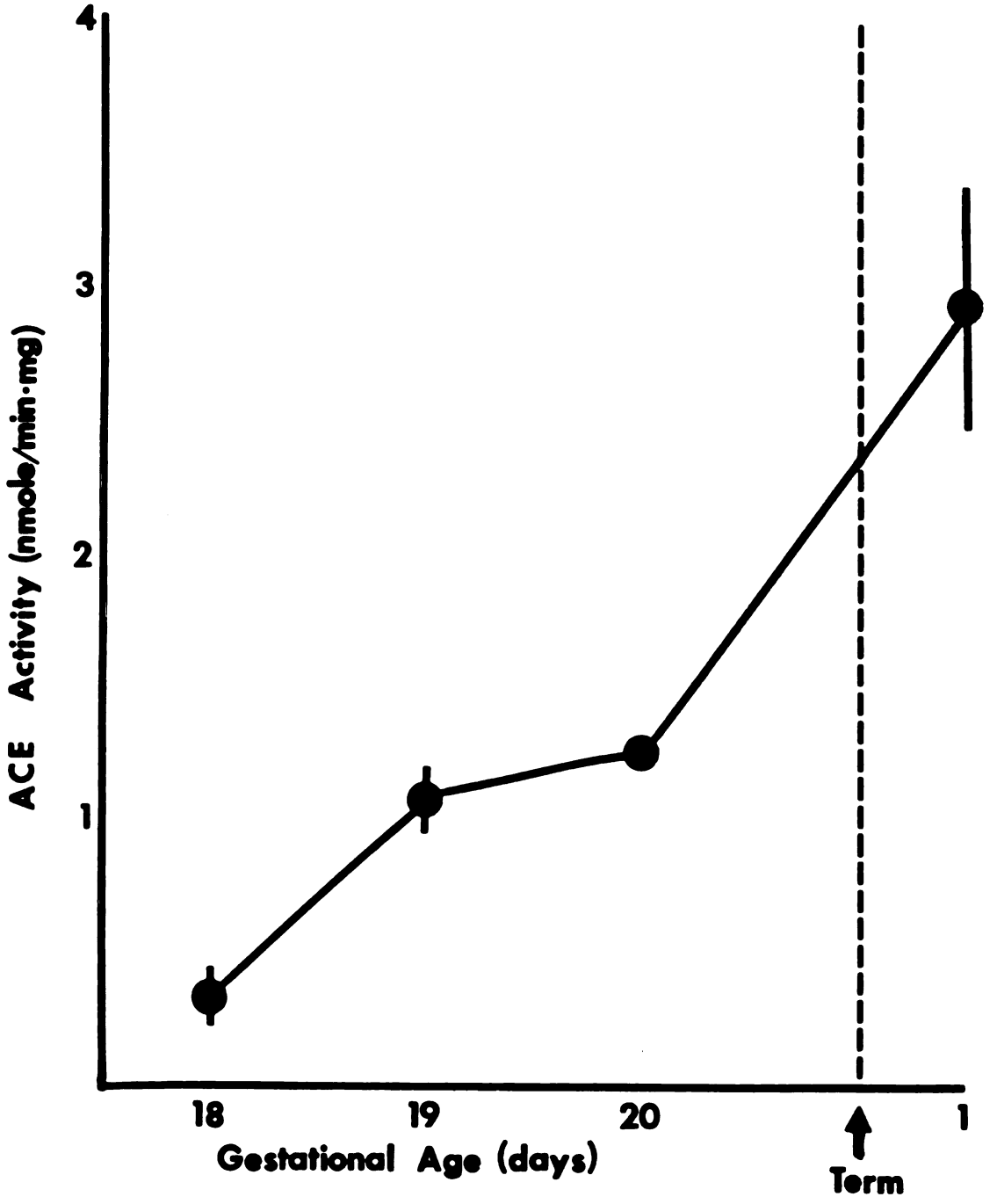

4
3
2
1
ACE Activity (nmole/min·mg)
18
19
20
1
Gestational Age (days)
Term

Figure 28

to differences in protein concentration (63 mg/g and 42 mg/g, respectively).

Spontaneous ventilation of near-term fetal rat lungs (day 19 of gestation) with room air did not affect lung wet weight or protein concentration (Table 6). ACE activity of ventilated lungs was not significantly different from non-ventilated lungs.

2. Kinetic description of fetal lung ACE

Incubation of fetal lung ACE with increasing concentrations of HHL resulted in progressively greater rates of generation of hippuric acid (Figure 29). The curve describing this substrate-dependence of ACE was hyperbolic and did not appear to approach saturation at concentrations of HHL less than 5.0 mM. Rearranging the data by the method of Eadie-Hofstee generated a straight line for the fetal enzyme (Figure 30). A plot of similar data for adult rat lung ACE resulted in a line parallel to that of the fetal enzyme. The points of intersection on the ordinate indicate a 4-fold greater Vmax of the adult enzyme compared to fetal ACE, whereas the similar slopes of the lines reflect similar affinities of the enzymes for HHL. No significant differences existed between the Km of fetal ACE and that of the adult.

I. Postnatal Maturation of Pulmonary Angiotensin-Converting Enzyme Activity in Cell-Free Preparations

The postnatal maturation of pulmonary ACE activity in the 600 x g supernatant fraction of lung homogenates proceeded in a linear fashion from 1-week of age through 6-weeks (Figure 31). Converting enzyme activity increased at a rate of approximately 0.12 μmol hippuric

TABLE 6

Effect of Room Air Breathing on Pulmonary Angiotensin-Converting Enzyme Activity of Near-Term Fetal Rats[a]

	Non-Ventilated[b]	Ventilated[c]
Lung Wt (gm)	0.14±0.01	0.14±0.01
Protein (mg/gm lung)	35.14±1.72	34.54±1.82
ACE Activity (nmoles hippuric acid/min·mg protein)	1.20±0.23	1.65±0.45

[a]Values represent mean ± S.E. of 4 determinations. Each litter of fetuses represented one determination.

[b]Near-term fetal rats were decapitated in utero and ventilation of the lungs prevented prior to enzyme assay.

[c]Fetuses were delivered by caesarian section and allowed to breathe room air for 30 minutes prior to decapitation and enzyme assay.

Figure 29. Substrate-dependence of angiotensin-converting enzyme of fetal (day 19 of gestation) rat lung. Each point represents mean activity of 3 enzyme preparations. Incubation was for 30 minutes at 37°C in the presence of varying concentrations of HHL.

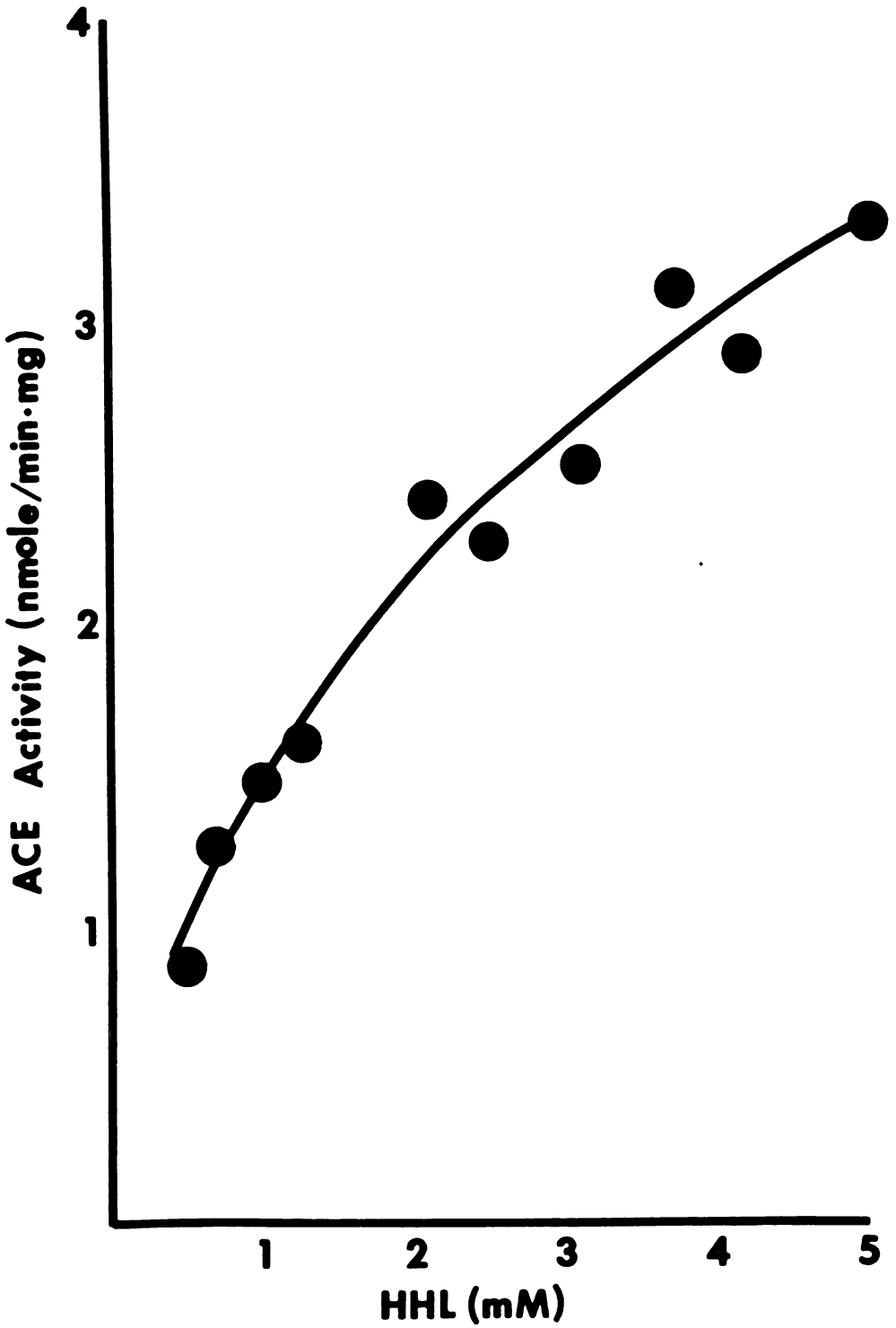


Figure 29

Figure 30. Eadie-Hofstee plot of fetal (day 19 of gestation) and adult rat lung converting enzyme activity. Each point represents mean of 3 determinations. ACE activity (V) is expressed as nmoles hippuric acid/min·mg protein and S depicts the concentration of HHL (mM) present in the incubation medium.

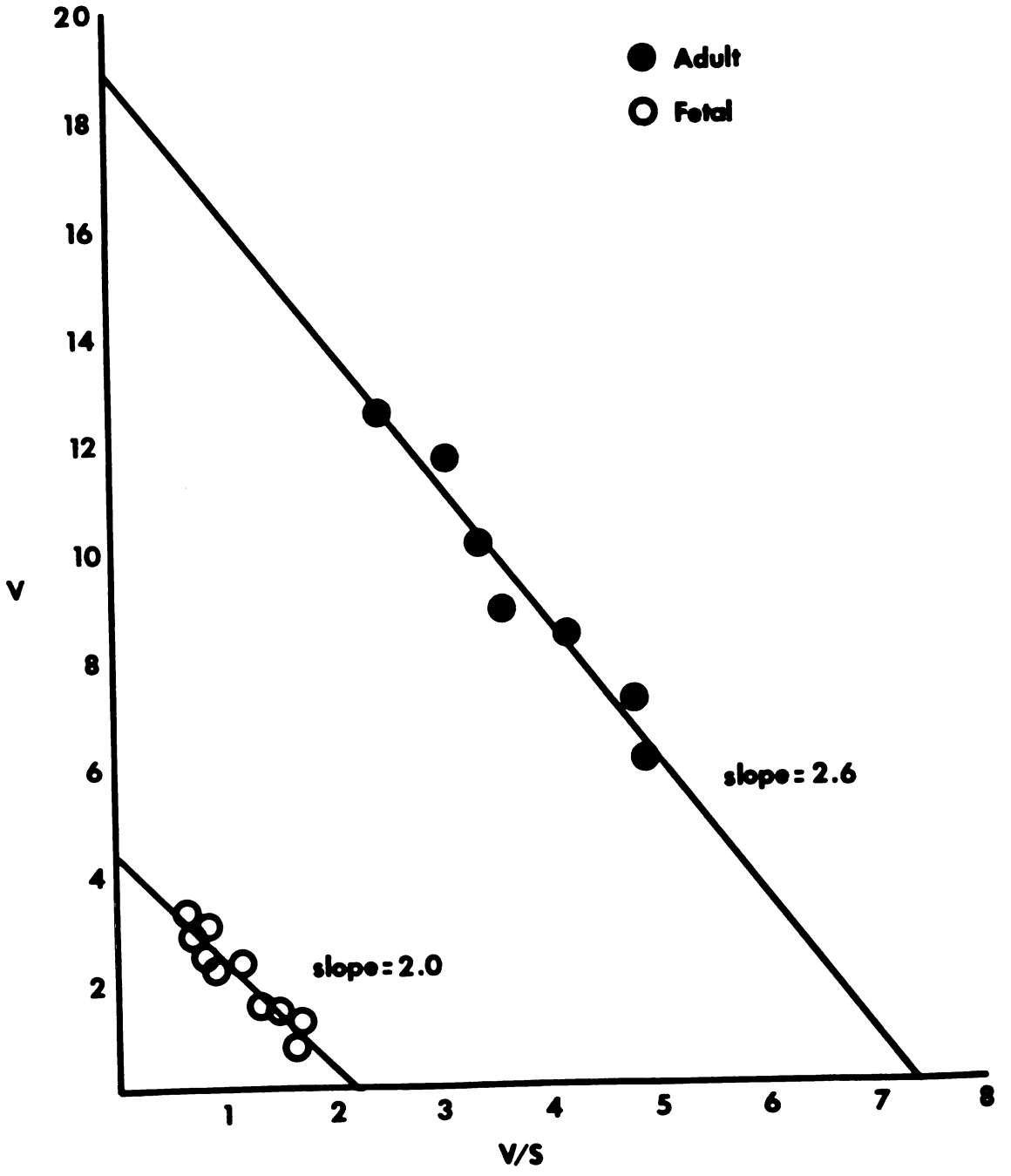

20
18
16
14
12
10
8
6
4
2
V
1
2
3
4
5
6
7
8
V/S
Adult
Fetal
slope = 2.6
slope = 2.0

Figure 30

Figure 31. Postnatal changes in pulmonary angiotensin-converting enzyme (ACE). Converting enzyme activity was expressed as the rate of production of hippuric acid from the hydrolysis of HHL (5.0 mM). Each point represents the mean ± S.E. of 4 determinations. Adult rats weighed 200-250 g.

