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ANGIOTENSIN-CONVERTING ENZYME IN LUNG AND KIDNEY

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Kendall Bruce Wallace

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ANGIOTENSIN-CONVERTING ENZYME IN LUNG AND KIDNEY OF DEVELOPING ANIMALS

By

Kendall Bruce Wallace

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ABSTRACT

Angiotensin-Converting Enzyme in Lung and Kidney
of Developing Animals

By

Kendall Bruce Wallace

Angiotensin-converting enzyme (ACE) catalyzes rapid conversion of angiotensin I (AI) to angiotensin II. Inasmuch as age-related differences exist in the renin-angiotensin system, and since ACE is the final catalytic component of this system, it was of interest to determine converting enzyme activity in developing animals. ACE activity was quantified spectrophotometrically by measurement of the hippuric acid liberated from hippuryl-L-histidyl-L-leucine (HHL) following incubation with the 20,000 x g supernatant of tissue homogenates. Rat pulmonary converting enzyme activity increased during the first 6 weeks postpartum in a biphasic manner. A similar age-dependent increase in ACE activity was observed in rat kidney, mouse kidney and mouse lung. Substrate affinity of all enzymes measured was similar, suggesting that the age-related difference was due to increased converting enzyme content of the older animals. Induction of fetal lung ACE activity was observed following maternal administration of aminophylline. The low activity of ACE in the newborn might function to limit AII production during the immediate postnatal period.

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INTRODUCTION

Angiotensin-converting enzyme (ACE) [peptidyldipeptide hydrolase, EC 3.4.15.1] was first isolated from horse plasma by Skeggs (169). Ng and Vane demonstrated, however, that the activity of this enzyme in plasma was insufficient to account for the generation of angiotensin II (AII) in vivo and postulated that the lung was the major site of angiotensin I (AI) conversion (136,137). Converting enzyme activity has subsequently been identified in nearly every tissue and specie studied (50,156). The localization of ACE in the vascular endothelium of lung, liver, adrenal cortex, pancreas, kidney and spleen suggests that this enzyme is directly accessible to circulating AI (28,160,163). However, only the lung has been shown to be capable of liberating significant quantities of AII into the venous effluent following addition of AI to blood perfusing the tissue (1,136,137,138). In tissues other than the lung, rapid uptake and/or metabolism of AII accounts for the small quantities of this octapeptide in the tissue effluent. Therefore, it appears that pulmonary converting enzyme is responsible for generation of AII destined for the systemic circulation whereas extrapulmonary ACE is involved in the production of AII utilized locally (1,141).

Age-related differences in plasma renin activity, plasma renin concentration and renin substrate concentration have been reported in

humans (90,113,151). Pohlova described similar changes in the components of the renin-angiotensin system in developing rats (152); however, ACE was not measured. Since ACE is the final catalyst involved in the generation of vasoactive AII, characterization of the renin-angiotensin system in developing rats remains incomplete.

The purpose of this investigation was to determine the age-dependent changes in converting enzyme activity in rats and mice. Since ACE of lung is active in liberating AII into the circulation (1,136,137,138) whereas the renal enzyme is involved in the local generation of AII (51,141), these two tissues were chosen as the source of ACE in this investigation.

Development of the Lung

Prenatal development of human lung can be divided into four stages: embryonic, pseudoglandular, canalicular and terminal sac period (139). An alveolar period follows after birth. During the pseudoglandular and canalicular stages, the epithelial cells of lung contain high concentrations of glycogen (177). At the stage of development of differentiation into type I and type II alveolar cells, the intracellular glycogen content diminishes dramatically in epithelial cells but persists in the mesoderm until term. Osmiophilic lamellar bodies appear in type II alveolar cells relatively late in gestation (31,109,110,187), and rapidly increase in number just prior to parturition (109). Peroxisomes, dense bodies containing H_2O_2-producing enzymes (148), appear in type II cells much earlier in development than do lamellar bodies and increase in number 3-fold by term (167).

Structural development of fetal lung in general, and its epithelial differentiation in particular, is accelerated by direct administration of corticosteroids (8). A similar induction of fetal lung maturation is observed following injection of L-thyroxine (188), whereas hypophysectomy retards epithelial differentiation (17).

Early in postnatal life, the lung acini subdivide into new alveoli (27) resulting in an increased number and surface area (but decreased mean diameter) of alveoli. Labelling of fibroblasts with [^{3}H]-thymidine reaches a peak during this period (106). Pulmonary maturation is accompanied by a 5-fold increase in collagen, as a proportion of total lung protein (20). Boyden and Tompsett (19) have reconstructed serial sections from newborn lung and concluded that budding starts in the peripheral saccules of the acinus and spreads proximally. New alveoli continue to appear until near the time of onset of puberty (44), with the majority of the increase occurring soon after birth (55).

Both available thoracic space and inspired oxygen concentration influence postnatal lung growth. Compensatory contralateral hypertrophy followed unilateral pneumonectomy (39,73). Mitotic activity and collagen synthesis per cell were maximal one week following removal of one lung from young rats (73), the wet weight of the remaining lung was equal to that of the combined weight of both control lungs. Compensatory lung growth was enhanced by hypoxia, retarded by hyperoxia, and unaffected by CO_2 breathing (22).

Fetal lungs contain a volume of liquid similar to that of the functional residual capacity of newborn lungs (135). This fluid

leaves the lung via the trachea to be swallowed or added to the amniotic liquid (140). The lung liquid is formed by transfer of solutes and water across the endothelium of pulmonary caillaries and the epithelium of fine air spaces (103). The permeability of the endothelium and epithelium are very different. The endothelium allows passage of large molecules such as antibodies into the interstitial space whereas the epithelium limits penetration by molecules having a diffusion radius greater than 0.55-0.60 nm, thus maintaining an osmotic gradient in favor of absorption to the interstitial space (18,140). Normand et al. demonstrated nonpolar, lipid soluble substances to penetrate the epithelium much more readily than polar solutes (140). Two pathways for solute transfer across the epithelium have been postulated (146): one for polar solutes, similating tight junctions between adjacent epithelial cells, and the other for nonpolar substances consisting of lipoid epithelial cell membranes. Within a few hours after birth, lung liquid is almost completely absorbed, as reflected by a large increase in pulmonary lymph flow (100). Egan and coworkers (57) have postulated an increase in epithelial permeability upon distension of the lung to account for the lung fluid absorption. This hypothesis is supported by the observation that in adult dogs, estimated pore radius varied from 0.9 nm at functional residual capacity to 4.0 nm at 90% total lung capacity (56).

Pulmonary surfactant appears relatively late in gestation, at about the same time as when the osmiophilic lamellar bodies are first seen (25,62,67,81,153,154). In several species it has been possible

to describe a period of surfactant storage in lung tissue followed by secretion into acini (76,81). The concentration of surfactant continues to increase until term (35,79). The major component of surfactant has been determined to be the saturated phosphatide, dipalmitoyl phosphatidyl choline (78,111). The major pathway of synthesis has been suggested to be mediated by choline phosphotransferase which catalyzes the transfer of a phosphoryl choline moiety from cytidine diphosphate choline to a 1,2-diglyceride molecule (67,68,69,80,108, 132,178,186). In fetal rat lungs, choline kinase and choline phosphotransferase activities increase sharply two days prior to term (70).

Several investigators have demonstrated stimulation of fetal lung maturation following maternal (48,49,124,125) or fetal (112,131,155) administration of corticosteroids. This acceleration of pulmonary maturation has been measured by both increased lung compliance and elevated concentrations of pulmonary surfactant. Induction of the appearance of pulmonary surfactant following corticosteroid treatment has been attributed to stimulation of choline phosphotransferase activity (71). Aminophylline has also been demonstrated to enhance the maturation of fetal rabbit lungs (104). Both aminophylline and hydrocortisone have been shown to cause similar changes in fetal lung cyclic AMP content, phosphodiesterase activity, and surfactant production when administered to pregnant rabbits (14). Barrett et al. (14) suggested a possible role of cAMP in mediating the development of pulmonary surfactant production.

Changes in pulmonary blood flow and vascular resistance play an important role in the development of circulatory changes at birth.

Fetal pulmonary blood flow accounts for only 7-10% of the total cardiac output (158), the balance of the output of the right heart reaching the systemic circulation through the ductus arteriosus and foramen ovale. This arrangement depends on a high pulmonary vascular resistance, due in large part to contriction of vascular smooth muscle. At birth, ventilation leads to a lowering of pulmonary vascular resistance and a concomitant increase in pulmonary blood flow (184). The rising left atrial pressure leads to closure of the foramen ovale while the increased PO_2 perfusing the ductus arteriosus causes constriction and eventual occlusion of this vessel (47,158). Both bradykinin and the prostaglandins have been implicated in the mediation of circulatory changes of the neonate at term. Pulmonary lymph flow, reflecting net filtration from lung capillaries, is twice as high in the mature fetal lamb than in the 2-day-old newborn (100). These observations omit the large immediate postnatal increase in pulmonary lymph flow due to lung liquid clearance. Lymphatic vessels were found to be more numerous and larger in diameter in the fetus than newborn and adult. Boyd et al. calculated a larger epithelial pore radius for the mature fetus than newborn lamb (18), however, pore area per unit path length was greater in the newborn. These data were interpreted to suggest that much of the pulmonary microcirculation in the fetus is intermittently shut off by closure of precapillary vessels. This would then lead to exposure of the remaining open capillaries to a substantial portion of the high inflow pressure explaining the large pore radius and high net rate of filtration in fetal lungs. In the newborn, the pulmonary vascular bed is no longer constricted resulting

in a larger capillary surface area and a lower mean perfusion pressure and capillary filtration rate. Study of arterial pressure flow curves lends some support to the possibility that recruitment of vessels accounts for a major portion of the decreased vascular resistance observed at birth (33,38).

Surfactant, a surface-active lipoprotein formed in type II alveolar cells, appears in increasing quantities during the final days of gestation (62,67). Maternal administration of glucocorticoids accelerates surfactant production (48,49,124,125), through stimulation of choline phosphotransferase activity (71). Aminophylline has been shown to cause similar changes in fetal lung biochemistry (14,104).

In view of the vast morphological changes which occur in the developing lung, it was speculated that these structural changes may be reflected as functional alterations in pulmonary metabolism. Inasmuch as the pulmonary endothelium is the site of angiotensin I conversion, differences in angiotensin-converting enzyme activity were anticipated to accompany pulmonary development. Aminophylline was administered to near-term pregnant rats in an attempt to accelerate the development of fetal lung ACE activity.

Renin-Angiotensin System

In 1898 Tigerstedt and Bergman observed that crude extracts of rabbit kidney when injected intravenously produced a pressor response (181). These investigators named the active component of the extract renin. The role of the kidney in hypertension remained controversial until 1934 when Goldblatt demonstrated diastolic hypertension to follow constriction of the renal artery (82). Page (147) and Braun-Menendez (21) in 1939 reported that renin was not the pressor substance

but acted as an enzyme which released the constrictor substance, angiotensin, from a circulating plasma globulin.

In 1954, Skeggs et al. (1970) resolved the vasoactive substance by countercurrent distribution into two pure components, angiotensin I and angiotensin II. They found that the decapeptide, angiotensin I, could be converted to the octapeptide, angiotensin II, by a heat-labile plasma activity that required halide or nitrate ions. Both peptides produced a pressor response in vivo, however, Helmer (92,93) identified angiotensin II as the true vasoconstrictor material by its action on the isolated rabbit aortic strip. The amino acid composition of AI was determined by Skeggs and his associates shortly thereafter (172,173).

The enzyme responsible for conversion of AI to AII was first isolated from horse plasma (121). The enzyme was found to cleave the dipeptide histidyl-leucine from the carboxy terminus of angiotensin I to form angiotensin II. EDTA inhibited the converting enzyme (169), providing the first evidence that the enzyme is a metalloprotein. Converting enzyme was described as being a carboxy-terminal dipeptidyl peptidase, following experiments where radioactive AII (159) and His-Leu (101,164) were isolated from pulmonary effluents following infusion of appropriately labelled AI. Transpulmonary infusion of an undecapeptide with an extra histidine residue in position 10 of AI, did not yield AII in vivo (138). Lentz et al. (121) isolated stoichiometric amounts of a dipeptide from their converting enzyme reaction mixture and identified it as His-Leu by the dinitrofluorobenzene technique. 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 Lentz and his associates to conclude that proline was the next amino acid, which was subsequently confirmed when they determined the entire sequence of equine angiotensin II (171).

In 1967, Ferreira and Vane (72) found that the smooth muscle dilator activity in the femoral arterial blood of cats was much greater following infusion of bradykinin into the aorta than into the right ventricle, suggesting pulmonary degradation of this polypeptide. That same year, Ng and Vane demonstrated AI to be far more potent when injected intravenously than intra-arterially (136). Over the next three years, these investigators studied angiotensin conversion in different tissus either in vivo with a blood-bathed organ technique (136,137,183) or in isolated perfused organs (1,13). The results of their studies suggested that the activity of converting enzyme in plasma was insufficient to account for the generation of AII in vivo, whereas a substantial percentage of AI conversion occurred during a single passage through the pulmonary circulation. Extrapulmonary conversion of AI was found to be far less significant than pulmonary conversion. It was concluded from these studies that the lung is the major site of generation of AII destined for the systemic circulation. In accordance with this postulate is the fact that angiotensin II

survives passage through the lung, unlike other tissues, with little degradation or extraction (96,118,137).

Experiments using the isolated lung preparation demonstrated that 75-99.9% of the injected bradykinin was degraded during a single transpulmonary passage (3,122). These findings provided the first evidence for a similarity in the site and rate of AI and bradykinin hydrolysis. During the following years several investigators questioned the validity of a single enzyme being responsible for both activities. Both angiotensin converting and bradykinin degrading activities are concentrated in the same subcellular fraction (4,9,165), and have been identified in a preparation of pulmonary plasma membranes (162). Ryan and co-workers have co-infused the vascular marker blue dextran with radiolabelled AI and bradykinin through perfused lungs (161,163). The radioactive metabolites emerged quantitatively and simultaneously with the dye indicating the occurance of both AI and bradykinin metabolism on the pulmonary vascular endothelium. Both enzyme activities have been shown to be inhibited similarly in vivo (36,60,85,107,179) and in vitro (4,10,105,166) by peptides isolated from the venom of Bothrops jararaca. The carboxy-terminal dipeptide of both substrates has been identified in the effluent following infusion of the substrate through isolated lungs (101,161). In partially purified preparations, bradykinin and angiotensin hydrolysis have been shown to be inhibited by chelating agents, suggesting both enzyme activities to be metalloproteins (4,12). As purification proceeds, the ratio of bradykininase to ACE remains constant, suggesting a similar enzyme moiety (182). The final product of purification, a single protein band, has been shown to hydrolyze both bradykinin and angiotensin (43,175). Pure

converting enzyme has been isolated from rabbit lung, hog lung, hog kidney and bovine kidney (52,145,175). All preparations catalyze hydrolysis of both AI and bradykinin. It is now fairly well established that a single, pure carboxyl-terminal dipeptidyl peptidase catalyzes angiotensin conversion and bradykinin degradation.

Angiotensin I conversion represents 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 (11,102,122). Ryan, Roblero and Stewart found that at least five peptide bonds were hydrolyzed when radioactive bradykinin was perfused through isolated rat lungs (161).

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 (169), and has been used as a definition of true converting enzyme. Although the hydrolysis of AI stops almost entirely in a chloride-free medium, that of bradykinin continues at about half the maximal rate (52,102,133). This anion requirement is best met with the halide (12,150,170) but varies with the substrate employed. In two preparations of pulmonary converting enzyme, as the concentration of chloride was progressively increased, conversion of AI also increased but bradykinin inactivation remained unchanged (2,166). Chloride has been described as an allosteric modifier of converting enzyme (34). Dorer et al. demonstrated chloride ion to significantly increase the maximal velocity and enzyme

affinity for bradykinin hydrolysis (52). The presence of chloride has been observed to alter the spectral properties of converting enzyme in the ultraviolet region (145), presumably reflecting a conformational change in the enzyme. Igic and co-workers (101) coupled converting enzyme covalently to Sepharose 4B to form a water-insoluble complex, and simulated organ perfusion by using a column filled 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 same system, these investigators demonstrated hydrolysis of neither peptide to be affected by the removal of chloride from the buffer system (102,133). Coupling of the pure enzyme to the column was suggested to 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. The hydrolysis of bradykinin was complete irregardless of enzyme conformation. It has been suggested that AI may have two possible orientations of binding to the enzyme, corresponding to low and high chloride concentrations (4,145). Only the latter orientation is suitable for AI hydrolysis to proceed. Bradykinin, however, has been suggested to bind with either enzyme protein configuration.