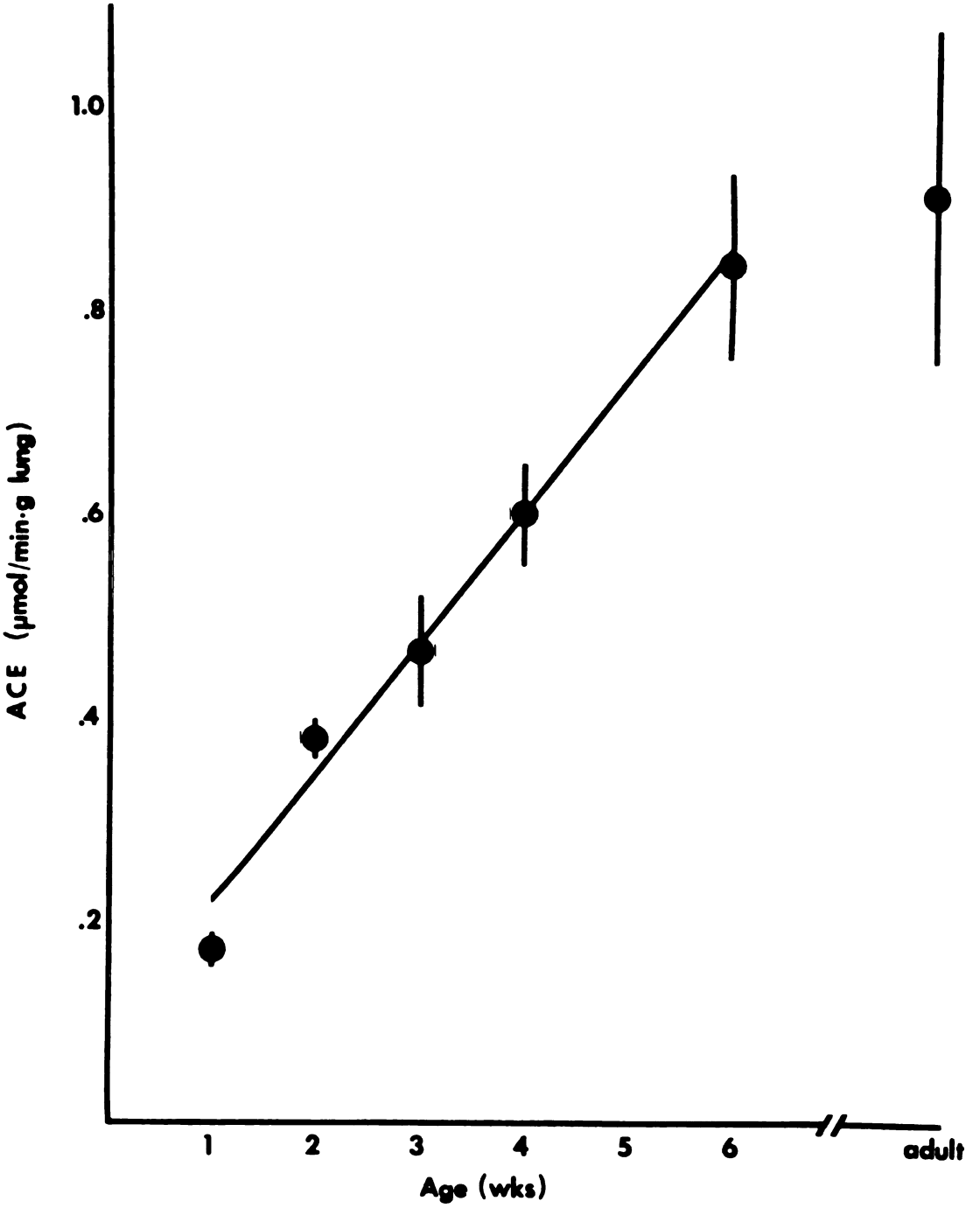


Figure 31

acid/min·g each week during this period. The activity of ACE in the 600 x g supernatant fraction of lungs from 6-week old rats was not different from that of adults (200-250 g).

A significant correlation existed between the postnatal development of pulmonary ACE activity and lung weight between 1-week of age and adult (Figure 32). The rate of increase in ACE activity was approximately 13 units/mg protein per gm wet lung wt (unit = μmole hippuric acid/min). Protein concentration did not change significantly with age (60-75 mg/g lung).

J. Age-Related Differences in Angiotensin I Metabolism by Isolated, Perfused Lungs

1. Disposition of AI in perfused lungs

A steady-state concentration of blue dextran in the effluent medium was achieved during the first 20 seconds of perfusion of lungs from adult rats (Figure 33). When ^{125}I-angiotensin I was coperfused with blue dextran, the radiolabel appeared simultaneously and quantitatively with the vascular marker in the effluent medium. Both the radioactivity and the dye reached concentrations in the effluent medium similar to that in the perfusate. When nonradioactive AI was perfused through similar lungs, only 40% of the perfused concentration of the peptide appeared in the effluent medium during the steady-state period, indicating that 60% of the AI was cleared from the circulation during a single transpulmonary passage. This removal of AI was attributed to the pulmonary circulation since greater than 95% of the perfused AI was recovered in the effluent medium when no lung was present in the chamber. The washout of AI from perfused lungs paralleled that for blue dextran (Figure 33).

Figure 32. Correlation of angiotensin-converting enzyme (ACE) activity with lung wet weight. Converting enzyme activity was estimated during incubation of lung homogenates with 5.0 mM HHL and expressed on the basis of lung protein. Each point represents a single determination. Data were fit to the best line calculated by linear regression, the method of least squares (Sokal and Rohlf, 1969).

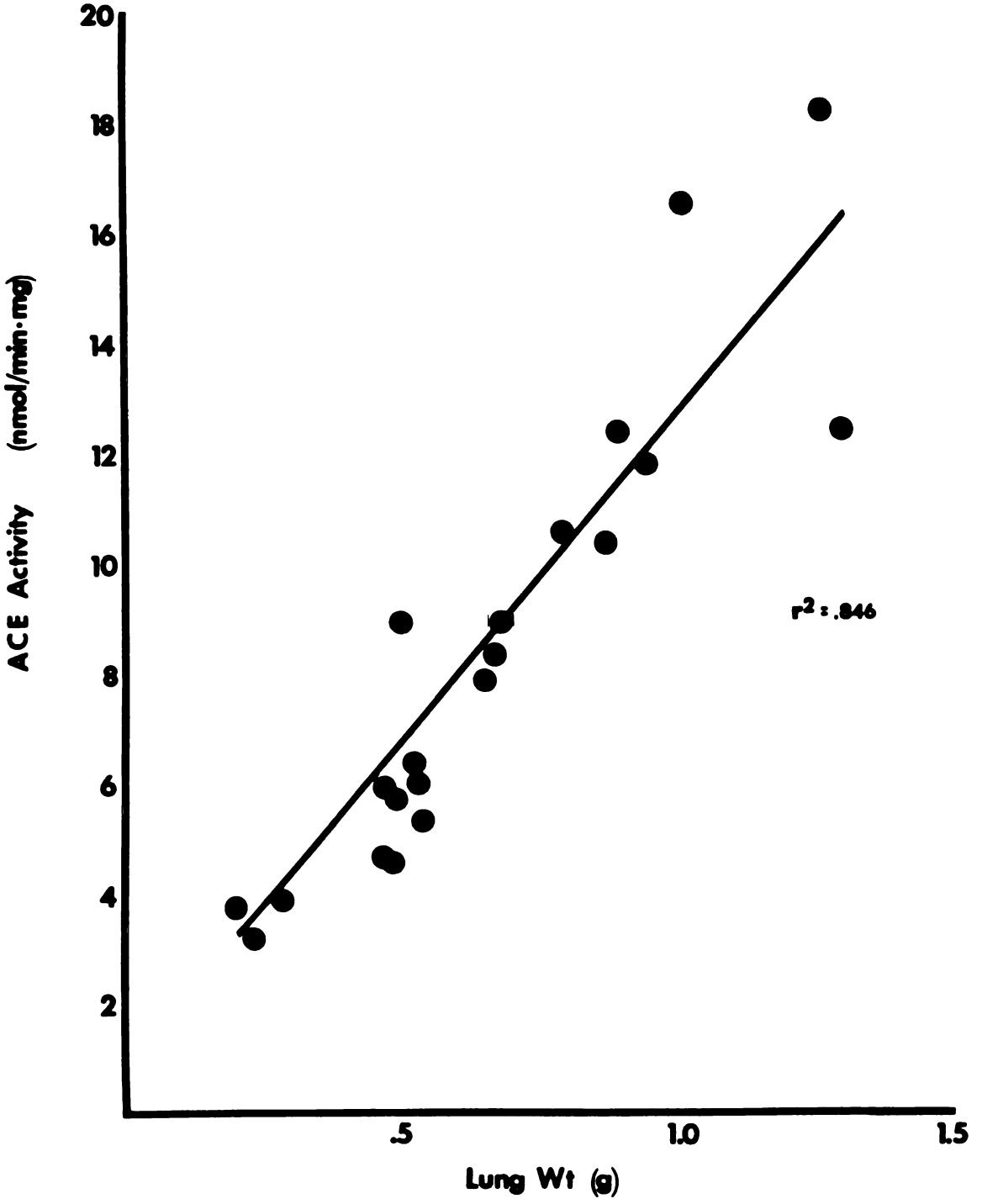

20
18
16
14
12
10
8
6
4
2
ACE Activity (nmol/min·mg)
$r^2 = .846$
.5
1.0
1.5
Lung Wt (g)

Figure 32

Figure 33. Time-course for the appearance of angiotensin and blue dextran in effluent medium from isolated, perfused lungs. Lungs from adult rats were perfused at 5.5 ml/min. Effluent concentrations are expressed as the fraction of the inflow concentration of perfused substance. Radiolabelled angiotensin was employed as a measurement of total angiotensin. Each point represents mean ± S.E. of 3 determinations. Vertical stippled line represents beginning of washout period and open symbols reflect the concentration of AI and blue dextran washed from the lung.

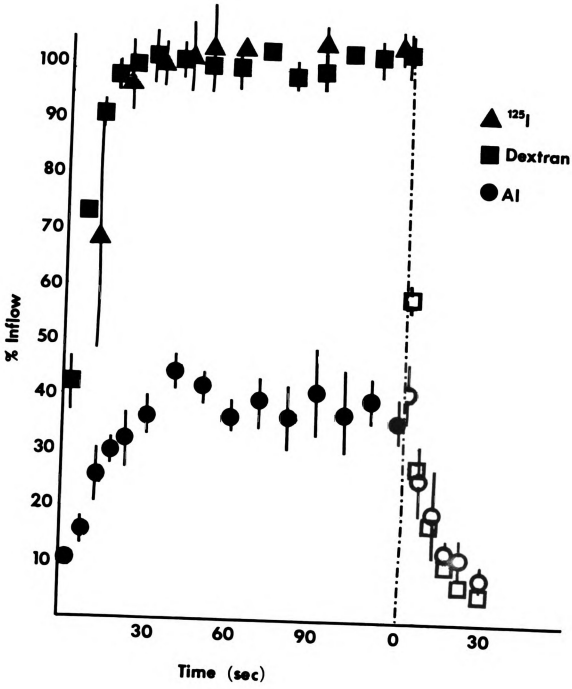

100
90
80
70
60
50
40
30
20
10
% Inflow
30
60
90
0
30
Time (sec)
125I
Dextran
AI

Figure 33

2. Comparison of fractional removal of AI by intact, developing lungs

Lung dry weight was 6-fold greater in adults than in rats 1-week of age (Table 7). Lungs from 7-day old rats weighed approximately 200 mg and possessed a slightly greater percent water content than did adult lungs (85.4% versus 84.0%). Adjustment of the rate of perfusion was such that no difference in the relative flow existed between adult and newborn lungs (27-30 ml/min·dry lung wt). The flow of the 95% O_2-5% CO_2 gas mixture was adjusted for each lung such as to maximize the tidal volume. Tidal volume ranged from 0.1-0.2 cc H_2O in newborns to 0.4-0.6 cc H_2O in adult rats. Under these conditions, lungs from adult rats cleared 51% of the perfused AI during a single pass whereas lungs from 7-day old rats were capable of removing only 35% of the peptide. No significant difference existed between the fraction of AI cleared by lungs from 21-day old rats compared to adults. The absolute rate of removal of AI (ng/min) by intact lungs from adult rats was significantly greater than that removed by lungs from 1- or 3-week old rats. This age-related difference in the rate of AI clearance was attributed primarily to the differences in the absolute rates (ml/min) of perfusion of these lungs.

3. Characterization of perfusion conditions

In the isolated, perfused lung model, accumulation of fluid by the lung is invariably associated with an increase in perfusion pressure during the course of the perfusion. No such changes in inflow pressure were observed during perfusion of the lungs. Similarly, the percent water content of lungs following perfusion was not different from that of non-perfused lungs (approximately 85%). A

TABLE 7

Clearance of Angiotensin I (AI) by Isolated, Perfused Rat Lungs[a]

Age	Lung Dry Wt (g)	% Lung Water	Flow Rate (ml/min·g Dry Wt)	AI Removal % Removal[b]	AI Removal (ng/min)
Adult	0.210±0.008	84.0±0.6	26.9±1.0	51.4±4.3	2.83±0.24
21 days	0.066±0.007[c]	85.1±0.4	29.6±1.0	47.5±3.0	1.19±0.08[c]
7 days	0.035±0.001[c]	85.4±0.3[c]	28.7±0.8	34.8±2.7[c]	0.35±0.03[c]

[a]Lungs were perfused in a single pass system as described in text. Values represent mean ± S.E. of 12 determinations.

[b]Removal was calculated from the fraction of perfused AI (1 ng/ml) appearing in the effluent medium.

[c]Significantly different from adult ($p<.05$).

disadvantage of the design of the perfusion chamber was that the absolute perfusion pressure was not quantifiable because the cannula in the pulmonary artery tended to twist, resulting in spuriously high estimates of perfusion pressure. However, since the position of the cannula did not change throughout the perfusion, it was valid to monitor changes in perfusion pressure during the experiment as evidence for the accumulation of lung fluids.

To evaluate the method of adjusting flow to lung mass, lungs from adult and 3-week old rats were perfused at the normalized flow rate with blue dextran and the volume of pulmonary vessels being perfused was estimated from the dilution of dye in the pulmonary circulation. No blue dye was detectable in the acidified protein precipitate of lung homogenates indicating complete recovery of the dye in the supernate with negligible protein binding of the dextran molecule. The absorbance at 700 nm was subtracted from that at 630 nm to normalize for the nonspecific absorbance due contaminating substances in the supernatant. The validity of this method was proven by adding known amounts of blue dextran to lung homogenates and correlating the difference in absorbance to the concentration of dye. Lungs from adult rats weighed approximately 2.5 times that of newborn lungs (Table 8). The perfused vascular volume of adult lungs was twice that of lungs from 21-day old rats, however, no differences existed in the relative volume of the pulmonary vasculature being perfused (0.14-0.17 ml/g, respectively). This similarity in the ratio of perfused vascular volume to lung weight indicates that normalization of flow was such that age-related differences in the fraction of blood vesssels being perfused were minimized.

TABLE 8

Vascular Volume of Perfused Lungs[a]

Age	Lung wt (g)	Vascular Volume[b] (ml)	Volume/wt (ml/g)
Adult	1.48±0.10	0.27±0.06	0.14±0.04
21-day	0.55±0.02[c]	0.13±0.02[c]	0.17±0.01

[a]Values represent mean ± S.E. of 6-10 determinations.

[b]Vascular volume which was perfused was determined using blue dextran as a vascular marker.

[c]Significantly different from adult ($p<.05$).

The specificity for the clearance of AI to result from the activity of converting enzyme was tested by coperfusing lungs from adult rats with AI and a specific inhibitor of ACE, SQ20,881. The fractional removal of AI by perfused lungs was progressively reduced by increasing concentrations of the inhibitor (Table 9). The clearance of AI was inhibited to 21% that of controls during perfusion with 10^{-6}M SQ20,881.

The fraction of perfused AI removed during a single passage through lungs of adult rats did not change when the concentration of AI in the inflow medium was varied over a 100,000-fold range (Table 10). Intact lungs removed 40% of the perfused AI when the concentration of the peptide in the perfusion medium was 10^{-9}M. This fractional removal of AI is not different from that when AI was perfused at 10^{-4}M.

The concentration of AI in the effluent medium did not change when the rate of substrate delivery was varied by increasing flow (Figure 34). Lungs perfused with 1 ng/ml AI at flows ranging from 1-10 ml/min removed similar fractions of the perfused peptide during a single pass (approximately 45%).

4. Correlation of AI removal to ACE content of developing lung

The concentration of protein (mg/g) in lungs was not different between adults and rats 7- and 21-days of age (Table 11). Converting enzyme activity, however, was 2-fold greater in the 600 x g supernatant fraction of lungs from adult rats compared to that of the two younger age groups. This age-related difference was more pronounced when ACE activity was expressed on the basis of lung weight rather than per mg tissue protein. No difference was observed for the

TABLE 9

Effect of SQ20,881 on the Removal of Angiotensin I (AI) by Isolated, Perfused Rat Lungs[a]

SQ20,881[b] mole/L	% AI Removal[c]	% Inhibition
0.00	57.6±4.2	---
10^{-9}	53.0±3.0	14:2±4.3
10^{-6}	11.0±3.4	79.2±7.0
10^{-3}	6.3±2.1	87.9±4.8

[a] Adult rat lungs were perfused as described in text. Values represent mean ± S.E. of 8 determinations.

[b] SQ20,881 was co-perfused with angiotensin I (1.0 ng/ml) through adult rat lungs.

[c] Removal was calculated from the fraction of perfused AI appearing in the effluent medium.

TABLE 10

Substrate-Dependence of AI Removal by Isolated, Perfused Lungs[a]

Inflow AI (g AI/ml perfusate)	% Removal[b]
10^{-9}	39.9±9.1
10^{-6}	33.1±5.1
10^{-4}	39.5±3.2

[a] Adult rat lungs were perfused with Krebs-bicarbonate buffer in a single pass system as described in text.

[b] Removal was calculated from the fraction of perfused AI appearing in the effluent medium. Values represent mean ± S.E. of 4 determinations.

Figure 34. Flow-dependence of AI metabolism by isolated, perfused lungs. The rate of perfusion of each lung was varied between 1.0 and 10 ml/min and the concentration of AI in the effluent medium quantified by radioimmunoassay. Each point represents a single determination.

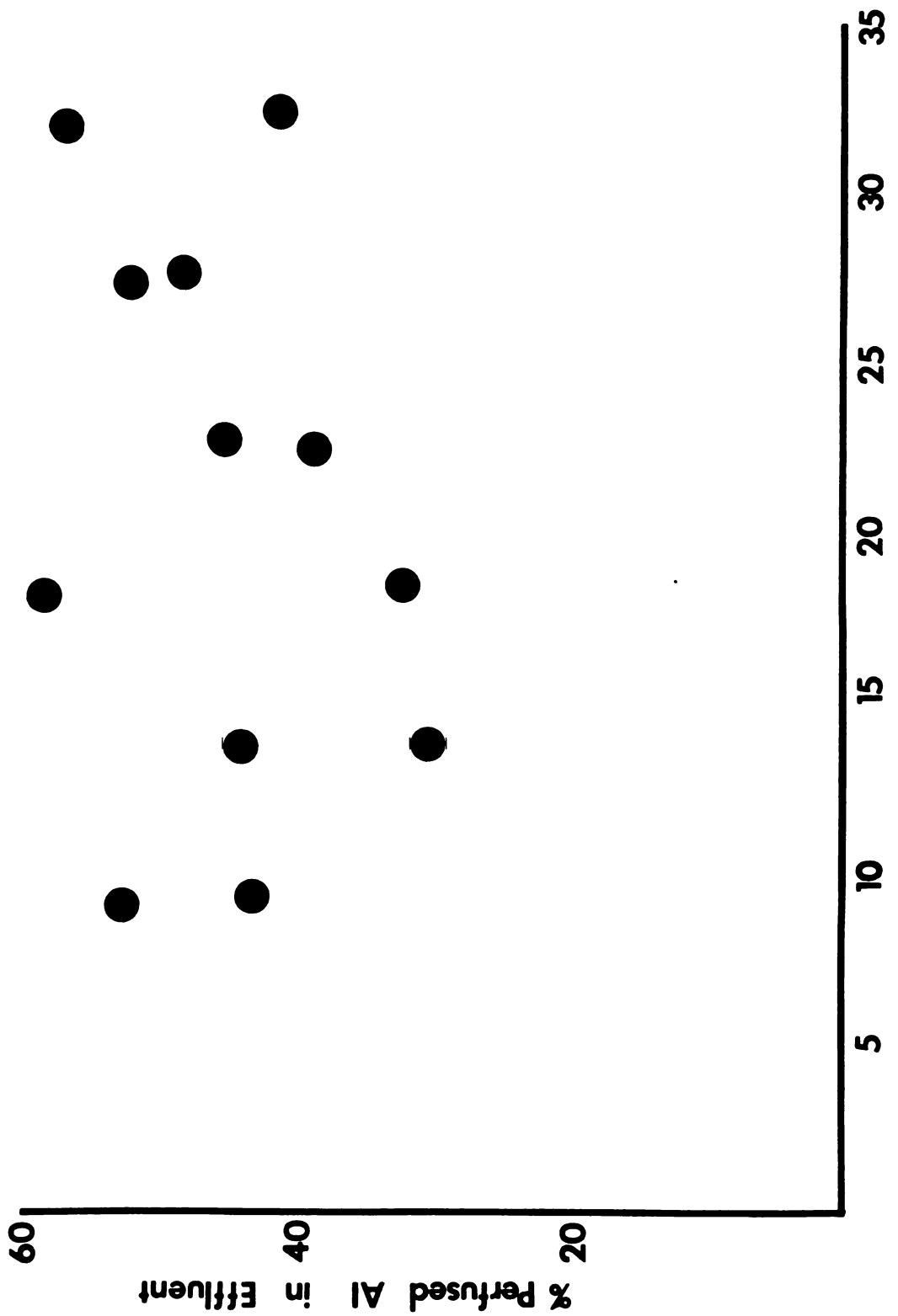


Figure 34

TABLE 11

Metabolism of Hippuryl-L-histidyl-L-leucine (HHL) by 600 x g Supernatant of Rat Lung Homogenates[a]

Age	Protein (mg/g)	nmol/min·mg protein[b]	nmol/min·g lung[b]
Adult	65.7±3.6	11.6±0.9	1129±110
21 days	67.6±3.0	5.7±0.9[c]	392± 75[c]
7 days	55.6±8.4	5.8±0.2[c]	301± 7[c]

[a]Values represent mean ± S.E. of 6-10 determinations.

[b]Converting enzyme activity was estimated from the rate of production of hippuric acid during incubation of lung homogenates with 5 mM HHL.

[c]Significantly different from adult ($p<.05$).

activity of ACE in the 600 x g supernatant of lungs from 7-day old rats compared to that from rats 3-weeks of age. The recovery of converting enzyme activity in the 600 x g supernatant fraction did not change with age (approximately 20-25% total tissue ACE activity) indicating no age-related difference in the solubilization of the enzyme from the membrane-bound complex.

DISCUSSION

All of the recognized biological functions of the renin-angiotensin system are exerted directly through angiotensin II or possibly indirectly through a few peptide fragments of AII. Therefore, regulation of the systemic arterial concentration of AII is of primary importance in understanding the function of this pressor system. Changes in angiotensinogen synthesis, renin secretion, angiotensin-converting enzyme activity, and AII inactivation rates must be integrated in some manner so as to regulate the steady-state concentration of AII in plasma under closely defined limits. Similarly, changes in the stoichiometry of the renin-angiotensin system which occur throughout maturation may predispose this system to age-related differences in the relative contributions played by each of its constituent enzymes in regulating plasma AII concentrations.

A. Angiotensin II

The changing stoichiometry of the renin-angiotensin system during development is reflected by age-related differences in the steady-state concentration of AII in circulating plasma (Figure 3). Blood samples collected from unanesthetized rats were used so as to avoid anesthesia-induced stimulation of renin secretion (Johnson and Malvin, 1975). The maturational changes in the concentration of AII, however, may not be selective to the octapeptide since most antibodies to AII

crossreact with the major peptide fragments of AII (Cain et al., 1969a,b,1970; Dietrich and Frishknecht, 1968a,b; Vallotton et al., 1967). Therefore, the development of AII described by radioimmunoassay may reflect changing concentrations of several peptide fragments in plasma. However, since AII antibodies crossreact only with the major fragments of AII (penta-, hexa-, and heptapeptides) and do not bind to most peptides with amino acid substitutions, it can be concluded that the measured peptides are AII or fragments generated by one or two consecutive amino acid cleavages. Inasmuch as the hydrolysis of AII by amino- and carboxypeptidases occurs very rapidly (Boyd et al., 1969; Cain et al., 1970), the immunologically active substances most likely represent AII and its fragments generated within 30 seconds prior to collection of the blood sample. Blood was collected into vials containing inhibitors of AII degradation to minimize peptide hydrolysis in vitro. In view of the biological activity of several of the larger peptide fragments of AII (Bravo et al., 1976; Bumpus, 1977; Freeman et al., 1975, 1976; Goodfriend and Peach, 1975; Steele et al., 1976), the immunologically active material may represent peptides possessing considerable pressor activity similar to that of AII. Therefore, it appears that the concentration of AII determined by radioimmunoassay provides valid comparisons of qualitative changes in the concentration of biologically active end-products of the renin-angiotensin system during development.

The concentration of AII increased following birth (Figure 3). Pipkin and coworkers (1974a) observed a similar age-related increase in plasma AII concentrations in sheep using bioassay to quantify AII.

Plasma AII concentration increased following birth reaching maximum values (839±96 pg/ml) in lambs 8 hr old. Inasmuch as the concentration of AII in plasma from similarly aged lambs delivered by caesarian section was not elevated above that of adult ewes (111±15 pg/ml), these investigators attributed the postnatal increase in plasma AII concentration to the trauma of spontaneous delivery (Pipkin et al., 1974a). However, this same group of investigators (Pipkin et al., 1971) found that AII concentration in plasma from rabbits was not maximum until 10-14 days postpartum (approximately 1.6 ng/ml). This transient increase suggests that the maturation of factors associated with the production and/or degradation of angiotensin were involved in mediating the steady-state concentration of AII in plasma of neonates. Since there was no evidence for a slower rate of inactivation of AII, it was concluded that the progressive increase in plasma AII concentrations was due to a greater rate of formation of the peptide (Pipkin et al., 1971). Our finding that in rats, maximum plasma AII concentrations are not attained until approximately 5-weeks following birth (0.7-1.0 ng/ml) supports the proposal that the postnatal increase in plasma AII is unrelated to the various processes associated with parturition.

Several investigators have reported that the concentration of AII in plasma of newborns is greater than that of adults (Lumbers and Reid, 1977; Pipkin and Smales, 1977; Pipkin et al., 1971, 1974a). In humans, there is no evidence for an immediate postnatal increase in the concentration of AII in plasma (Lumbers and Reid, 1977; Pipkin and Smales, 1977; Pipkin and Symonds, 1977; Pipkin et al., 1976). A

decrease in plasma AII occurs after 8 hr in sheep and between 10 and 24 days in rabbits. In rats, plasma AII concentration decreases between 6- and 8-weeks postpartum (Figure 3). This species difference in the age at which plasma AII is maximum may be due to the relative immaturity of rats and rabbits at birth (Tanner, 1962). In contrast to rats and rabbits, the immediate postnatal increase in plasma AII of sheep is most likely due to the stimulation of renin secretion induced by blood loss during parturition (Pipkin et al., 1974a).

B. Renin-Substrate Reaction

The postnatal changes in the steady-state concentration of AII in plasma may result from age-related differences in either the rate of formation of AII and/or changes in the rate of degradation of this peptide. Since AII availability in adults is regulated primarily by its rate of generation (Hodge et al., 1967; Sambhi and Barrett, 1968), age-related differences in the rate of formation of AII are the most likely candidates responsible for the changes in plasma AII concentrations during development. The renin-substrate reaction has classically been assumed to be the rate-limiting step in the generation of AII in adults. Accordingly, changes in plasma renin activity have been correlated with proportional changes in circulating AII (Barrett et al., 1977; Haber et al., 1969; Lijnen et al., 1978; Sealey et al., 1972; Soveri and Fyhrquist, 1977). Plasma renin activity of rats increased during the first two weeks postpartum (Figure 4). Solomon and coworkers (1977) reported comparable changes in PRA of Charles-Rivers rats following birth, with the maximum PRA occurring between 1 and 3 weeks postpartum. Similar postnatal increases in PRA

have been reported to occur during the first 3 days in humans (Godard et al., 1976; Hayduk et al., 1972; Kotchen et al., 1972). The greater PRA of newborn rats compared to adults agrees well with that reported for all other species examined (Dillon and Ryness, 1975; Granger et al., 1971; Mott, 1975; Pipkin and Smales, 1977; Pohlova et al., 1973; Sassard et al., 1975). Renin activity (PRA) is a measure of the renin-substrate reaction in vivo, the rate of which is dependent on both the amount of renin present and the concentration of angiotensinogen (Gould et al., 1966; Poulson, 1971a; Reid et al., 1973; Skinner, 1967). Therefore, the enhanced rate of generation of AI in newborn plasma may reflect a greater concentration of renin and/or substrate in plasma of young animals.

The renin-substrate reaction in rat plasma was found to conform closely with that predicted by classical Michaelis-Menten reaction kinetics (Figure 21). Similar results were obtained using plasma from hogs and dogs wherein the observed velocity of the renin-substrate reaction was superimposed on the theoretical curve derived from the first-order velocity equation (Figure 17). Although the Michaelis-Menten equation appears to accurately predict the velocity of the renin-substrate reaction in all species of plasma, the calculated Km values are not quantitatively exact. This is due to the fact that the small amounts of endogenous angiotensinogen in the renin sample were not accounted for. Since the concentration of angiotensinogen was not quantified, it was necessary to analyze the data assuming endogenous substrate to be negligible. Handling the data in this fashion shifts the ordinate of the substrate-dependence curves an undetermined

distance to the right and thereby yields estimates of Km less than the actual values. The error introduced by not accounting for the endogenous angiotensinogen is not certain and varies with the different enzyme preparations.

Although it was not possible to quantify the endogenous angiotensinogen concentration, qualitative estimates of the degree of error can be made by examining the closeness of fit of the data to the theoretical velocity curves and double-reciprocal plots. Plotted in double-reciprocal fashion, the kinetic data accurately described a straight line. The largest deviations of the data from linearity occurred at the lower substrate concentrations. This would be expected because of the greater relative contamination by endogenous angiotensinogen at lower exogenous substrate concentrations. However, statistical handling of the data was by a method which emphasizes the velocities at high substrate concentrations rather than points more distant from the origin of the double-reciprocal plots (Wilkinson, 1961). Accordingly, this method of data analysis minimizes the error due to endogenous angiotensinogen. Furthermore, contaminating substrate present in the renin sample would tend to retard the rate at which the reaction velocity approaches zero and thereby deflect the double-reciprocal plot downwards away from the straight line. Since this was not evident in any of the plots of the kinetic data, it was concluded that the concentration of endogenous angiotensinogen was insufficient to affect the kinetic estimates of Km and Vmax. This assumption is supported by the relative sensitivity of the double-reciprocal plot to small errors in substrate concentration (Lineweaver and Burk, 1934).

Finally, the fact that the data were superimposed on the theoretical curve derived by substitution of the estimated Km and Vmax into the Michaelis-Menten equation, provides further evidence that the error in the calculations was negligible. Had the endogenous angiotensinogen concentration been significant, the data would have fallen above and to the left of that predicted by the theoretical curve. Fitting the data by this procedure is a conventional test of the accuracy of the calculated kinetic parameters.

Therefore, although the estimated Km of the renin-substrate reaction is not exact, the error in the calculation appears to be small. Since the intercept of the ordinate in the equation describing the double-reciprocal plot does not contain a substrate term;

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} ,$$

the calculated Vmax is not influenced by residual angiotensinogen in the renin sample. This follows from the fact that Vmax is estimated under conditions of saturating substrate, and thus the small amounts of contaminating endogenous angiotensinogen exerts minimal influence on the total substrate concentration.