Converting enzyme has been estimated to constitute approximately 0.1% of total lung protein (43). The molecular weight of the native enzyme has been estimated to be approximately 130,000 (43,175). Much higher estimates of molecular weight have been obtained by gel filtration and were attributed to the large oligosaccharide content of the enzyme. The results from experiments using purified ACE indicate that the enzyme contains a single long polypeptide chain and is a highly

asymmetric molecule (175). The enzyme is rich in aromatic amino acids, contains a substantial number of half-cystine residues and a high content of hydrophobic residues. Converting enzyme has been suggested to possess a moderate degree of hydrophobicity (89), and contains one molar equivalent of bound zinc (43,175). The metal requirement for activity can be fulfilled by other divalent metals, including cobalt or manganese (4,12,41). Converting enzyme requires a free carboxyl group of the substrate (58), will not act on D-amino acid residues (65,142,144), and will not cleave a peptide bond consisting of the imino group of a prolyl residue (58), which explains why liberated angiotensin II is not further degraded by converting enzyme (190). 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 than angiotensin I (52).

Converting enzyme has been identified in nearly every tissue (42, 99,156) and specie (50) studied. Considerable indirect evidence has accumulated suggesting this enzyme to be a constituent of the vascular endothelium. ACE activity has been found in the particulate fraction of homogenates (9) and membranes enriched in converting enzyme activity have been prepared from lung tissue (163,165). Further support for the situation of the enzyme on the vascular surface of endothelial cells comes from the co-emergence in perfused lung effluents of radioactive metabolites of AI and bradykinin with the vascular marker, dextran blue (161,163). Ryan et al. (160) immunized goats with pure hog lung converting enzyme, conjugated the antibody to microperoxidase,

and studied the distribution of oxidized diaminobenzidine by electron microscopy after application of the conjugate peroxide and dye to rat lung sections. The heaviest electron-dense deposits were found along the luminal surface of the endothelial cells and their caveolae in capillaries and venules; demonstrating directly that ACE is a component of the vascular endothelial surface in opposition to the pulmonary circulation. Subsequently, fluorescein-labelled antibody to pure converting enzyme has been found to be concentrated in the vascular endothelium of rabbit lung, liver, adrenal cortex, pancreas and spleen (28). Renal ACE has been identified in the glomerular tuft and the brush border of proximal tubules (28,185,192). Carone et al. (32) have identified bradykininase activity on the proximal but not the distal tubule of the intact kidney.

In addition to the similar morphological localization of converting enzyme in various tissues, several other factors have been described which suggest a marked resemblance of ACE from different sources. Both lung and kidney ACE have been found to be concentrated in the microsomal subcellular fraction (9,64,65). Lanzillo and Fanburg observed pulmonary ACE from rat, hog and rabbit to migrate identically on polyacrylamide gels (116), suggesting equivalent molecular weight and subunit structure of the three enzymes. The value for the molecular weight they obtained agrees well with that estimated for ACE from hog, human and guinea pig plasma (98,119,145) and from rabbit lung (43) and bovine kidney cortex (145). These investigators also compared the kinetic and inhibitory properties of converting enzyme from guinea pig lung and serum (117). They found both enzymes to possess similar substrate affinity using either AI or HHL as substrate. When AI,

bradykinin or a tetrapeptide was added to the enzyme preparation, no difference in the inhibitory properties were observed for lung and serum converting enzymes. Immunologic studies have demonstrated crossreactivity between ACE prepared from hog kidney, lung and plasma (64) and between pulmonary converting enzyme of hog and rat (160) and rat, rabbit, guinea pig and dog (37).

Converting enzyme has been studied in various pulmonary diseases. Lieberman (123) and Oparil et al. (143) found elevated levels of ACE activity in serum and plasma of patients with sarcoidosis. The elevated serum ACE activity in patients with active sarcoidosis declined during daily prednisone therapy (123). Converting enzyme activity was depressed in patients suffering from several other pulmonary diseases such as chronic obstructive pulmonary disease, shock lung, cystic fibrosis, lung cancer and tuberculosis. These investigators postulated the altered blood converting enzyme activity during various pulmonary pathologies to imply a relationship between the pulmonary and plasma enzymes and suggested the plasma enzyme to derive from pulmonary endothelial ACE.

Within the last few years, the usefulness of converting enzyme inhibitors in treating renin-dependent hypertension has been recognized (24,61,77,115,129,130). The pentapeptide isolated from Bothrops jararaca lowers the elevated arterial blood pressure in rats with renovascular hypertension (115), however the nonapeptide SQ20,881 has a longer-lasting effect (15,60,61). The elevated blood pressure induced by renal artery stenosis is prevented by intravenous injection of SQ20,881 (129). This inhibitor also ameliorates the compensatory

rise in systemic arterial blood pressure in response to hypotension induced by endotoxin or hemorrhagic shock (63,66). These results are indicative of a role played by the converting enzyme as a component of the renin-angiotensin system in altering arterial blood pressure under these patho-physiological conditions.

Changes in the components of the renin-angiotensin system have been observed during development. Plasma renin activity (PRA) has been observed to be elevated in newborn puppies as compared to adult dogs (84). A similar age-dependent decrease in PRA has been observed in humans (90,113,151). Kotchen et al. also observed renin substrate concentration to be greater in newborn than adult human plasma (151). Pholova and Jelinek (152) described changes in the renin-angiotensin system in developing rats. These investigators noted that in rats 40 days and older, kidney weight was greater in males than females. Sex differences were also observed for renin substrate concentration and renal renin and renal angiotensinase activities. They showed that renin substrate concentration remained relatively unchanged or increased with age. Plasma angiotensin II half-life decreased with age, as did the PRA.

A striking maturation-dependent increase in ACE activity has been observed in rat testicular fluid (40) and in lungs of sheep (91). Friedli, Kent and Olley (75) have demonstrated pulmonary bradykininase activity to be undetectable in prenatal sheep, low in term fetuses and significantly higher in newborn lambs. Bradykininase activity was greatest in lungs from adult ewes.

Several different methods have been employed to assay converting enzyme. Bioassays using isolated smooth muscle or the pressor response

in intact animals and radioimmunoassays have been widely used to study conversion *in vivo*. The chemical measurement of His-Leu liberated from angiotensin I has been used to characterize ACE activity in broken cell preparations. The His-Leu can be quantitated as a radioactive product of appropriately labelled AI (99), by a fluormetric technique (34,150), or by an increase in ninhydrin-reactive material (54). The advantage of these assays is that they employ a physiological substrate and therefore provide a meaningful comparison of kinetic parameters for pure enzymes from different sources. However, a major disadvantage is that they 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 (119). A second type of chemical assay employs as substrate a model tripeptide protected at its amino-terminus. Blocking groups include benzyloxycarbonyl, hippuryl and *tert*-butyloxycarbonyl. The presence of the blocking group protects the peptide from degradation by contaminating aminopeptidase activities. HHL exemplifies the advantages of a model substrate. Neither it nor hippuric acid is destroyed by extraneous activities in crude lung homogenates (41), so that measurement of the latter provides a true assessment of enzyme content. However, since the model compound is an artificial 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 data. Cushman and Cheung (40) have compared the rate of HHL hydrolysis with that of AI during purification of converting enzyme. The relative rate of cleavage of the tripeptide and AI did not change throughout purification, suggesting a single enzyme to be

active in the hydrolysis of both peptides. Angiotensin I and HHL hydrolyzing activities have been coeluted from standard disc-gel electrophoresis and from gradient centrifugation (175). Finally, both hydrolyzing activities have been observed to exhibit similar inhibition properties when studied in vitro (117). Therefore, it appears that HHL is indeed a representative substrate of converting enzyme and that data obtained from studies using this tripeptide as substrate provide valid qualitative physiological relevance.