The quantitative inaccuracies intrinsic to this method of data analysis are not unique to this investigation. Several other investigators have examined the kinetics of the renin-substrate reaction and neglected to address the problem of contaminating angiotensinogen present in their enzymes preparation (Blaquier et al., 1967; Carvalho et al., 1975; Haas and Goldblatt, 1967; Nasjletti et al., 1971a; Pickens et al., 1965; Poulsen, 1971a; Reid et al., 1973;

Rosenthal et al., 1971; Skinner et al., 1969, 1975b). The close agreement of the data with that predicted by Michaelis-Menten kinetics suggests a similar small error in their estimates of Km and Vmax for the reaction.

The fact that the relative concentration of angiotensinogen in rat and dog plasma was less than that required for 1/2 maximum velocity (Table 2) suggests that the rate of generation of AI in these two species is dependent, at least partially, on the concentration of endogenous angiotensinogen in plasma. Using techniques similar to those employed in these experiments, Reid and associates (1973) found that endogenous angiotensinogen in dog plasma was 83% of the Km for homologous renin (PAC = 650 ng/ml, Km = 775 ng/ml). Poulson (1971a) estimated that angiotensinogen in plasma from salt-depeleted rats was approximately 27% that required for half-maximal velocity of the renin-substrate reaction. These values agree well with those for dog (74%) and rat (19%) reported in Table 2. In contrast, the endogenous concentration of substrate in hog plasma was 7-fold greater than the respective Km suggesting that in vivo, hog renin is saturated with angiotensinogen and thus the rate of production of AI in this species is limited primarily by the amount of renin present in plasma.

A similar substrate-dependence of plasma renin activity has been observed in rats, dogs, hogs, rabbits, sheep, and man. Helmer and Judson (1967) observed that plasma angiotensinogen concentration was elevated 3- to 4-fold in human pregnancy. Similar increases in angiotensinogen were observed in women receiving contraceptive steroids. The elevated concentration of angiotensinogen resulted in

greater rates of generation of AI than would be predicted from PRC, indicating a substrate-induced increase in the specific activity of renin (PRA/PRC) in these patients. Similarly, the elevated plasma angiotensinogen concentration during estrus in rats is accompanied by a decrease in PRC whereas PRA remains unchanged (Nasjletti et al., 1971a). Reid and coworkers (1973) found that the decreased plasma angiotensinogen concentration following adrenalectomy in dogs produced a much greater decrease in PRA than PRC. Conversely, administration of dexamethasone caused a large increase in both plasma angiotensinogen and PRA, however, PRC was not changed. Caloric deprivation in rabbits resulted in reciprocal changes in PRC and angiotensinogen whereas PRA did not change, which conforms with a substrate-dependence of the renin reaction in this species also (Nocenti et al., 1975).

In dogs, the estimated physiological concentration of angiotensinogen is near the Km of renin (Reid et al., 1973). Rosenthal and coworkers (1971) obtained indirect evidence that angiotensinogen concentrations were sufficient to sustain near maximum rates of generation of AI in normal human plasma. This is in contrast to several other reports which describe the endogenous angiotensinogen concentration to be at or below that required for half-maximal velocity of the renin-substrate reaction in humans (Pickens et al., 1965; Skinner et al., 1969, 1972, 1975b). Inasmuch as discordant results are obtained when heterologous angiotensinogen is employed in kinetic determinations (Dahlheim et al., 1970b; Rosenthal et al., 1971; Skinner, 1967; Stockigt et al., 1971), it is imperative that homologous substrate be used in order to obtain data representative of the renin-substrate reaction as it occurs in vivo. The apparent

difference in the reactions using the different species of angiotensinogen are most likely due to the differing affinities of renin for the different substrates (Rosenthal et al., 1971; Skinner et al., 1975b).

The concentration of angiotensinogen reported in Table 2 is relative to that in plasma obtained from bilaterally nephrectomized hogs, dogs, and rats. Since plasma angiotensinogen concentration is increased 5-fold in rats 48 hrs following bilateral nephrectomy (Montague, 1968), the ratio of substrate to Km is an overestimate of that present in normal plasma. Therefore, the limitation of PRA by angiotensinogen in dogs and rats is more profound than that indicated in Table 2. Since both k_{app} and Vmax were calculated using the same sample of renin, the ratio of these values is an index of the relative rate of generation of AI in vivo and may be referred to as the specific activity of renin. The limiting substrate concentration in rat and dog plasma is reflected by the low specific activities of these renins. In constrast, the near substrate saturation in hog plasma explains the high specific activity of hog renin (91% maximum).

Inasmuch as different techniques were used to stimulate renin secretion in dogs, hogs, and rats, it is not appropriate to make comparisons of PRC between species using the values of Vmax reported in Table 1. Similarly, comparison of Km values is not relevant since different preparations of angiotensinogen were employed as homologous substrate for each species.

From Figure 17 it can be seen that saturation of both hog and rat renin is possible using large volumes of unextracted plasma from

nephrectomized animals as homologous angiotensinogen. Substrate-saturation of rat renin is also demonstrated by lack of a change in the rate of generation of AI at high volumes of angiotensinogen (Figure 6) and the correlation of AI production with the volume of renin present in the incubation medium (Figure 7). The conformity of the renin-substrate reaction with zero-order kinetics (Figure 8) is indirect evidence for the saturation of renin with angiotensinogen. In contrast, it was not possible to saturate dog renin with angiotensinogen from nephrectomized dog plasma (Figure 17). This inability to increase substrate concentration sufficiently to saturate the enzyme may be due to the relatively high PRC of this particular preparation of plasma (Table 1). Alternatively, a relative dilution of angiotensinogen in nephrectomized dog plasma could account for these results. This inability to sufficiently raise plasma angiotensinogen concentration in dogs following nephrectomy may explain the high Km of dog renin (Km = 418 μl) compared to that of rats and hogs.

These data suggest that it is relatively difficult to measure PRC in dogs using unextracted plasma as substrate. It may be possible to saturate dog renin using purified preparations of angiotensinogen. However, the data using preparations of angiotensinogen partially purified by ammonium sulfate precipiation (Green and Bumpus, 1954; Nasjletti and Masson, 1971b; Skeggs et al., 1963) suggest that certain methods of concentrating angiotensinogen may alter the kinetics with which the substrate interacts with renin. Partial purification of angiotensinogen reduced the Km 5-fold for rat and 2-fold for dog renin (Figures 11 and 16, respectively). The observation that the rate of generation of AI when rat renin was incubated with 25 μl purified

angiotensinogen was several-fold greater than the calculated Vmax (Figure 6) indicates that the use of purified plasma as substrate may give spuriously high values for PRC.

The renin-substrate reaction of all species examined followed first-order kinetics. The substrate-dependence of renin suggests that changes in PRA may reflect not only changes in PRC, but may also be due to alterations in the concentration of angiotensinogen in plasma. Furthermore, these findings emphasize the importance of controlling substrate concentration during measurement of PRC.

The rate of generation of AI was constant at all substrate concentrations examined in all species. This linear time course for product formation conforms with the velocity equation describing reaction kinetics zero-order with respect to substrate (Figure 8). Such tests for AI production have been used as criteria for saturation of renin with a particular preparation of angiotensinogen, which is then interpreted to indicate appropriate conditions for quantifying PRC (Regoli, 1970; Schneider et al., 1968; Wathen et al., 1965). Most authors do not report the criterion used to insure substrate saturation in their PRC determinations. Although the reaction velocity was proportional to the concentration of renin, experiments were not performed by these investigators to test the dependence of the proportionality constant on angiotensinogen concentration. The observation of increased reaction rates with increasing substrate concentrations indicates that k_{app} is not a true zero-order rate constant, rather it represents $(k_p)\frac{[S]}{Km + [S]}$. Accordingly, k_{app} can be defined as the initial velocity of the reaction. The contribution of

substrate (S) to k_{app} demonstrates the necessity for controlling the incubation conditions such that [S]>>Km where the equation reduces to k_{app} = kp. Therefore, changing the source of angiotensinogen may introduce large errors in PRC determinations due to differences in substrate concentrations of plasma. This situation is demonstrated by the 25% variability in PRC measurements using the same sample of rat renin incubated with two different samples of homologous angiotensinogen (Barrett et al., 1976).

Comparison of the kinetics of the renin-substrate reaction of newborn rats with that of adults indicates that both processes conform with classical Michaelis-Menten kinetics (Figure 21). The Vmax of the reaction in newborn plasma was 5-fold greater than that of adults (Table 3) reflecting the substantially larger PRC of immature rats. Similar age-related differences in PRC have been reported in dogs, humans, and sheep (Granger et al., 1971; Pipkin et al., 1974b; Hayduk et al., 1972). Inasmuch as no difference existed between the Km of newborn renin and that of adults (Table 3), it was concluded that the manner in which renin interacts with angiotensinogen does not change with age. This conclusion is based on the validity of the estimates of Km which have been shown to be influenced by the endogenous substrate concentration. However, the error intrinsic to these estimates appears to be negligible as reflected by the close agreement of the data with that predicted by theoretical Michaelis-Menten kinetics (Figures 20 and 21). Furthermore, the age-related increase in plasma angiotensinogen concentration (Pohlova and Jelinek, 1974) would tend to further minimize the difference between the Km of

newborn and adult renin. The endogenous angiotensinogen concentration is less in newborn plasma compared to adults whereas the reverse situation is true of the estimated values for Km (109 and 74 µl, respectively). Assuming that the degree of shift of the velocity curve is proportional to the concentration of endogenous angiotensinogen, the higher plasma substrate concentration of adult rats would tend to increase the actual Km of adult renin by a greater increment than the increase in newborn Km after accounting for the endogenous angiotensinogen. This then would further reduce the small differences between the estimated values for Km. Therefore, although quantitatively inexact, the conclusion that the affinity of renin for angiotensinogen does not change with age appears to be valid.

Since the same sample of pooled plasma was used as substrate for both enzymes, the similar values for Km reflect similar affinities of renin for substrate. It is possible, however, that the molecular structure of angiotensinogen changes during development, which may lead to alterations in the kinetics of the renin-substrate reaction. The difficulty in nephrectomizing adequate numbers of 7-day old rats to obtain sufficient plasma for use as homologous angiotensinogen prevented the investigation of this possibility.

The kinetic parameters calculated for renin from normal adult rats (Tables 3 and 4) were different from those using plasma from renin-stimulated rats (Tables 1 and 2). Sodium restriction resulted in a doubling of Vmax compared to that of unanesthetized normal adults (0.485 ng/min versus 0.239 ng/min, respectively). This finding

conforms with that predicted from the effect of sodium on renin secretion mediated by the macula densa (Davis, 1973; Vander, 1969). This increase in PRC results in a decrease in the relative concentration of angiotensinogen from 50% Km in controls to 19% Km in salt-restricted rats. Poulsen (1971a) noted a similar reduction in plasma angiotensinogen during salt-restriction. This reduction in plasma angiotensinogen together with the increase in PRC may account for the reduction in the specific activity of renin during salt deprivation (k_{app}/V_{max} = 16% compared to 33% in controls).

Estimation of the concentration of substrate in plasma revealed that the relative concentration of angiotensinogen was significantly less in plasma from 7-day old rats compared to adults (Table 4). In view of the substrate-dependence of renin (Figure 21), this difference in angiotensinogen would predict a lower specific activity of renin in newborn plasma. This is supported by the 2-fold greater k_{app}/V_{max} in adult rats compared to newborns (Table 4). It is concluded that the elevated PRA of newborn rats is due exclusively to the greater PRC. The lower plasma angiotensinogen in newborns functions to reduce the age-related differences in PRA to values less than that predicted from the differences in PRC.

The rate of generation of AI from endogenous substrate during incubation of plasma <u>in vitro</u> is defined as the renin activity. Renin concentration is determined under identical conditions with the exception of the addition of exogenous angiotensinogen to the reaction mixture. Assuming the additional substrate is saturating to endogenous renin, the rate of AI production is theoretically proportional to

the concentration of renin in the sample. Therefore, the consistently greater PRC than PRA observed during development (Figure 22) indicates limiting amounts of endogenous angiotensinogen available for maximum rates of AI formation in newborns. Convergence of PRA and PRC with age suggests that the increase in the relative concentration of substrate (angiotensinogen/PRC) is transient. This agrees with the reported age-related increase in plasma angiotensinogen concentration in male rats (Pohlova and Jelinek, 1974). The progressive increase in substrate concentration accounts for the age-related increase in the specific activity of renin (Figure 23).

The quantitative difference between PRA/PRC (Figure 23) and k_{app}/V_{max} (Table 4) can be explained by the difference in methods used to determine PRC and Vmax. The maximum velocity is a theoretical entity calculated by estimating the limit of substrate saturation. Since absolute substrate saturation is not obtainable experimentally, Vmax is invariably greater than the observed maximum velocity and thus the calculated specific activity (k_{app}/V_{max}) is consistently less than that observed (PRA/PRC). Alternatively, the discrepancy between PRA/PRC and k_{app}/V_{max} may be due to alterations in the catalytic properties of angiotensinogen following purification. Kinetic estimation of Vmax was performed using unextracted plasma as a source of substrate. In contrast, PRC determinations were carried out under conditions of saturating concentrations of a purified preparation of angiotensinogen. Since purification of angiotensinogen results in a reduction of the Michaelis-Menten constant for the reaction (Figures 11 and 16), this preparation is apparently a poor representative

substrate for renin. Accordingly, quantitative inferences concerning PRC measurements are not appropriate, and thus k_{app}/V_{max} is probably a more accurate indication of the substrate-dependence of renin *in vivo*.

The age-related differences in plasma angiotensinogen may result from several factors. Since angiotensinogen secretion by liver is regulated by the rate of *de novo* synthesis (Bing, 1972; Nasjletti and Masson, 1971a, 1972, 1973), an age-related increase in hepatic protein turnover could explain the increase in plasma angiotensinogen. Greengard (1974) has reviewed the literature establishing the functional immaturity of liver at birth. The differences in angiotensinogen may also be mediated by age-related increases in the density of estrogen receptors in liver (Eisenfeld *et al.*, 1976; Krakoff and Eisenfeld, 1977) which provides the tissue with a progressively greater capacity for stimulation of angiotensinogen synthesis. A concomitant increase in estrogen and pituitary hormone secretion at puberty would complement this age-related increase in angiotensinogen secretion in females. Although the concentration of adrenocortical hormone receptors in liver does not change during development (Eisenfeld *et al.*, 1976; Feldman, 1974; Krakoff and Eisenfeld, 1977), age-related changes in adrenal hormone secretion could also contribute to greater rates of angiotensinogen secretion.

Alternatively, the increase in plasma angiotensinogen with age may reflect a lesser rate of consumption of substrate by renin in older animals. This decreased rate of metabolism may contribute to an age-related increase in renin substrate after 3 weeks. However, the

increasing PRA during the first couple of weeks following birth is not consistent with an increase in angiotensinogen concentration during this early period of development (Pohlova and Jelinek, 1974).

Since AII stimulates angiotensinogen synthesis directly (Blair-West et al., 1974; Carretero and Gross, 1967b; Nasjletti and Masson, 1973), the lower renin substrate concentration in newborns may reflect the lower plasma AII concentration in rats less than 3-weeks of age (Figure 3). Conversely, the higher concentration of AII in adult plasma may contribute to the greater plasma angiotensinogen concentrations observed in mature rats.

Despite these age-related changes in plasma angiotensinogen, the fact that the postnatal pattern of PRC parallels that of PRA suggests that PRC is the predominant factor responsible for changes in PRA. The increasing plasma angiotensinogen may complement PRC in bringing about the increase in PRA during the first 2-weeks following birth. However, the continued increase in angiotensinogen throughout development (Pohlova and Jelinek, 1974) is not sufficient to prevent the decrease in PRA after 3 weeks. This age-related increase in renin substrate may, however, function to diminish the decline in PRA with age by increasing the specific activity of renin.