RATIONALE

Angiotensinogen, an α_2-globulin of hepatic origin, contains an active tetradecapeptide (renin substrate) side-chain from which the decapeptide angiotensin I is liberated. Cleavage of AI from angiotensinogen is catalyzed by the highly labile plasma enzyme, renin. Renin is synthesized and released from the juxtaglomerular apparatus of the kidney in response to various stimuli. Circulating AI possesses negligible pressor or aldosterone releasing activity. However, AI is rapidly converted to the highly vasoactive octapeptide AII during passage through several tissues. The enzyme responsible for AI hydrolysis (ACE) has been identified on the vascular endothelium, directly accessible to circulating AI, in almost every tissue. Converting enzyme from different sources possess similar structural, kinetic and immunoreactive characteristics, however only the pulmonary enzyme is capable of liberating AII into the systemic circulation. Extrapulmonary AI conversion appears to function in the production of AII utilized locally.

Age-dependent differences have been described for the various components of this system, however only semiquantitative and discontinuous data are available for the development of ACE. Inasmuch as converting enzyme is the final catalyst of the renin-angiotensin system (which may play a major role in establishing blood pressure in

the neonate), it was of interest to quantify ACE activity in developing animals. Since converting enzyme of lung is active in liberating AII into the systemic circulation, the development of this enzyme activity was of primary concern. In contrast, renal ACE is involved in the local generation of AII and therefore the maturation of this enzyme activity was compared to that of the lung. Kinetic analysis of enzymatic changes provided not only a comparison of enzyme structure in the various tissues, but also a mechanistic description of the parameters involved. Aminophylline, a phosphodiesterase inhibitor known to accelerate pulmonary epithelial maturation, was injected into pregnant rats in an attempt to enhance the development of fetal lung converting enzyme activity.

METHODS AND MATERIALS

Experiments were conducted on fetal, newborn, and adult Sprague-Dawley rats and Swiss-Webster mice (Spartan Research Animals, Inc., Haslett, Michigan). Animals 4 days and older were delivered to the laboratory at least one day prior to their use. Suckling animals were housed with 6-8 pups per lactating female. Nonsuckling animals (21 days and older) were housed 3 animals per cage. Near-term pregnant rats were purchased, housed in separate cages, and allowed to deliver spontaneously. Following parturition, the activity of lung converting enzyme was determined in the pups at 1, 2 and 3 days of age. Timed pregnant female rats were purchased on day 18 of gestation and housed in separate cages until day 20 (one day prior to normal parturition) at which time the pregnant mothers were sacrificed, the fetuses decapitated *in utero*, and fetal lung ACE measured.

Animals were killed by decapitation and the lungs and kidneys immediately removed and washed in cold NaCl (0.9%). The tissues were then weighed and homogenized (Polytron, Brinkmann Instruments) in 4 volumes of cold potassium phosphate-sodium chloride buffer (pH 8.3). The homogenate was centrifuged (Sorvall Inc.) for 20 minutes at 20,000 x g and 4°C. The supernatant was filtered through Whatman No. 2 paper and stored at 4°C. The filtrate was assayed the following day for converting enzyme activity.

Activity of the converting enzyme was determined by a modification of the method described by Cushman and Cheung (41). The enzyme incubation was initiated by addition of 0.10 ml of the filtrate to 0.15 ml hippuryl-L-histidyl-L-leucine (HHL). The HHL was purchased from Vega-Fox Biochemicals and prepared in potassium phosphate-sodium chloride buffer (pH 8.3). The final concentration of HHL in the incubation medium was 5.0 mM under standard assay conditions. Following a 30 minute incubation (Dubnoff Metabolic Shaking Incubator) at 37°C, 0.25 ml 1 N HCl was added to stop the reaction. The hippuric acid formed was extracted into 1.5 ml ethyl acetate by vortex mixing for 15 seconds and centrifuging at 2,000 x g (International Equipment Company) for 10 minutes. A 1.0 ml aliquot of the ethyl acetate phase was then evaporated at 40°C under nitrogen. The residual hippuric acid was dissolved in 1 M NaCl (3.0 ml) and allowed to stand at room temperature for 30 minutes. The optical density of the solution was then determined at 228 nM (Beckman DB-GT Spectrophotometer). The molar extinction coefficient for hippuric acid (E_{228}) is 9.8 mM^{-1} cm^{-1} and the fraction of hippuric acid extracted was 0.91 (41). Converting enzyme activity was expressed as nmoles hippuric acid liberated per min per mg tissue protein. Protein was determined by the method of Lowry (127).

This method for estimating converting enzyme was validated by addition of ACE inhibitors to the reaction mixture. Ethylenediaminetetraacetic acid (EDTA) and $CuSO_4$, bradykinin and [Asp^1, Ile^5]-AI (Beckman Instruments, Inc.), and the nonapeptide SQ20,881 (E.R. Squibb & Sons, Inc.) were each added to the incubation medium prior to the

addition of enzyme. Inhibition was expressed as percent hippuric acid formed in the presence of inhibitor as compared to control.

Blood was collected directly from the severed neck vessels into chilled vials containing 0.5 M ethylenediamine-tetraacetic acid (EDTA). Blood was separated by centrifugation at 1,000 x g for 15 minutes and the plasma analyzed by radioimmunoassay (RIA) for AI, renin and renin substrate concentrations (87). The RIA employed a specific antibody to pure [Asp^1, Ile^5]-AI. Plasma AI concentration was determined by addition of antibody, I^{125}-[Asp^1, Ile^5]-AI and the converting enzyme inhibitors dimercaprol (BAL) and 8-hydroxyquinoline (8-HQ) to the plasma sample. Following an 18 hour incubation at 4°C, the free and antibody bound AI were separated in a 10% charcoal suspension (pH 7.4). Endogenous plasma AI concentration was calculated from the relative amounts of bound and unbound radiolabelled AI determined by gamma scintillation counting of the fractions. Plasma renin concentration was estimated by measurement of AI generated during incubation of the plasma sample with high renin substrate plasma prepared from 24 hour bilaterally nephrectomized rats. Renin substrate concentration was estimated from the amount of AI generated following a 24 hour incubation of the plasma sample with high renin plasma. Plasma containing high renin activity was obtained from rats maintained on a sodium deficient diet for 3 weeks then dehydrated overnight prior to obtaining the blood sample.

Plasma AI and renin substrate concentration were expressed as ng AI/ml and plasma renin concentration was expressed as ng AI liberated/ml/hr incubation.

Induction of fetal lung maturation was achieved by maternal administration of aminophylline (10 mg/kg, i.m.) twice daily beginning on day 14 of gestation. On day 19, the pregnant female rats were killed, the fetuses decapitated *in utero* and fetal lung ACE activity determined.

Statistical significance was determined by analysis of variance (176). The 0.05 level of probability was used as the criterion of significance.

RESULTS

In characterization of this method for estimating ACE activity, optimal assay conditions were established for each enzyme preparation. When the 20,000 x g supernate of rat lung homogenate was incubated in the presence of 5.0 mM HHL, the rate of hippuric acid formation was constant up to 60 minutes (Figure 1). However, when enzyme activity exceeded 20 nmoles/min, HHL hydrolysis was no longer a linear function of time. The hyperbolic relationship between hippuric acid formation and time of incubation observed with high enzyme activities was interpreted to suggest substrate depletion at the longer time points. It was therefore necessary to perform preliminary 30 and 60 minute incubations of each enzyme preparation to obtain proper protein dilution to yield maximal activity yet exhibit a linear time dependence. It was then this enzyme dilution that was used in the determination of ACE activity under standard assay conditions.

The rate of hippuric acid formation, at enzyme activities less than 25 nmoles/min, was directly proportional to the amount of protein present in the incubation medium ($r^2 > .95$) and was independent of the potassium phosphate concentration in the buffer system (Figure 2). The similarity between the lines generated from protein dilution in the various buffer concentrations suggests the absence of any effect of phosphate ion on the activity of ACE. However,

Figure 1. Effect of incubation time on the activity of converting enzyme of adult rat lung. Incubation was carried out at 37°C in the presence of 5.0 mM HHL, 0.5 mg protein, and potassium phosphate-sodium chloride buffer (pH 8.3). Each point represents mean ± S.E. of 3 enzyme preparations. Circles without error bars indicate that the standard error was within the radius of the circle.