The age-related changes in PRC may result from a variety of factors involving renin secretion and/or its clearance from the circulation, the net change depending on the relative contribution of each effector. Therefore, the profile of factors influencing the increase in PRC shortly following birth is probably markedly different from that after 3-weeks when PRC decreases throughout the remainder of development (Figure 22).

Pipkin and coworkers (1974a) attributed the enhanced activity of the renin-angiotensin system following birth to reflect increased rates of renin secretion mediated by the reduction in blood volume and the increased release of adrenocortical hormones during parturition. However, the continued increase in PRC for 2-3 days following birth in humans (Godard et al., 1976; Hayduk et al., 1972; Kotchen et al., 1972) and for 2-3 weeks in rats (Figure 22) suggests that factors unrelated to the stress of delivery are responsible for the initial increase in PRC.

Park and associates (1978) correlated the rate of renin secretion with the concentration of renin in kidney slices prior to incubation. Considering the age-related increase in renal renin content (Figure 24 and 25; Solomon et al., 1977), it is possible that the initial increase in PRC may reflect an age-related increase in renin available for secretion. This is supported by the observation of Pipkin and coworkers (1971) that maximum plasma AII concentrations occurred at a time during development when the relative kidney weight (g/kg body weight) was maximum. The fact that renal renin concentration does not parallel total renin content suggests that the postnatal increase in RRC/g is due to more selective induction of renin synthesis than a general increase in functional kidney mass.

An alternate explanation for an increase in renin secretion concerns the topography of juxtaglomerular apparatus (JGA) and the age-related changes in distribution of renal blood flow. Mature rats possess a greater granulation and renin content of superficial glomeruli than glomeruli in juxtamedullary regions (Faarup, 1971;

Flamenbaum and Hamburger, 1974; Rouffignac et al., 1974). At birth renal blood flow is distributed primarily to juxtamedullary nephrons, however, intrarenal blood flow is distributed primarily to outer cortical glomeruli of adults (Aschinberg et al., 1975; Jose et al., 1971; Olbing et al., 1973). This pattern of development of blood flow distribution is comparable to the centrifugal pattern of histologic and functional development of kidneys (Aschinberg et al., 1975). Therefore, the initial increase in PRC of newborn rats may reflect an age-related increase in perfusion of superficial juxtaglomerular apparatus allowing for the release and removal of renin in the renal venous plasma. Non-perfused JGA of immature kidneys would not be expected to contribute to the production of renin for release into the circulation. In rats, mean arterial blood pressure increases from 14 mmHg at birth to approximately 100 mmHg in rats greater than 3-weeks old (Adolph, 1957; Dawes, 1961). The postnatal increase in arterial blood pressure of rats proceeds in a curvilinear fashion from 22 mmHg at 2 days to 110 mmHg in rats 5-weeks of age (Litchfield, 1958). Accordingly, the development of increased perfusion pressures and the consequent inhibition of renin release mediated by the juxtaglomerular cells in the afferent arteriole may account for the increase in renal renin content observed after the second week postpartum (Figure 24) and the decrease in PRC observed after 3 weeks (Figure 22). It may be more than coincidental that the increase in relative renal blood flow (ml/g kidney) in rats occurs at about the same time during development as the decrease in PRC (Aperia and Herin, 1975).

The postnatal changes in PRC may also reflect age-related changes in the rate of delivery and/or transport of sodium across the macula

densa cells (Davis, 1973; Nash et al., 1968; Vander, 1969). Glomerular filtration rate (GFR) of newborn infants is approximately 1/3 that of adults (Loggie et al., 1975). In rats, GFR increases at a faster rate than total kidney mass during the first 20 days postpartum (Dlouha, 1976; Horster and Levy, 1970). Spitzer and Brandis (1974) demonstrated that single nephron GFR developed from the juxtamedullary glomeruli towards the periphery of the kidney suggesting that the increase in GFR was secondary to the centrifugal pattern of development of blood flow. The dependence of GFR on renal perfusion pressure in newborns is reflected by the proportionate changes in arterial blood pressure and GFR in newborn puppies (Kleinmann and Lubbe, 1972). The lower GFR in newborns would be expected to result in less filtration of sodium and thus a decreased sodium load delivered to the macula densa of the distal tubule. However, correlation of proximal tubular salt reabsorption with proximal tubular length in developing dogs (Spitzer and Brandis, 1974) suggests that sodium reabsorption in newborns is less than that in adults, leading to a greater proportion of the filtered sodium reaching the macula densa of younger animals. This then may act to counter the decreased filtration of sodium in newborns and thus provide for similar rates of delivery of sodium to the macula densa of newborn and adult kidneys. The filtered sodium load is also dependent on the plasma sodium concentration. In 20- to 24-day old rats, plasma sodium is approximately 143 mEq/L (Solomon et al., 1977, 1978). Sodium concentration decreases to 140 mEq/L in rats 25- to 39-days of age then increases to 145 mEq/L in plasma of rats older than 6 weeks. The age-related changes in plasma sodium concentration may be due to changes in dietary sodium and may be a factor

involved in regulating renin secretion. However, the age-related changes in plasma sodium parallel that of PRA suggesting that renin activity determines the plasma sodium concentration rather than vice versa. An increase in delivery of sodium to the macula densa during development would function to bring about an age-related increase in the inhibition of renin secretion which may complement the increased renal perfusion pressure in causing the postnatal decrease in PRC after 3 weeks (Figure 22).

Postnatal changes in the sympathetic nervous system could also explain the differences in PRC. Friedman and coworkers (1968) found that the autonomic nervous system of rabbits was functionally undeveloped at birth. Responses to nerve stimulation increased in intensity following birth indicating a gradual development of autonomic influences. In contrast, Bailie and associates (1979) interpreted the alterations in PRA of newborn piglets by administration of propranolol and isoproterenol to indicate the existence of a functional neural control of renin secretion to these immature kidneys. Comline and Silver (1966) found that the neural control of catecholamine release from adrenal glands develops late during gestation or shortly after birth in lambs. Since the vasoconstrictor response to intra-arterial injections of norepinephrine, epinephrine, tyramine, and AII is less in newborn puppies compared to adult dogs (Boatman et al., 1965), the relatively undeveloped nature of the autonomic nervous system at birth may be related to an insensitivity of the receptor mechanisms. The fact that administration of norepinephrine to immature lambs does not affect PRA (Pipkin and Symonds, 1977), suggests

that the undeveloped neural influences on renin secretion are at least partially due to a decreased sensitivity of receptor-sites. Aoi and Weinberger (1976) found that the stimulatory effect of norepinephrine on renin release from kidney slices increased in an age-related manner suggesting a deficiency of β-adrenergic receptors in newborn kidneys. Accordingly, age-related increases in receptor density and/or nerve activity may be involved in the increase in PRC shortly following birth. However, age-related increases in β-adrenergic stimulation of renin secretion would oppose the decrease in PRC after 3 weeks.

A final mechanism controlling renin release which may contribute to the postnatal changes in PRC is the direct negative feedback effect of AII (Beckerhoff et al., 1972; Cain et al., 1971; Low and Oparil, 1975; Naftilan and Oparil, 1978; Shade et al., 1973; Vander et al., 1965). In animals with elevated PRA, administration of the AII antagonist saralsin causes a large increase in renin release (Freeman et al., 1973; Mimran et al., 1974; Satoh and Zimmerman, 1975; Slick et al., 1975), whereas this drug does not affect renin secretion in animals with normal PRA (Davis, 1975; Freeman et al., 1973; Ishikawa and Hollenberg, 1975; Satoh and Zimmerman, 1975). It appears, therefore, that inhibition of renin secretion by AII is greatest under conditions of enhanced AII production. Thus, the low plasma AII concentration in rats less than 3-weeks of age (Figure 3) may affect minimal inhibition of renin secretion, thereby leading to the elevated PRC in these animals. The continued increase in plasma AII would have the converse effects, causing maximum inhibition of renin secretion and thereby resulting in the lower PRC in animals greater than 3-weeks old.

Aoi and Weinberger (1976) observed that the basal rate of renin secretion from slices prepared from kidneys of 6-week old rats was half that from rats 4-months old, indicating that the postnatal decline in renin release is intrinsic to the kidney. Therefore, extrarenal influences such as sympathetic nerves, electrolyte composition or pressure changes may act only secondarily to modify the changes in renin secretion.

Alternatively, factors effecting the clearance of renin from the circulation may contribute to the postnatal changes in PRC. Solomon and coworkers (1977) found that the rate of disappearance of renin from plasma of nephrectomized newborn rats (less than 2-weeks old) was less than that of nephrectomized adults ($T_{\frac{1}{2}}$ = 25 min and 18 min, respectively). Inactivation of renin in plasma from rats 4-6 week old proceeded at a rate similar to that of adults. These data support a role for the hepatic metabolism of renin in determining the postnatal decrease in PRC. However, these age-related differences in renin inactivation cannot explain the initial increase in PRC during the first 3 weeks postpartum.

C. Angiotensin-Converting Enzyme

Regardless of the mechanisms responsible for the postnatal changes in PRC, changes in PRA following birth would be expected to be accompanied by proportionate changes in plasma AI and AII concentrations, assuming a constant rate of conversion of AI and degradation of AII (Figure 1). In contrast, the concentration of AI in plasma did not change appreciably throughout development (Figure 26), indicating that the steady-state concentration of AI was not determined solely by its

rate of formation. Rather, age-related changes in ACE and/or angiotensinase activity may contribute to the regulation of plasma AI, and possibly AII, concentrations. In particular, the inverse relationship between PRA and plasma AII concentration between 3 and 6 weeks postpartum suggests a role of ACE and/or angiotensin degrading enzymes in regulating the steady-state concentration of AII in plasma.

The possible limiting effect of ACE on the rate of production of AII in immature animals was first proposed by Pipkin and coworkers (1974b) who found normal concentrations of AII in fetal lambs despite a 7-fold elevation of PRA. These investigators concluded that conversion of AI in fetal lungs may be the rate-limiting step in the generation of AII. This is supported by the finding that the enhanced activity of plasma ACE in children with respiratory distress (Mattioli et al., 1975) is accompanied by a 2-fold increase in systemic arterial AII concentration (Pipkin and Smales, 1977).

Hebert and coworkers (1972) provided the first evidence for a decreased ability of immature lungs to convert AI to AII by comparing the systemic blood pressure response to pulmonary arterial injections of AI and bradykinin. Injection of AI into fetal pulmonary arteries resulted in conversion of only 19% of the peptide, whereas lungs from adult ewes converted 44% of the injected AI. The pulmonary vasculature of lambs less than 24 hours of age activated 22% of the AI. In contrast, Friedli and coworkers (1973) using similar techniques were unable to detect bradykinin conversion in fetal lambs.

Kokubu and coworkers (1977) found that ACE activity was first detectable in homogenates of fetal rabbit lungs at 24 days of

gestation (term = 32 days) and increased thereafter to term, when converting enzyme activity was half that of adults. Pulmonary ACE activity increased rapidly following birth, reaching adult values by the third day postpartum. It was speculated that this rapid postnatal increase in enzyme activity may be mediated in some way by the onset of ventilation. Pulmonary converting enzyme activity in rats also developed late during gestation (Figure 28). Since enzyme incubations were carried out in the presence of saturating concentrations of HHL (Figure 29), this increase in activity can be equated with an increase in ACE concentration. The largest increase in ACE activity occurred between day 21 in utero and 24 hr postpartum, suggesting a possible stimulatory effect of ventilation. However, since pulmonary converting enzyme activity of near term fetal rats allowed to breathe room air for 30 min was not different from that of littermates decapitated in utero (Table 6), it is concluded that factors other than ventilation and associated changes in plasma pH, P_{aCO_2}, P_{aO_2}, and P_{AO_2} must be responsible for the immediate increase in ACE activity at birth. It is possible that the increase in protein concentration following birth may reflect ventilatory-induced clearance of alveolar fluid. The rapid clearance of fetal lung fluids upon ventilation is reflected by the slight decrease in lung wet weight immediately following birth (Figure 27). Clearing the lung of nonspecific alveolar fluid proteins would tend to increase the specific activity (activity/mg protein) of most pulmonary enzymes and thus potentiate the increase in ACE activity expressed per mg lung protein.

The validity of estimating ACE using a synthetic tripeptide as substrate is questionable since the reaction may represent non-specific catalysis by peptidases other than converting enzyme. However, comparison of rate of hydrolysis of HHL with that of AI demonstrated that the relative rates of cleavage of the tripeptide and AI did not change throughout purification of ACE, suggesting that a single enzyme was active in the hydrolysis of both peptides (Cushman and Cheung, 1972). Further support for HHL as an appropriate substrate for ACE was gained through coelution of HHL and AI hydrolyzing activities from standard disc-gel electrophoresis and from gradient centrifugation (Soffer et al., 1974). Similar enzyme inhibition characteristics have been obtained using HHL and AI as substrates (Lanzillo and Fanburg, 1976b). Quantitation of hippuric acid is not influenced by extraneous enzyme activities since neither HHL nor hippuric acid is degraded by peptidases in crude lung homogenates (Cushman and Cheung, 1971a). Therefore, measurement of hippuric acid liberated from the hydrolysis of HHL appears to provide a true assessment of ACE content, regardless of the fate of His-Leu.

More direct identification of ACE in fetal lungs was observed by Wigger and Stalcup (1978) using a fluorescein-conjugated antibody to purified rabbit lung ACE. Slices from various fetal rabbit tissues were incubated with the fluorescent antibody and the fluorescence observed by electron microscopy. Fluorescence was associated primarily with the luminaml plasma membrane of endothelial cells from lung, kidney, gut, liver, heart, brain, skeletal muscle, and placenta. The only types of epithelial cells which exhibited fluorescence were the brush border of renal proximal tubules, collecting tubules, ducts

of Bellini and papillary epithelium and the brush-border of intestinal epithelium. Caldwell and associates (1976) described a similar distribution of immunologically reactive ACE in adult rabbits. Binding of the labelled antibody to endothelium was not detectable until the third trimester (day 17 gestation) and occurred at a similar age for all tissues examined (Wigger and Stalcup, 1978). Fluorescence was detectable in coronary vessels as early as day 14 of gestation. In contrast, ACE of the epithelioid tissues was not observable until days 22-26 (term = 32 days). The intensity of fluorescence increased from the day of appearance throughout gestation in all tissues examined, suggesting an age-related increase in the distribution and density of ACE.

Perfusion of lungs isolated from fetal rabbits at 22 days of gestation demonstrated that only one-third of the perfused AI or bradykinin was removed during a single pass through the pulmonary circulation (Stalcup et al., 1978). Lungs from 1-week old rabbits removed 80% of the perfused AI. The fraction of peptide cleared was not changed when either flow or substrate concentration was varied. The age-related increase in fractional removal of both peptides proceeded at faster rates than the increase in lung or body weight, suggesting a specific induction of this metabolic activity.

The limited ability of fetal rabbit lungs to remove AI (Stalcup et al., 1978) despite the presence of considerable immunoreactive ACE (Wigger and Stalcup, 1978) was interpreted to indicate that factors other than enzyme density limited the degree of AI conversion. Ryan and coworkers (1975, 1976) described numerous surface projections and indentations ("caveolae intracellulares") in the pulmonary endothelium

which were densely lined with immunoreactive converting enzyme. Stalcup and associates (1978) observed that the number of these cytoplasmic projections and caveolae in capillaries of 19-day old fetal rabbit lungs was much less than that in lungs from rabbits at 26-days of gestational age, suggesting an age-related increase in the structural complexity of the pulmonary vascular surface. This increase endothelial infoldings and surface adaptations, together with the proliferation in the number of patent pulmonary capillaries during this period (Cassin et al., 1964; Cook et al., 1963; West et al., 1975), suggests a progressive increase in the vascular surface area available for exposure of ACE to circulating AI. Therefore, the unspecialized nature of pulmonary endothelial cells in utero has been postulated as one factor which, by minimizing the surface area exposed to the vascular lumen, may contribute to the decreased conversion of AI in lungs from fetal rabbits (Stalcup et al., 1978).

The dissociation between PRA and AII early during postnatal development can be explained by the age-related increase in ACE activity during this period (Wallace et al., 1978). Converting enzyme activity of the 600 x g supernatant of lung homogenates increased in a linear fashion from 1-week to 6-weeks of age (Figure 31). This in contrast to the biphasic increase in ACE activity of the 20,000 x g supernatant fraction of lung homogenates reported earlier (Wallace et al., 1978). Localization of ACE in endothelial cell membranes has been fairly well established by subcellular fractionation (Lanzillo and Fanburg, 1974; Ryan and Smith, 1971; Ryan et al., 1972; Sander and Huggins, 1971) and by direct electron microscopy (Caldwell et al., 1976; Ryan et al., 1975). Therefore, the differences in the development

of converting enzyme in the two supernatant fractions can be explained by relative differences in the solubilization of the enzyme from membrane-bound complexes. The fact that a greater fraction of the total ACE activity was recovered in the 600 x g supernatant (20-25%) compared to the 20,000 x g supernate (17.5%) demonstrates the quantitative differences in the results using these two fractions. However, despite these differences, the qualitative increase in pulmonary ACE activity during development is evident in both cell-free preparations. Kokubu and coworkers (1977) reported a similar age-related increased ACE activity of homogenates of rabbit lungs, however, the postnatal increase in enzyme activity was much less profound and reached adult values earlier than that in rats.

The age-related increase in ACE activity may relfect either; 1) an increase in the amount of active enzyme due to increased protein synthesis, removal of competitive inhibitors, or addition of essential activators, or 2) further activation of pre-existing enzyme by removal of noncompetitive inhibitors or addition of nonessential activators. The fact that the affinity of ACE for HHL was similar for both fetal and adult enzyme (Figure 30) suggests that the increased converting enzyme activity results from a greater amount of active enzyme in lungs from adult rats. Whether this activation represents <u>de novo</u> synthesis was not distingiushed from derepression of existing enzyme or allosteric activation. However, correlation of ACE activity with lung weight (Figure 32) suggests that the increase most likely reflects a general increase in the functional mass of the lung.

A similarity in converting enzyme isolated from various sources has been postulated. Lanzillo and Fanburg (1976a) observed comparable molecular weight and subunit structure of ACE from rat, rabbit and hog lung. These investigators also found that lung and serum converting enzyme in guinea-pig possessed similar substrate affinity and inhibition characteristics (1976b). Immunologic studies have demonstrated cross-reactivity between hog kidney, lung and plasma converting enzymes (Erdos and Oshima, 1974; Oshima et al., 1974), between hog and rat lung ACE (Lanzillo and Fanburg, 1976a), and between the pulmonary converting enzyme of rat, rabbit, guinea pig and dog (Conroy et al., 1976). The molecular weight of human plasma converting enzyme was estimated by gradient centrifugation to be 150,000 (Lee et al., 1971b), which is similar to the values obtained for rat, rabbit, guinea pig and hog lung ACE (Lanzillo and Fanburg, 1976a; Lee et al., 1971a). The Km value reported for fetal rat ACE agrees with that found for ACE from other sources (Cushman and Cheung, 1971a; Das and Soffer, 1975; Lanzillo and Fanburg, 1976b). The increasing converting enzyme activity with age without a concomitant change in substrate affinity implies that the similarity in the catalytic properties of the enzyme prepared from the various tissues persists throughout development (Wallace et al., 1978).

Estimation of enzyme activity using broken cell preparations (Kokobu et al., 1977; Wallace et al., 1978) does not take into account age-related differences in total tissue perfusion, blood flow distribution, or availability of the enzyme for interaction with substrate. Disruption of cellular integrity may also affect enzyme activity by altering the enzyme-membrane complex. Furthermore, cell lysis may

release intracellular enzymes not normally exposed to the cell surface of intact tissue. An example of this situation is the rapid metabolism of AII by broken cell preparations of lung (Bumpus et al., 1961; 1968; Itskovitz and Miller, 1967; Matsunaga et al., 1969). In contrast, AII survives passage through intact lungs with very little degradation or extraction (Aiken and Vane, 1970; Biron et al., 1968, 1969; Hodge et al., 1967; Leary and Ledingham, 1969; Ng and Vane, 1968). Therefore, the age-related increase in ACE activity of lung homogenates may not be a valid indication of that which occurs in the intact developing animal. Accordingly, results using the isolated, perfused lung model may provide more accurate evidence for the contribution of ACE to the generation of AII in vivo.

Metabolism of AI by isolated, perfused lungs has previously been examined in dogs (Aiken and Vane, 1970; Bakhle et al., 1969; Fanburg and Glazier, 1973) and in rats (Ryan et al., 1970, 1972). The susceptibility of this metabolic process to competitive inhibitors of AI hydrolysis indicates that the removal of AI by perfused lungs is specific to the activity of ACE (Aiken and Vane, 1970; Bakhle et al., 1969; Stalcup and coworkers, 1978). The complete recovery of AI and AII following perfusion with AI (Ryan et al., 1970, 1972; Fanburg and Glazier, 1973) contraindicates metabolism of AI by enzymes other than ACE. Therefore, it is generally accepted that the removal of circulating AI by intact lungs represents conversion of AI to AII accompanied by the quantitative appearance of newly generated AII in the pulmonary venous effluent.

Perfusion of lungs from adult rats with 1 ng/ml AI resulted in only 40% of the peptide appearing in the pulmonary venous effluent (Figure 33), indicating that 60% of the AI was metabolized during a single passage. Since greater than 95% of the AI was recovered in the effluent medium when no lung was present in the chamber, this removal was attributed to pulmonary metabolism. The similar distribution of ^{125}I and blue dextran in the pulmonary circulation and the coemergence of AI and the vascular marker in the effluent medium during the washout period suggests that AI was metabolized without leaving the pulmonary vascular space. This is in agreement with the observations of Ryan and coworkers (1970, 1972) who coperfused blue dextran and radiolabelled AI or bradykinin through intact rat lungs and found that the radiolabelled metabolites emerged simultaneously and quantitatively with the blue dye. Fanburg and Glazier (1973) found that the volume of distribution of AI and AII in isolated, perfused dog lungs was no larger than that of indocyanine green, providing further support for the hypothesis that the conversion of AI to AII occurs exclusively within the pulmonary vascular space. Localization of ACE on the luminal aspect of pulmonary endothelium (Caldwell et al., 1976; Ryan et al., 1975, 1976; Wigger and Stalcup, 1978) suggests direct exposure of converting enzyme to the luminal contents of pulmonary vessels. This direct apposition of ACE to circulating AI provides for the rapid and efficient conversion of AI to AII with very little retention of either peptide. The negligible extraction or further metabolism of AII by the intact pulmonary vasculature adds to the economy of lung as the locus for the generation of AII destined for the systemic

circulation (Aiken and Vane, 1970; Biron et al., 1968, 1969; Hodge et al., 1967; Leary and Ledingham, 1969; Ng and Vane, 1968; Osborn et al., 1969). This is in contrast to extrapulmonary tissues which rapidly remove and metabolize circulating AII (Aiken and Vane, 1970; DiSalvo and Montefusco, 1971; Ng and Vane, 1967, 1968; Oates and Stokes, 1974).

Removal of AI by perfused rat lungs was examined at a time during perfusion (2 min) when AI metabolism was in a steady-state (Figure 33). Evaluation of AI clearance at this time prevented problems associated with age-related differences in the rate of dilution of AI in the pulmonary circulation and thus simplified the interpretation of the results. Under steady-state conditions, the rate of AI metabolism is dependent solely on the rate of delivery of substrate and the availability of ACE. Since enzyme availability is the product of enzyme concentration and vascular surface area, the rate of perfusion was adjusted to lung mass in an attempt to minimize age-related differences in the relative volume of pulmonary vessels (ml/g) which were perfused with substrate. Cross and associates (1959) found that cardiac output of lambs 20- to 60-days old was similar to that of adults when expressed on the basis of body weight. Since lung wt/body wt ratio decreases with age (Hamosh et al., 1978; Kotas et al., 1971), the relative rate of perfusion of newborn lungs in vivo (ml/min·g) is greater than that of adults. Therefore, normalization of flow to lung mass underestimated that which occurs physiologically and may account for the decreased removal of AI by newborn lungs compared to adults. However, in view of the flow-independence of AI removal by

adult lungs (Figure 34), it is not likely that the age-related differences in AI metabolism were due to the different rates of perfusion. Because of the fragility of the 7-day old lungs, it was not possible to further increase flow through newborn lungs without causing tissue disruption and accumulation of lung fluids. Normalization of flow to lung weight resulted in similar relative volumes (ml/g) being perfused (Table 8) and thereby provided for similar rates of delivery of AI (ng/min) per unit pulmonary vascular volume. Assuming that the ratio of volume to vascular surface area was constant under these conditions, age-related differences in AI metabolism most likely reflect differences in enzyme density within the pulmonary endothelium.

Perfusion of lungs at flows normalized to tissue mass revealed an age-related increase in the ability to clear AI from the circulation (Table 7). Lungs from adult rats removed 51% of the perfused AI whereas lungs from 7-day old rats were capable of clearing only 35% of the AI. The percent removal of AI by lungs of 3-week old rats (48%) was not significantly less than that of adults. When AI removal was expressed as the rate of clearance (ng/min), a more striking age-related increase was observed. However, this difference was attributed primarily to the greater flows (ml/min) used to perfuse the larger lungs.

The lower metabolic activity of newborn lungs may be explained by 1) less converting enzyme available for catalysis, 2) differences in the relative rate of substrate delivery, 3) shunting of flow through metabolically less active vessels, and/or 4) by enzymes other than ACE involved in the hydrolysis of AI by adult lungs. It is unlikely that

the observed differences in AI metabolism were due to edema since neither the percent water content nor perfusion pressure, both of which invariably increase with the accumulation of lung fluids, changed throughout the perfusion period. The slightly greater percent water content of 7-day old lungs, although statistically significant, was probably not physiologically important. Furthermore, this greater hydration of newborn lungs would be expected to enhance the removal of AI via increased diffusion of the peptide into the enlarged extracellular water space.

Ventilation of the various lungs with 95% O_2-5% CO_2 was adjusted so as to maximize the tidal volume. The rate of ventilation was similar for all lungs (30 strokes/min). It is possible that age-related differences in tissue oxygenation may have influenced the results, however, we did not observe any differences in pH of the effluent medium collected from the various aged lungs (pH 7.4-7.7). Roth and Gillis (1978) noted that lungs statically inflated with room air removed a smaller fraction of the perfused mescaline, 5-hydroxytryptamine (5-HT), and norepinephrine (NE) than lungs ventilated with room air. Ventilation with 95% O_2-5% CO_2 mixture did not increase the removal of these substances by intact rabbit lungs. The differences in metabolism were attributed not to differences in gas tensions but resulted from pH changes accompanying ventilation with room air. The pH of the effluent medium from lungs ventilated with 95% O_2-5% CO_2 was markedly less than that of room air-ventilated lungs (pH 7.5 versus pH 8.2, respectively). It was concluded that these pulmonary metabolic processes are rather independent of tissue oxygenation *per se* but are

markedly affected by pH changes associated with ventilation. The relatively constant pH of the effluent medium of lungs from different aged rats ventilated with 95% O_2-5% CO_2 indicates that this method of ventilation was adequate to avoid changes in AI metabolism due to pH effects.

Shunting of flow through a patent ductus arteriosus could explain the decreased ability of newborn lungs to remove AI. However, perfusion of lungs from 7-day old rats with either a concentrated solution of blue dextran or a synthetic plasticizer failed to reveal any shunting of flow through the ductus into the aorta. Furthermore, care was taken to advance the pulmonary arterial cannula as far as possible without bypassing the bifuration of the main pulmonary artery to minimize the possibility of perfusing through the ductus arteriosus.

Nonspecific hydrolysis of AI by amino- carboxy-, and/or endopeptidases may account for the greater metabolism of this peptide by adult lung. However, the near-complete recovery of perfused AI as AI plus AII in the pulmonary venous effluent from isolated lungs (Fanburg and Glazier, 1973; Ryan et al., 1972) suggests that hydrolysis of AI by nonspecific peptidases is not a significant factor. Cleavage of the amino terminus of AI has been observed in intact lung (Barrett and Sambhi, 1971; Oparil et al., 1970). Whether such nonspecific hydrolyses of AI alter the immunoreactivity of this peptide is not certain. We have tested the AI-antibody used in our laboratory and found it to be specific in descriminating between AI and des-Asp^1-AI. Although metabolism of AI by aminopeptidases may account for the disappearance of immunologically active AI across the lung, such enzymes probably

contribute negligibly to the total metabolism of AI in lung. Since aminopeptidase enzymes would also hydrolyze AII, the lack of inactivation of AII by intact lung confirms the insiginficant role of aminopeptidases in the pulmonary metabolism of AI (Bakhle et al., 1969; Biron et al., 1969; Fanburg and Glazier, 1973; Hodge et al., 1967). The near-complete inhibition of AI removal by coperfusing with a specific competitive inhibitor of ACE (Table 9) demonstrates the specificity with which this metabolism represents converting enzyme activity.

Therefore, the decreased ability of newborn lungs to clear circulating AI most likely reflects differences in substrate delivery or enzyme availability. Since adjustment of flow was such that no difference in the relative rate of perfusion existed (Table 7) and since all lungs were perfused with same concentration of AI (1 ng/ml), the relative rate of delivery of substrate was similar for all ages examined. Thus, enzyme availability appears to be the primary factor restricting AI metabolism by immature lungs.

The validity of normalizing flow to minimize differences in the volume of vessels being perfused is demonstrated by the similar relative vascular volume (ml/g) perfused in newborn lungs compared to adults (Table 8). It was not possible to measure the perfused vascular volume of lungs from 7-day old rats using this method due to the small size and thus small amounts of blue dextran trapped within the pulmonary vasculature. The similarity in the relative volume of vessels being perfused suggests that the differences in enzyme availability do not represent a smaller fraction of the total vascular volume being perfused in newborn lungs. Changes in vascular volume

could reflect changes in the diameter and/or the number of vessels being perfused. Since vascular volume is not necessarily proportional to surface area, the results cannot be interpeted on the basis of enzyme availability per unit endothelial surface area.

The age-related increase in ACE availability may represent an increase in enzyme concentration within the pulmonary endothelium and/or a greater vascular surface area exposed to circulating AI. Differences in enzyme density are demonstrated by the age-related increase in ACE concentration of lung homogenates (Table 11). Converting enzyme activity of homogenates of lungs from adult rats was 2-fold greater than that of 7-day old lungs when expressed on the basis of lung protein. This difference was more profound when enzyme activity was expressed per g lung due to the slightly greater protein concentration of adult lungs. However, enzyme density alone cannot account for the differences in the ability of intact lungs to metabolize AI. Despite the 2-fold greater ACE content of adult lungs compared to lungs from 21-day old rats (Table 11), no difference was observed in the fraction of AI removed by isolated, perfused lungs (Table 7).