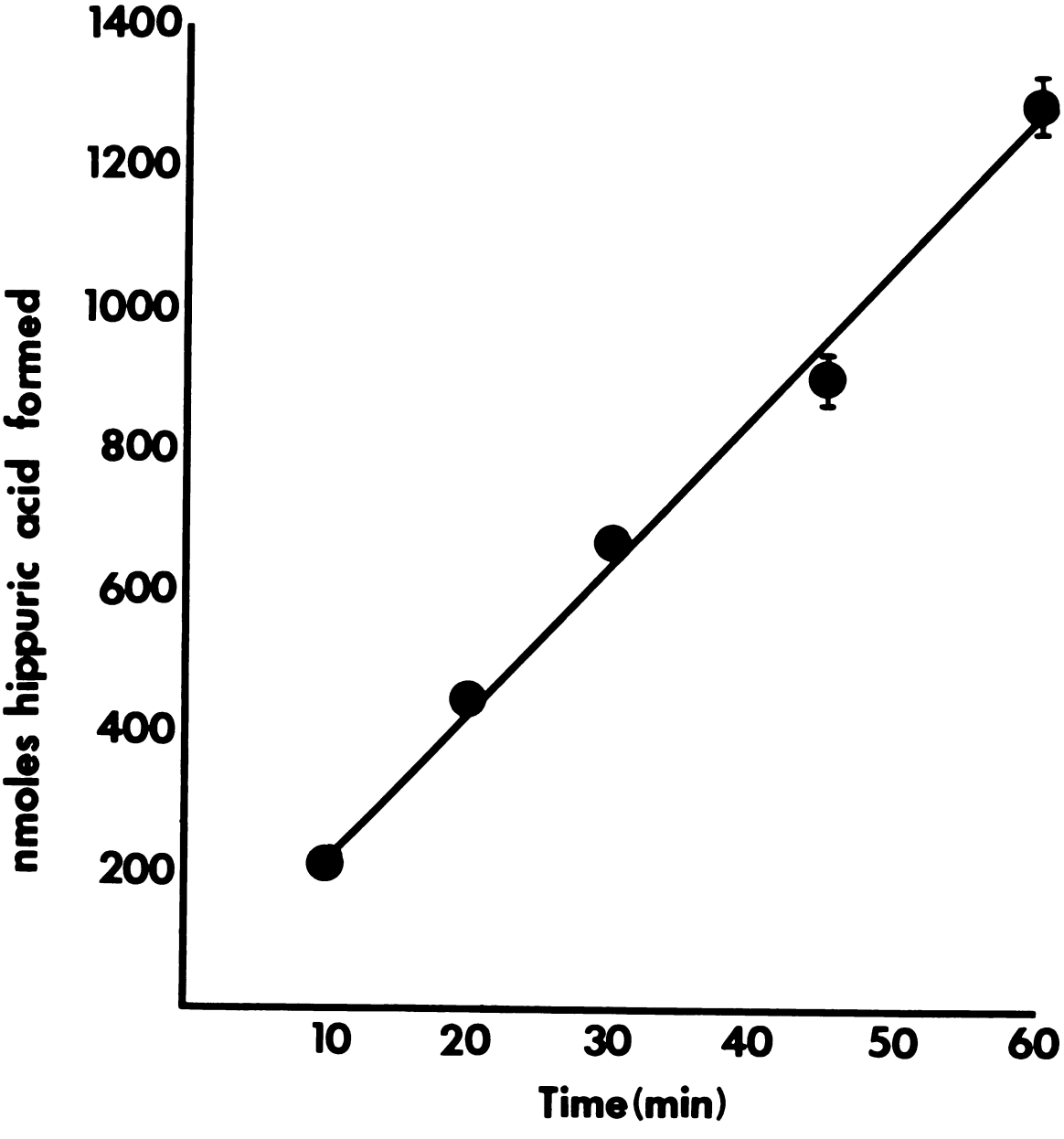
1400
1200
1000
800
600
400
200
nmoles hippuric acid formed
10
20
30
40
50
60
Time (min)

Figure 1

Figure 2. Effect of enzyme dilution on the activity of adult rat lung. Incubation was carried out at 37°C for 30 minutes in the presence of 5.0 mM HHL. Protein dilution was performed on enzymes prepared in different concentrations of phosphate in the buffer system. Each point represents the mean of 3 determinations. Values of r^2 represent the correlation coefficient for linearity.

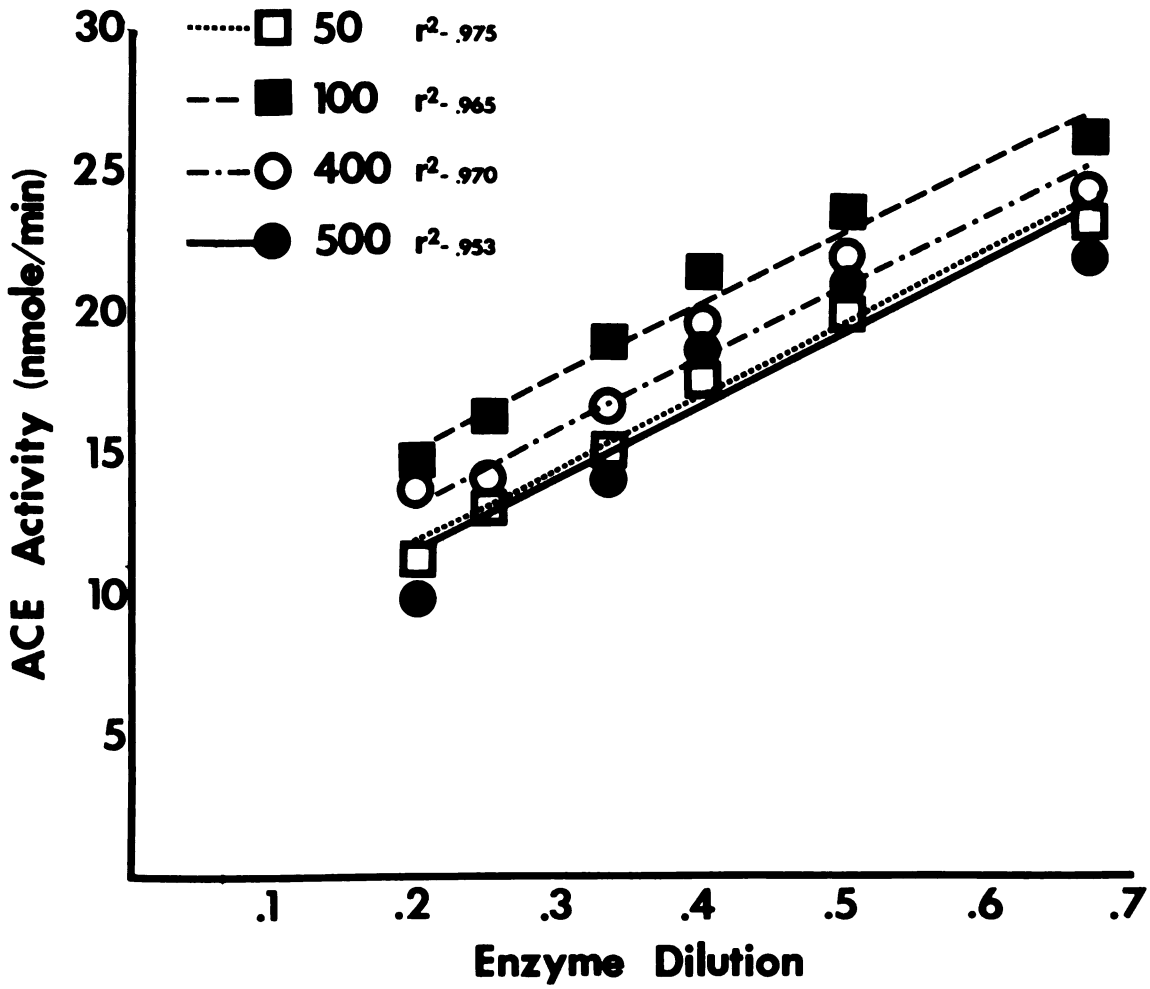
30
25
20
15
10
5
ACE Activity (nmole/min)
50 r2-.975
100 r2-.965
400 r2-.970
500 r2-.953
.1
.2
.3
.4
.5
.6
.7
Enzyme Dilution

Figure 2

when chloride was omitted from the buffer, converting enzyme activity was less when incubated in 100 mM than 500 mM potassium phosphate (Table 1). ACE activity was depressed 95% following removal of chloride from the 100 mM potassium phosphate buffer whereas more than 50% of the activity persisted in the absence of chloride from the 500 mM phosphate buffer.

Standard ACE inhibitors, each with different mechanisms of action, were tested for their effect on the rate of HHL hydrolysis by rat lung converting enzyme (Table 2). Enzyme activity, when incubated in the presence of 5.0 mM HHL, was inhibited 50% by $CuSO_4$, EDTA, bradykinin, and angiotensin I at concentrations ranging from 10-100 μM. The most potent inhibitor of hippuric acid formation was the nonapeptide, SQ20,881 (I_{50} = 110 pM).

ACE activity in crude rat lung homogenates was stable for at least 6 months when stored at 0-4°C in potassium phosphate-sodium chloride buffer (pH 8.3). The enzyme was readily denatured following preincubation at 50°C.

Development of converting enzyme activity of rat lung appeared to be biphasic (Figure 3). Enzyme activity in lung of prenatal rats was no different than 1 day old animals. Converting enzyme activity increased 4-fold by day 10 and remained relatively constant until 28 days of age at which time a second steep increase in activity occurred, reaching adult values by day 40. Protein content of the lung did not change significantly over the various ages measured. Converting enzyme activity in males was not different than females between 10 and 40 days of age.

TABLE 1

Effect of Chloride on Angiotensin-Converting Enzyme Activity[a]

	ACE Activity (nmoles/min)		
	300 mM NaCl[d]	Chloride-free[e]	%Δ[f]
Concentrated Buffer[b]	717.6	368.2	48.7
Dilute Buffer[c]	811.7	39.7	95.1

[a]Values represent mean of 3 determinations of ACE activity in adult rat lung.

[b]Enzyme preparation and incubation was carried out in 500 mM potassium phosphate buffer (pH 8.3).

[c]Enzyme preparation and incubation was carried out in 100 mM potassium phosphate buffer (pH 8.3).

[d]Sodium chloride was added to the buffer prior to enzyme preparation and incubation.

[e]Enzyme preparation and incubation was carried out in the absence of sodium chloride.

[f]Percent ACE activity lost by the omittance of NaCl from the buffer system $\left[1 - \frac{\text{ACE (chloride-free buffer)}}{\text{ACE (300 mM NaCl buffer)}}\right] \times 100$.

TABLE 2

Inhibition of the Angiotensin-Converting Enzyme[a]

Inhibitor	I_{50}[b]
$CuSO_4$	15 M
EDTA	35 M
SQ20,881	110 pM
AI	40 M
Bradykinin	55 M

[a]Adult rat lung was incubated for 30 minutes at 37°C in the presence of 5 mM HHL.

[b]Values represent mean of 3 determinations.

Figure 3. Converting enzyme activity of rat lung at various stages of development. Assay conditions were as described in the text. Each point represents mean ± S.E. of at least 3 determinations in animals of mixed sexes. Litter mates (up to 21 days postpartum) were pooled to represent one determination. Converting enzyme activity of fetal lung (20 days of gestation) is plotted on the ordinate (day zero).

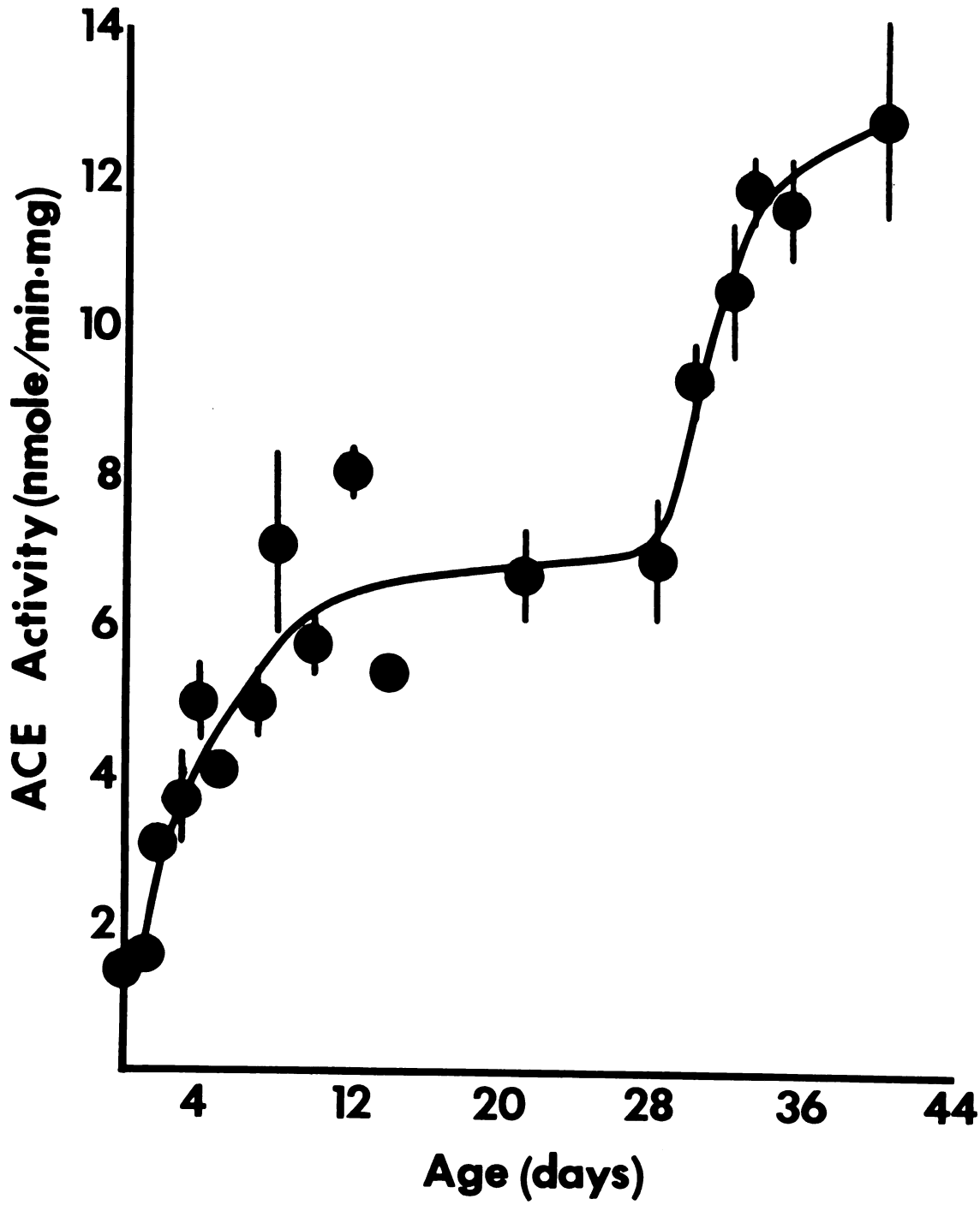

Figure 3

An age-dependent increase in converting enzyme activity was also observed for rat kidney, mouse kidney, and mouse lung (Table 3). Lung and kidney weight were significantly greater in adult than newborn in both animal species; however, there was no difference in lung or kidney protein concentration. Rat lung converting enzyme activity increased 3-fold between 4 days of age and adult. Enzyme activity of rat kidney was 8-fold greater in adult than newborn but was still less than the activity in newborn rat lung. In mouse kidney, converting enzyme activity was markedly greater than in rat kidney. The activity of converting enzyme of mouse kidney increased 2-fold between 7 days and adult which was comparable to the increase in converting enzyme activity of mouse lung.

Enzyme activity was markedly greater in adult than newborn rat lung over a wide range of substrate concentrations (Figure 4). Both newborn and adult enzyme exhibited a hyperbolic relationship between the rate of hippuric acid production and the concentration of HHL in the incubation medium. When the data from Figure 4 were arranged on an Eadie-Hofstee plot (Figure 5), near parallel lines were generated. The different points of intersection of the ordinate suggest a 3-fold difference in maximal activity of converting enzyme between newborn and adult rat lung.