In contrast to <u>in vitro</u> measurements, it is highly unlikely that substrate approaches saturating concentrations with respect to ACE in the isolated, perfused lung. Increasing AI to concentrations 100,000-fold greater than that normally perfused did not affect the fraction of AI removed by adult lungs (Table 10). Similarly, increasing the rate of substrate delivery by altering the rate of perfusion did not change the fractional removal of AI (Figure 34). These results taken together suggest that AI metabolism by intact lung is a substrate-limited

process, as opposed to an enzyme-limited system. This is in accordance with the reported kinetics of converting enzyme. Measurements of the Michaelis-Menten constant (Km) of ACE for AI range from 20×10^{-6}M to 78×10^{-6}M (Cushman and Cheung, 1972; Das and Soffer, 1975; Das et al., 1977; Dorer et al., 1974; Huggins et al., 1970; Lanzillo and Fanburg, 1976b; Lee et al., 1971b). Inasmuch as the concentration of AI perfused through lungs (1×10^{-9}M) was approximately 1/50,000 the Km, the rate of conversion was probably not limited by enzyme. Apparently, sufficient ACE is present for an almost instantaneous conversion of physiological concentrations of perfused AI. Accordingly, Oparil and coworkers (1970,1971) found that greater than 50% of the AI was converted when perfused through lungs at 2,000-10,000 times greater than the physiological concentration. Barrett and Sambhi (1971) reported that 80% of an injected dose (35 ng) of AI was converted during a single transpulmonary passage and that conversion decreased to 20% as the dose of AI was increased to 200 µg. Fanburg and Glazier (1973) recovered maximum concentrations of AII in pulmonary venous effluent when the dose of AI injected into a dog lung model exceeded 1 mg. Injection of AI at higher doses did not increase the recoverable AII suggesting that ACE was saturated at this very high substrate concentration. No attempt was made to estimate the actual concentration of AI in the pulmonary circulation following bolus injections of AI, and therefore these data cannot be quantitatively extrapolated to the continuous infusion procedures employed in the present investigation.

In view of the flow- or substrate-limited kinetics of AI metabolism by intact lungs, it is highly unlikely that the decreased ability

of newborn lungs to remove AI was due to saturation of limited amounts of enzyme with excess substrate. Therefore, factors other than absolute enzyme density (or concentration) probably contribute to limiting the ability of immature lungs to clear circulating AI.

The vascular surface area exposed to the circulation is another factor contributing to enzyme availability. The constant fractional removal of AI at increasing rates of flow suggests a proportionate increase in the amount of enzyme exposed to the substrate. This increased enzyme availability most likely reflects a greater vascular surface area being perfused at higher flows. It was not possible to distinguish between increased surface area resulting from an increased diameter of the vessels being perfused or recruitment of vessels at the higher flows. Fanburg and Glazier (1973) observed proportionate increases in AI conversion when the vascular volume of dog lungs was increased by raising venous pressure and keeping the transit time constant. Conversely, lowering venous pressure resulted in less conversion of AI to AII. The rate of conversion of AI was also correlated with mean transit time. These investigators interpreted the greater conversion of AI at high venous pressures to result from an increased vascular surface area, and consequently a greater amount of ACE exposed to the circulating substrate.

In summary, the age-related increase in the ability of intact lung to metabolize AI agrees with the increase in ACE activity of lung homogenates. However, factors other than enzyme density which affect the availability of ACE are implicated in contributing to the decreased ability of immature lungs to remove AI from the circulation. A possible explanation for the increased enzyme availability during

development was proposed by Stalcup and associates (1978) who found that despite the presence of considerable ACE in lungs of fetal rabbits at 17 days of gestation, the ability of these lungs to remove AI and bradykinin was markedly depressed. The observation of a primitive, smooth-surfaced endothelium containing few caveolae and cytoplasmic projections in lungs from younger fetuses compared to those at term suggested that an age-related increase in endothelial infoldings and specialized surface structures may bring about a greater vascular surface area (relative to vascular volume), and thus a greater amount of ACE exposed to circulating AI. Accordingly, a rapid, postnatal increase in the structural development of the pulmonary endothelium in rats could explain the inability of ACE concentration alone to account for the reduced capacity of newborn lungs to clear AI from the circulation. In the newborn, the pulmonary endothelial surface available for interaction with substrate may be limited relative to the volume of vessels perfused thus permiting AI to pass through the intact lung unmetabolized via streaming of AI past the enzyme on the primitive, unspecialized vascular surface. The age-related increase in AI removal by intact lungs may be attributed to both an increase in ACE content and a progressively greater vascular surface area.

Regardless of the factors mediating the limited ability of immature lungs to metabolize AI, this decreased conversion of AI to AII in newborns may function to limit the steady-state concentrations of AII in plasma. The dissociation between plasma AII concentration and the rate of generation of AI in immature rats supports this hypothesis.

The reciprocal relationship between PRA and AII between 3- and 6-weeks postpartum is rather conclusive evidence for factors other than renin being the rate-determining step in the regulation of the steady-state concentration of AII in plasma during this period of development. Since ACE is the final catalyst in the generation of AII (Figure 1), the continued increase in AII concentration after 3 weeks may be explained by the age-related increase in conversion of AI to AII.

Alternatively, the dissociation between PRA and plasma AII concentrations early during development may be mediated by age-related changes in the rate of inactivation of AII. Only two reports exist to date describing the postnatal development of AII degrading enzyme activities. Pipkin (1972) found that the inactivation of AII injected into the hepatic portal vein of newborn rabbits (11-24 days) was less than that of adults. It was suggested that this lower rate of inactivation of AII may contribute to the elevated plasma AII concentrations of newborn rabbits. The relatively decreased capacity to inactivate AII may reflect the metabolic immaturity of liver at birth (Greengard, 1974). Pohlova and Jelinek (1974) observed a similar decreased angiotensinase activity in homogenates of newborn rat kidneys. The biological half-life ($T\frac{1}{2}$) of AII incubated with homogenates of kidneys from 1-day old rats was approximately 11 min compared to a $T\frac{1}{2}$ of 7 min in adult kidneys. This age-related increase in renal angiotensinase activity occurred progressively throughout development being less at 20-days than at 40-days postpartum. Therefore, the age-related changes in both the renal and hepatic inactivation of AII suggest that the circulating half-life of newly formed AII is longer in newborns than adults. The increase in angiotensinase activity

during development would tend to bring about an age-related decrease in plasma AII concentrations. This effect may contribute to the decrease in plasma AII observed after the sixth week postpartum (Figure 3). However, the progressive increase in angiotensinase activity from birth through day 40 (Pohlova and Jelinek, 1974) contradicts the early postnatal increase in AII concentration observed between 3- and 6-weeks postpartum.

The available evidence indicates that the only factor capable of accounting for the dissociation between PRA and AII is an age-related increase in the rate of conversion of AI to AII by the pulmonary circulation. The inability of PRA to account for the changes in plasma AII concentrations is evident from the far greater AII/PRA of 6-week old rats compared to that of 3-week old and adult rats. Although no significant difference existed for the fractional removal of AI between 3-week old rats and adults (Table 7), the progressive increase in pulmonary blood flow (Cross et al., 1959) would tend to unmask age-related differences in the absolute rate of liberation of AII (ng/min) into the pulmonary venous effluent and subsequently the amount of AII entering systemic circulation (Table 7). In rats, as in most species, the relative blood volume per kg body weight decreases with age (Constable, 1963; Garcia, 1957). This age-related decrease in blood volume would complement the increased rates of conversion of AI in increasing the circulating concentration of AII in systemic arterial plasma. Therefore, the increased availability of pulmonary ACE along with the greater perfusion of developing lungs appear to be predominant factors regulating the steady-state concentration of AII in plasma of rats between 3- and 6-weeks postpartum.

SUMMARY

Since all of the recognized biological effects of the renin-angiotensin system are exerted through angiotensin II, regulation of the steady-state concentration of AII is of central importance in understanding the relevance of this system. The concentration of angiotensin II in plasma changes in a non-linear fashion during development (Figure 3). Plasma AII concentration decreases between birth and 3-weeks postpartum then increases dramatically to maximum values in rats 5- to 6weeks of age. The concentration of AII subsequently declines to adult values by 8-weeks postpartum. Since the renin-angiotensin system is a composite of several enzyme activities, the steady-state concentration of AII reflects the cumulative effects of individual changes in renin, ACE, and angiotensinase activities. Therefore, age-related changes in AII are manifested through integration of the development of each of the component enzymes. The directional changes in AII most likely parallel the age-related changes in the rate-limiting component of the renin-angiotensin system.

The initial decrease in plasma AII concentration during the first 3-weeks postpartum is difficult to explain by the age-related changes in renin or ACE. Plasma renin activity increases following birth to maximum values at 2-weeks of age (Figure 4). Since PRC also increases during this time (Figure 22), the increase in PRA is most likely due to a greater concentration of renin in plasma. In view of the

first-order kinetics of the renin-substrate reaction (Figure 21), the increase in plasma angiotensinogen concentration (Table 4) complements PRC in bringing about the age-related increase in PRA. The increase in PRC may reflect greater rates of renin secretion mediated by a gradual development of renal innervation. Alternatively, the increase in PRC could be secondary to the decrease in plasma AII during this period (Figure 3), thereby exerting progressively less inhibition of renin secretion. Regardless of the mechanism responsible for the changes in PRC, the age-related increase in PRA during the first 3 weeks would tend to increase the concentration of circulating AII. Similarly, the age-related increase in pulmonary AI conversion (Figures 28 and 31; Table 7) is inconsistent with the decrease in plasma AII between birth and 3-weeks of age. The decrease in AII concentration may be attributed to the progressive increase in renal angiotensinase activity reported in rats.

The dramatic increase in the steady-state concentration of AII between 3-and 6-weeks following birth (Figure 3) indicates a rapid transition in the rate-limiting component of the renin-angiotensin system. The continued increase in angiotenase activity does not agree with the increase in plasma AII. Furthermore, the decrease in PRA during this period of development (Figure 22) is inconsistent with the increasing AII concentration. The decrease in PRA is most likely due to the decreasing PRC (Figure 22). Age-related increases in renal renin content (Figure 25) may reflect a progressive decrease in renin secretion after 3-weeks of age. The age-related decrease in renin secretion may be intrinsic to the kidney or may be mediated by

progressively greater renal arterial blood pressure and/or greater rates of delivery of sodium to the macula densa. Alternatively, the increasing plasma AII concentration (Figure 3) may exert progressively greater inhibition of renin release. An age-related increase in renin metabolism has also been implicated in the postnatal decrease in PRC. The decrease in PRA between 3- and 6-weeks postpartum (Figure 22) predicts a progressively slower rate of formation of AI and consequently AII during this period of development. The only evidence consistent with the increase in plasma AII concentration between 3- and 6-weeks postpartum is the continued increase in pulmonary ACE activity (Figure 31). Age-related increases in the concentration of ACE along with greater pulmonary vascular surface area and pulmonary blood flow indicates a progressively greater rate of conversion of AI to AII. Therefore, in contrast to the classical view that PRA is the rate-determining reaction of the renin-angiotensin system in adults, it appears that ACE limits the availability of AII in rats during the third to sixth week of development.

The subsequent rapid decline in plasma AII concentration (Figure 3) along with the continued increase in pulmonary ACE content (Figure 31) suggests a second transition in the rate-limiting step involved in the formation of AII. The inverse relationship between ACE and AII after 6 weeks indicates that converting enzyme probably does not limit AII production in older animals. The continued increase in renal angiotensinase activity may account for the rapid turnover of AII in plasma at this time. Alternatively, the low PRA may limit AII formation through slower rates of generation of AI. However, since PRA

does not change appreciably between 3- and 6-weeks of age (Figure 22), the decreasing plasma AII probably does not solely reflect changes in AI production. PRC, on the other hand, continues to decrease throughout development (Figure 22) and may act indirectly to determine the rate of formation of AII. It is possible that the decrease in AII between 3- and 6-weeks of age may reflect a gradual transition from ACE to PRA as the rate-limiting component of the renin-angiotensin system. The causative factors mediating this transition, however, are not understood.

In view of the vasopressor and adrenal steroidogenic effects of AII, the age-related changes in plasma AII concentration may exert important influences of the homeostasis and body fluid composition of developing animals. The low concentration of AII in plasma during the first 3-weeks of life (Figure 3) may not be physiologically beneficial, rather it may reflect the immaturity of the renin-angiotensin system immediately following birth. This in turn may be a consequence of the relative immaturity of rats at birth compared to most species. In more precocious animals, plasma AII concentration increases immediately following birth, whereas in rats, AII concentration does not increase until after the third week postpartum (Figure 3). The postnatal increase in AII (Figure 3) may be responsible for the greater sodium retention and larger extracellular fluid volume of neonates compared to adults. The greater reactivity of the renin-angiotensin system in immature animals suggests that this system may contribute a greater influence in the homeostasis of newborns compared to adults. Accordingly, the subsequent decrease in plasma AII

concentration (Figure 3) may reflect a gradual decrease in the importance of the renin-angiotensin system in the control of resting blood pressure and body fluid composition. It is possible that other mechanisms such as antidiuretic hormone and the autonomic nervous system gradually assume the major role in regulating the resting blood pressure and body fluid composition in older animals.

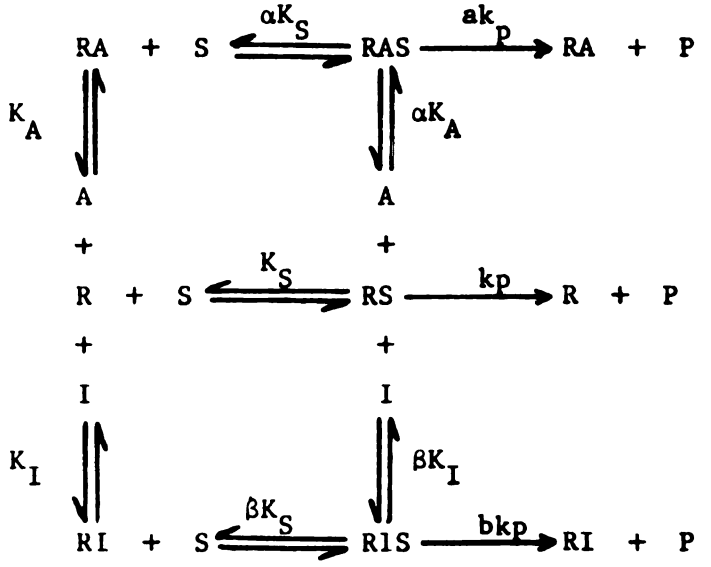
Description of the stoichiomtery of the renin-angiotensin provides an indication of the primary factors responsible for regulating the steady-state concentration of AII in plasma. Although the renin-angiotensin system appears to be functional shortly following birth, the age-related transitions in the rate-limiting reaction predict quantitative differences in the response of this system to alterations in normal body fluid homeostasis. An appreciation of the transition from ACE to PRA as the primary component controlling AII turnover allows for more efficacious treatment of anomalies in the renin-angiotensin system by directing attention to the responsible factor. Accordingly, the evidence provides for more accurate predictions of the utlimate effect of either pharmacological or physiological intervention on the steady-state concentration of circulating AII during development.

APPENDIX A

APPENDIX A

Derivation of the Reaction Kinetics of the Renin-Substrate Interaction.

Beginning with the following reaction mechanism as a workable hypothesis,



where R represents renin, S is angiotensinogen, A is an undefined non-essential activator, I is a mixed-type inhibitor, and P represents angiotensin I. The equilibrium constants (K) are the ratios of the respective reverse and forward reactions at each step. Assuming the catalytic rate constants (kp) are unidirectional and that quasi-equilibrium conditions prevail for each enzyme species, it is possible to derive the velocity equation to predict the overall rate of generation of AI (P).

If the catalytic process is not affected by either activators or inhibitors then the reaction would proceed via a first-order mechanism and could be described by the classical Michaelis-Menten equation,

$$V = \frac{Vmax\ [S]}{K_S + [S]}$$

where $Vmax = kp[R_{total}]$ and kp is the true first-order rate constant.

If this reaction were saturated with substrate ($[S] >> K_S$), the rate of product formation would not be dependent on [S] and the velocity equation would reduce to:

$$V = \frac{Vmax\ [S]}{[S]} = Vmax = kp[R_{total}]$$

Under these conditions $kp[R_{total}]$ would be the pseudo-zero-order rate constant and the velocity of the reaction would be reflective of enzyme concentration. Conventional methods for measuring plasma renin concentration are based on the assumption that this type of reaction kinetics prevails.