The relative dependence of adult and newborn mouse lung converting enzyme on HHL concentration was similar to that seen in rat tissue. Both enzymes displayed first order reaction rates as a function of substrate concentration, with adult enzyme activity being greater than newborn (Figure 6). Representation of this data

TABLE 3

Angiotensin-Converting Enzyme Activity in Newborn and Adult[a]

	RAT[b]				MOUSE[b]			
	LUNG		KIDNEY		LUNG		KIDNEY	
	Newborn (4 days)	Adult	Newborn (4 days)	Adult	Newborn (7 days)	Adult	Newborn (7 days)	Adult
Tissue Wt (gm)	0.19± .01[c]	1.36± .09	0.12± .01[c]	2.25± .22	0.10± .01[c]	0.28± .01	0.06± .01[c]	0.80± .05
Protein (mg/gm tissue)	65.25±1.26	64.18±2.48	57.38±4.91	54.25±1.64	68.57±1.90	68.04±2.78	71.81±3.24	65.23±1.38
ACE Activity (nmoles/min·mg protein)	4.08± .15[c]	14.25±2.00	0.21± 0.8[c]	1.64± .44	4.30± .17[c]	9.49±1.35	3.61± .43[c]	6.27± .21

[a]Values represent mean ± S.E. (n=3).

[b]Animals were of mixed sexes.

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

Figure 4. Converting enzyme activity of adult and newborn (4 days) rat lung as functions of substrate concentration. Each point represents mean activity of 3 enzyme preparations. Incubation was for 30 minutes at 37°C.

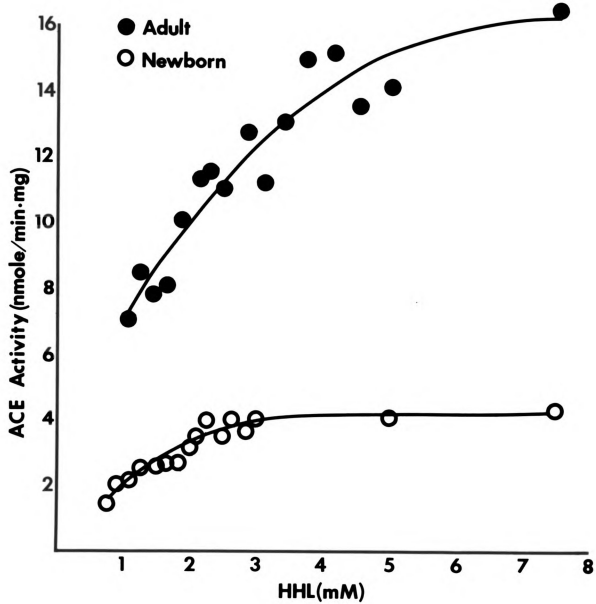
Adult
Newborn
ACE Activity (nmole/min·mg)
HHL(mM)
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8

Figure 4

Figure 5. Eadie-Hofstee plot of converting enzyme activity of newborn (4 days) and adult rat lung. Each point represents mean of 3 determinations. Converting enzyme activity (V) is expressed as nmoles/min·mg protein and S depicts HHL concentration (mM) present in the incubation medium. The best fit line was determined by linear regression, the method of least squares.

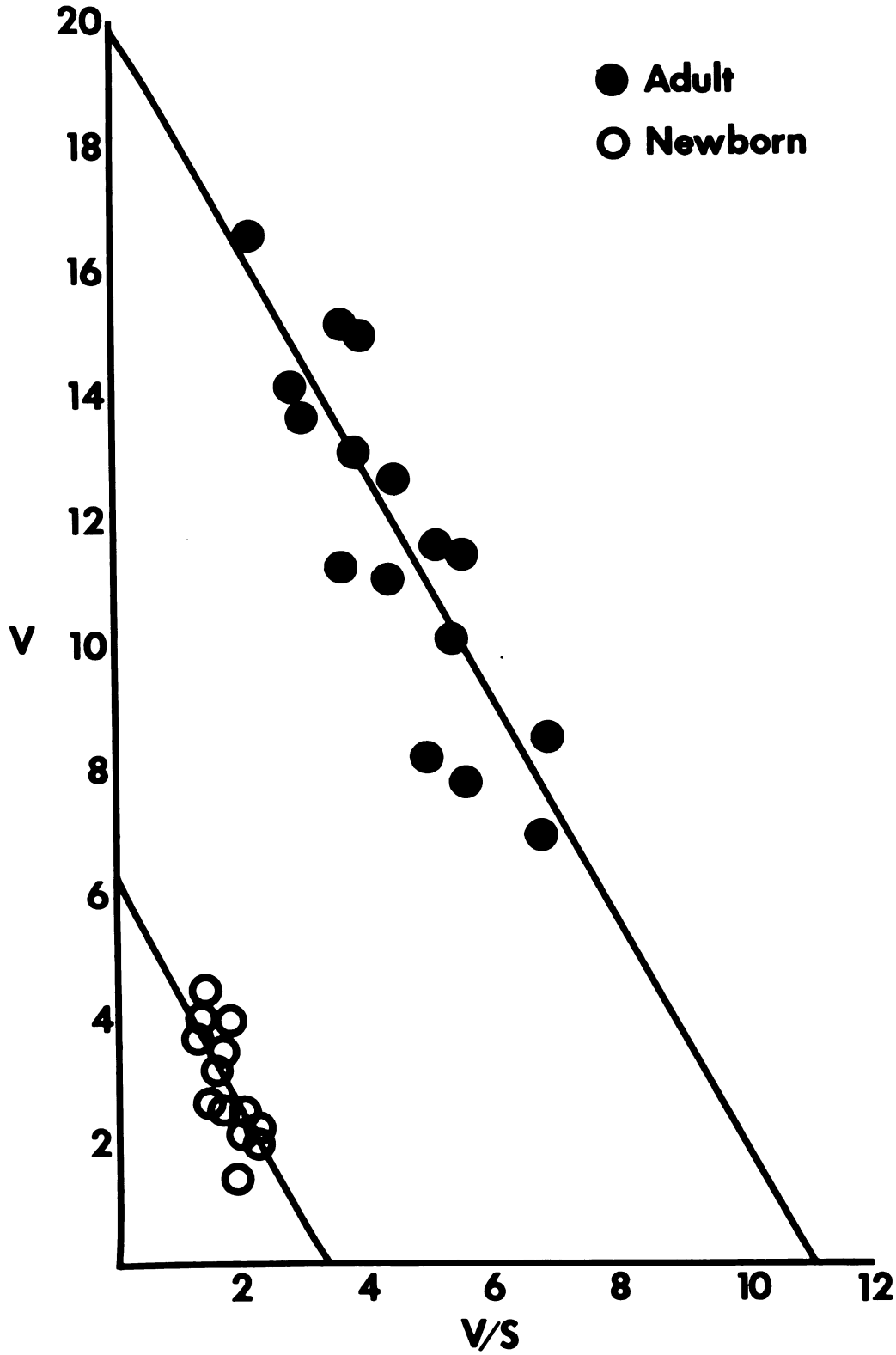

Figure 5

Figure 6. Converting enzyme activity of newborn (7 days) and adult mouse lung as functions of substrate concentration. Each point represents mean activity of 3 enzyme preparations. Incubation was for 30 minutes at 37°C.