In contrast, if either activators and/or inhibitors of the renin-substrate reaction are present, the net rate of AI generation becomes a complex function of [R], [S], [A], and [I]. The velocity equation can be derived under the assumption that rapid equilibrium conditions prevail (Segel, 1975).

The net rate of AI production is predicted by,

$$v = kp[RS] + akp[RAS] + bkp[RIS]$$

and, $[R_{total}] = [R_t] = [R] + [RS] + [RA] + [RI] + [RAS] + [RIS]$

Therefore, $$\frac{v}{R_t} = \frac{kp[RS] + akp[RAS] + bkp[RIS]}{[R] + [RS] + [RA] + [RI] + [RAS] + [RIS]}$$

Since, $$[RS] = \frac{[S]}{K_S}$$

$$[RA] = \frac{[A]}{K_A}$$

$$[RI] = \frac{[I]}{K_I}$$

$$[RAS] = \frac{[A]\,[S]}{\alpha K_S\, K_A}$$

$$[RIS] = \frac{[I]\,[S]}{\beta K_S\, K_I}$$

substituting for each species of R yields;

$$\frac{v}{[R_t]} = \frac{kp\,\frac{[S]}{K_S} + akp\,\frac{[A]\,[S]}{\alpha K_A\, K_S} + bkp\,\frac{[I]\,[S]}{\beta K_I\, K_S}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[I]}{K_I} + \frac{[A]\,[S]}{\alpha K_S\, K_A} + \frac{[I]\,[S]}{\beta K_S\, K_I}}$$

where $[R_{free}] = 1$

since $kp[R_t] = Vmax$, the equation becomes;

$$\frac{v}{Vmax} = \frac{\frac{[S]}{K_S} + a\frac{[A]\,[S]}{\alpha K_A\, K_S} + b\frac{[I]\,[S]}{\beta K_S\, K_I}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[I]}{K_I} + \frac{[A]\,[S]}{\alpha K_A\, K_S} + \frac{[I]\,[S]}{\beta K_S\, K_I}}$$

Factoring the numerator by [S] and K_S yields;

$$\frac{v}{Vmax} = \frac{\frac{[S]}{K_S}\left(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I}\right)}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[I]}{K_I} + \frac{[A]\,[S]}{\alpha K_S K_A} + \frac{[I]\,[S]}{\beta K_S K_I}}$$

Multiplying both numerator and denominator by K_S gives;

$$\frac{v}{Vmax} = \frac{[S]\ (1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}{K_S + [S] + \frac{K_S[A]}{K_A} + \frac{K_S[I]}{K_I} + \frac{[A]\ [S]}{\alpha K_A} + \frac{[I]\ [S]}{\beta K_I}}$$

factoring the denominator by [S] and K_S yields;

$$\frac{v}{Vmax} = \frac{[S]\ (1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}{K_S(1 + \frac{[A]}{K_A} + \frac{[I]}{K_I}) + [S](1 + \frac{[A]}{\alpha K_A} + \frac{[I]}{\beta K_I})}$$

Then rearranging to a form similar to that of Michaelis-Menten equation yields;

$$v = \frac{Vmax\ [S]}{K_S\frac{(1 + \frac{[A]}{K_A} + \frac{[I]}{K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})} + [S]\frac{(1 + \frac{[A]}{\alpha K_A} + \frac{[I]}{\beta K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}}$$

where $\frac{(1 + \frac{[A]}{K_A} + \frac{[I]}{K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}$ = a slope factor

and $\frac{(1 + \frac{[A]}{\alpha K_A} + \frac{[I]}{\beta K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}$ = an intercept factor

In simpler terms, setting the slope factor equal to M and the intercept factor equal to Y gives the equation in a form similar to that of the classical Michaelis-Menten equation:

$$V = \frac{Vmax\ [S]}{K_S(M) + [S](Y)} \quad \text{and} \quad \frac{1}{v} = \frac{K_S(M)}{Vmax}\left(\frac{1}{S}\right) + \frac{Y}{Vmax}$$

Thus, a double-reciprocal plot of such data will generate a straight line having a slope of $\frac{K_S}{Vmax}(M)$ and intercepting the ordinate at $\frac{Y}{Vmax}$. Thus, K_S apparent would be equal to $K_S\ (\frac{M}{Y})$ (Lineweaver and Burke, 1934).

Assumptions of the above derivation include:

1) rapid equilibrium of renin with S, A, and I. Therefore, the rate-limiting step is kp.

2) dissociation of complexes to form product is unidirectional during initial period of reaction during which measurements are made (kp is irreversible).

3) binding of activator and inhibitor to enzyme affects subsequent binding of substrate by a factor of α and β, respectively.

4) dissociation of activated and inhibited substrate complexes to form product proceeds at a rate of akp and bkp, respectively. The constants a and b are proportionality constants relating to the dissociation of the affected complex (kp).

APPENDIX B

APPENDIX B

Prediction of the Equilibrium Constant for the Renin-Substrate Reaction in Plasma.

Considering the velocity equation derived in Appendix A:

$$V = \frac{d[P]}{dt} = \frac{Vmax\ [S]}{K_S \frac{(1 + \frac{[A]}{K_A} + \frac{[I]}{K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})} + [S]\frac{(1 + \frac{[A]}{\alpha K_A} + \frac{[I]}{\beta K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}} \quad (1)$$

taking the reciprocal

$$\frac{1}{v} = \frac{K_S \frac{(1 + \frac{[A]}{K_A} + \frac{[I]}{K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})} + [S]\frac{(1 + \frac{[A]}{\alpha K_A} + \frac{[I]}{\beta K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}}{Vmax\ [S]} \quad (2)$$

and,

$$\frac{1}{v} = \frac{K_S}{Vmax} \frac{(1 + \frac{[A]}{K_A} + \frac{[I]}{K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})} \cdot \frac{1}{[S]} + \frac{1}{Vmax} \cdot \frac{(1 + \frac{[A]}{\alpha K_A} + \frac{[I]}{\beta K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})} \quad (3)$$

if we express

$$M = \frac{(1 + \frac{[A]}{K_A} + \frac{[I]}{K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}$$

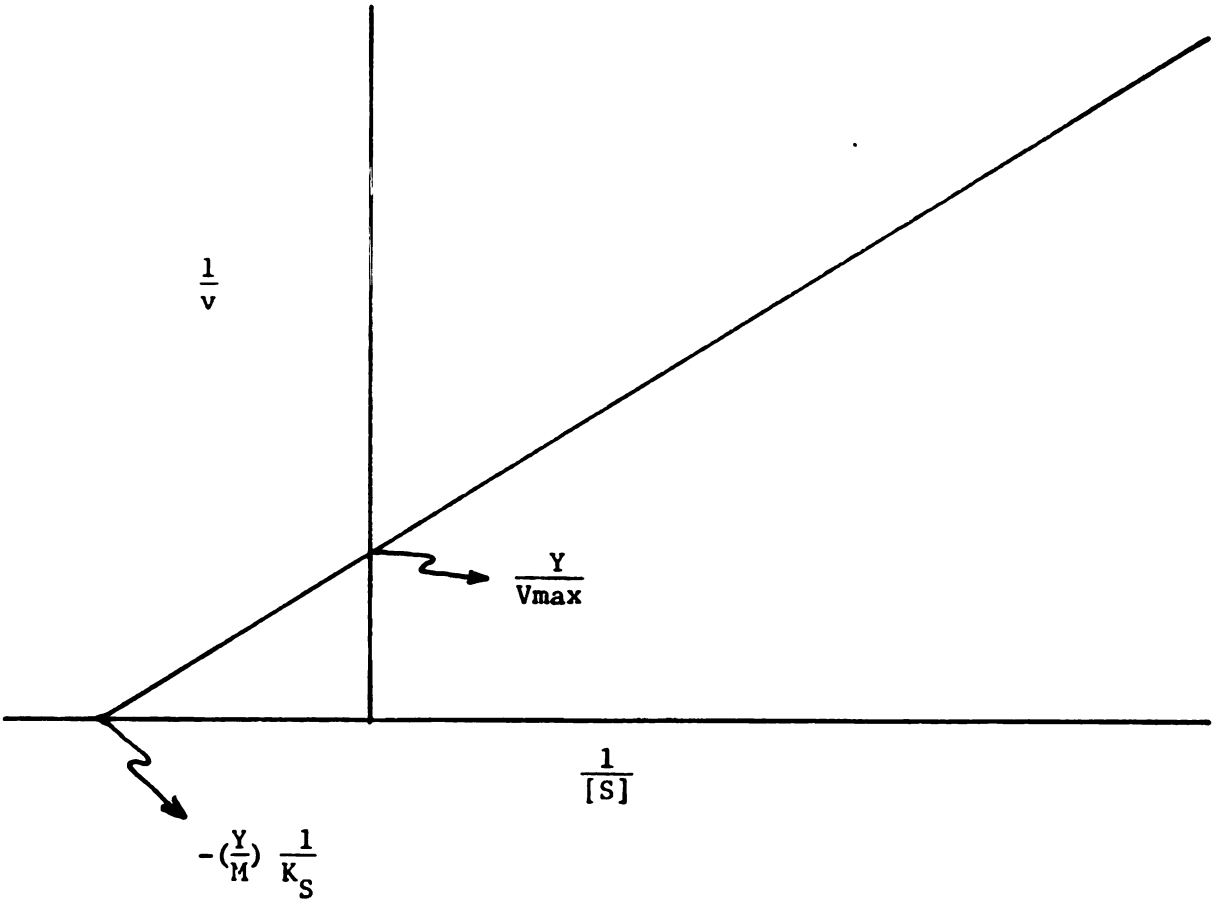
and

$$Y = \frac{(1 + \frac{[A]}{\alpha K_A} + \frac{[I]}{\beta K_I})}{(1 + a\frac{[A]}{\alpha K_A} + b\frac{[I]}{\beta K_I})}$$

then,

$$\frac{1}{v} = \frac{Km}{Vmax}(M)\ \frac{1}{[S]} + \frac{1}{Vmax}(Y) \qquad (4)$$

describes a lines having a slope of $\frac{Km}{Vmax}(M)$ and intercepting the ordinate at $\frac{Y}{Vmax}$. The intercept of the abscissa would then be $\frac{Y/Vmax}{(M)\ K_S/Vmax} = -(\frac{Y}{M})\frac{1}{K_S}$.



From equation (3) the only means by which a single straight line can be generated is if the bracketed terms (M and Y) are constant when substrate is varied. This only occurs when Y = M = 1.0. Alteration of [A], [I], K_A, K_I, a, b, α, or β would necessarily result in a disproportionate change in both the Y and M terms due to the proportionality constants of the respective denominators.

Therefore, if the data plotted in this reciprocal fashion generate a straight line, it can be concluded that neither activators nor inhibitors of the renin reaction are present in the substrate sample. Similarly, effectors present in the plasma renin sample would ultimately affect the kinetics such as to generate a non-linear reciprocal plot.

A straight line generated by such reciprocal plotting can be interpreted as indicating a true first-order reaction process described by the equation:

$$\frac{1}{v} = \frac{Km}{Vmax}\ \frac{1}{[S]} + \frac{1}{Vmax}$$

and

$$v = \frac{Vmax\ [S]}{K_S + [S]}$$

Since $v = \frac{d[P]}{dt}$ and $Vmax = kp[R_t]$, the velocity equation can be rewritten as,

$$\frac{d[P]}{dt} = \frac{kp[R_t]\ [S]}{K_S + [S]}$$

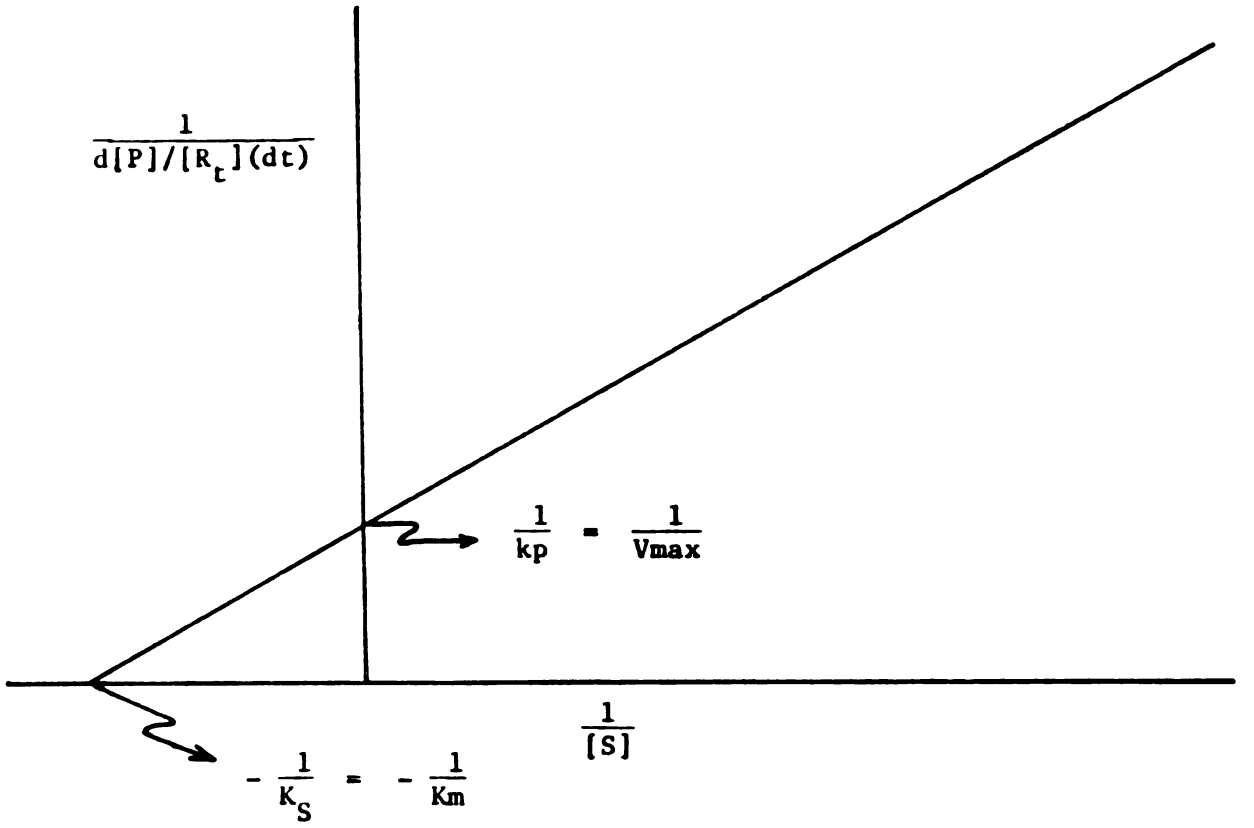
Factoring both sides by $[R_t]$,

$$\frac{d[P]}{[R_t](dt)} = \frac{kp\ [S]}{K_S + [S]}$$

and taking the reciprocal of both sides of the equation gives;

$$\frac{1}{d[P]/[R_t](dt)} = \frac{K_S + [S]}{kp\ [S]} = \frac{K_S}{kp}\ \frac{1}{[S]} + \frac{1}{kp}$$

Therefore, a double-reciprocal plot of such data would generate a line having a slope of $\frac{K_S}{kp}$ and intercepting the ordinate at $\frac{1}{kp}$. The intercept of the abscissa would then be $-\frac{1/kp}{K_S/kp} = -\frac{1}{K_S}$.



From this plot, K_S represents the classical Michaelis-Menten constant (Km) for the renin-substrate reaction and kp is the maximum rate of AI generation (Vmax) when normalized to the amount of enzyme added to the reaction mixture. Since $Vmax = kp[R_t]$, the measured Vmax reflects the actual plasma renin concentration.

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