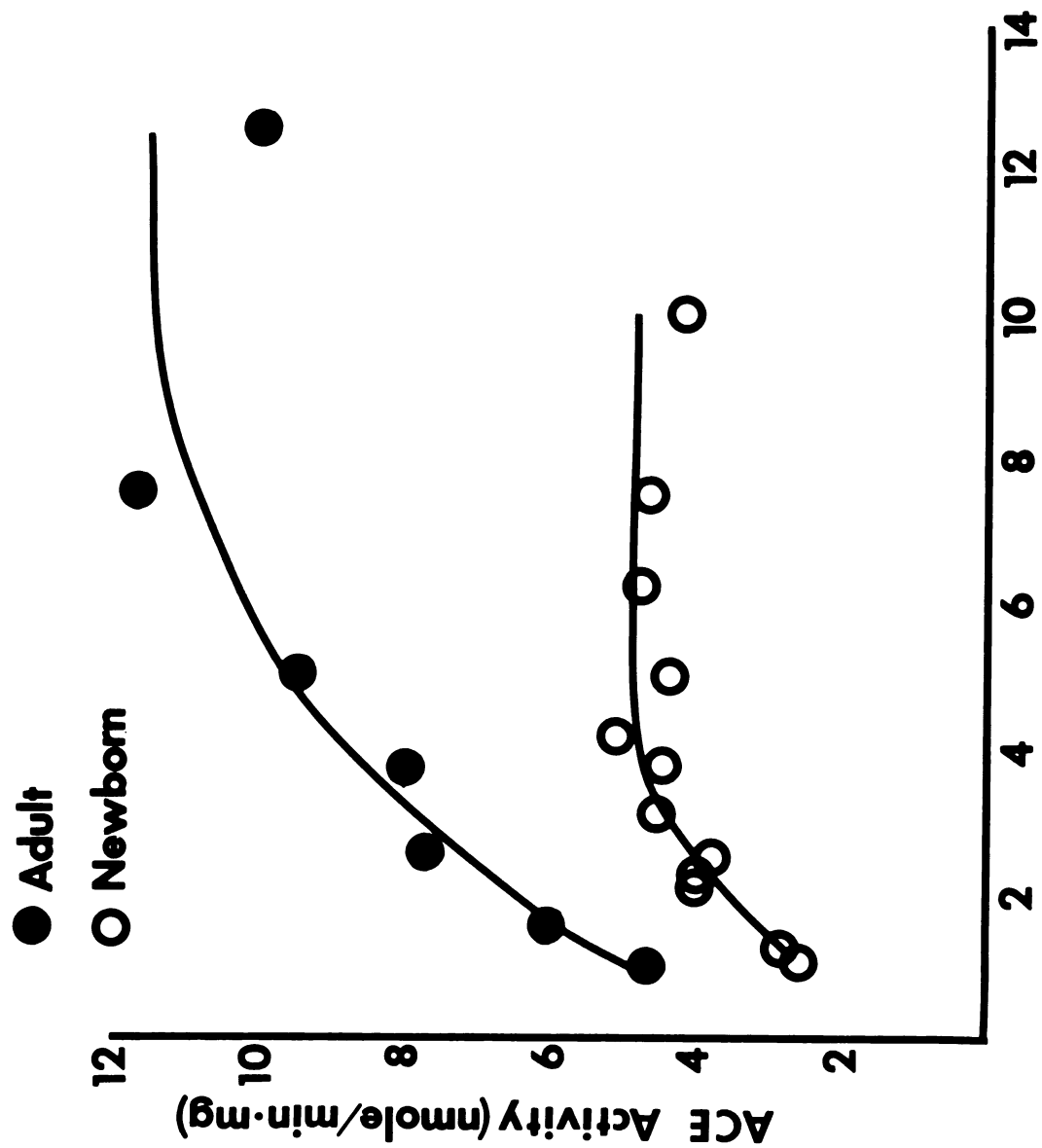
● Adult
○ Newborn
ACE Activity (nmole/min·mg)
12
10
8
6
4
2
2
4
6
8
10
12
14
HHL(mM)

Figure 6

on an Eadie-Hofstee plot (Figure 7) gave rise to 2 lines of equal slope, suggesting similar substrate affinity with 2-fold greater maximal rate of adult lung converting enzyme.

Similarly, mouse kidney converting enzyme activity was greater in adult than newborn, both activities being a hyperbolic function of HHL concentration (Figure 8). The magnitude of the difference in maximal converting enzyme activity was estimated from an Eadie-Hofstee plot of the data (Figure 9). The lines generated from adult and newborn enzyme activities were parallel.

The kinetic parameters obtained from the Eadie-Hofstee plots of the data are summarized in Table 4. In all tissues examined, Vmax for converting enzyme was greater in adult than newborn. The affinity (Km) of the enzyme for HHL was similar in all enzyme preparations measured.

Developmental changes in plasma renin and renin substrate concentrations were also observed (Table 5). The concentration of renin in the plasma of 4 day old rats was significantly greater than adult animals. The elevated plasma renin was accompanied by a concomitant decrease in renin substrate concentration in the newborn, however plasma AI was not different than adult.

Induction of fetal lung maturation by maternal administration of aminophylline resulted in an increase in fetal lung weight (Table 6). The increased tissue weight with no change in protein concentration suggests an increase in the total protein content of aminophylline treated fetal lungs. Concurrent with the increased protein content was an increase in ACE activity of the treated fetal lungs.

Figure 7. Eadie-Hofstee plot of converting enzyme activity of adult and newborn (7 days) mouse lung. Each point represents mean of 3 determinations. Converting enzyme activity (V) is expressed as nmoles/min·mg protein and S depicts the concentration of HHL (mM) present in the incubation medium. The best fit line was determined by linear regression, the method of least squares.

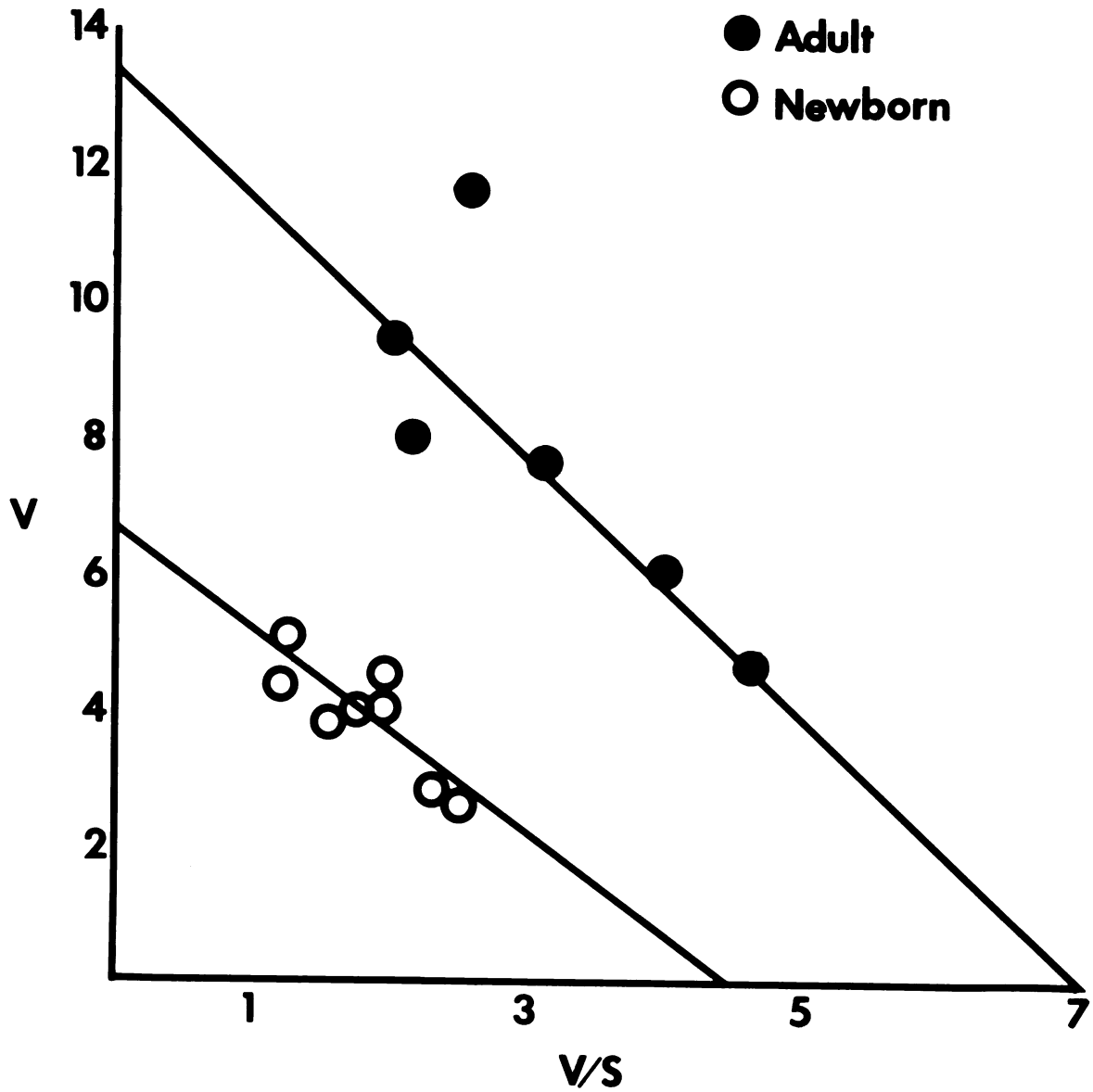

Figure 7

Figure 8. Converting enzyme activity of newborn (7 days) and adult mouse kidney as functions of substrate concentration. Each point represents mean activity of 3 enzyme preparations. Incubation was for 30 minutes at 37°C.

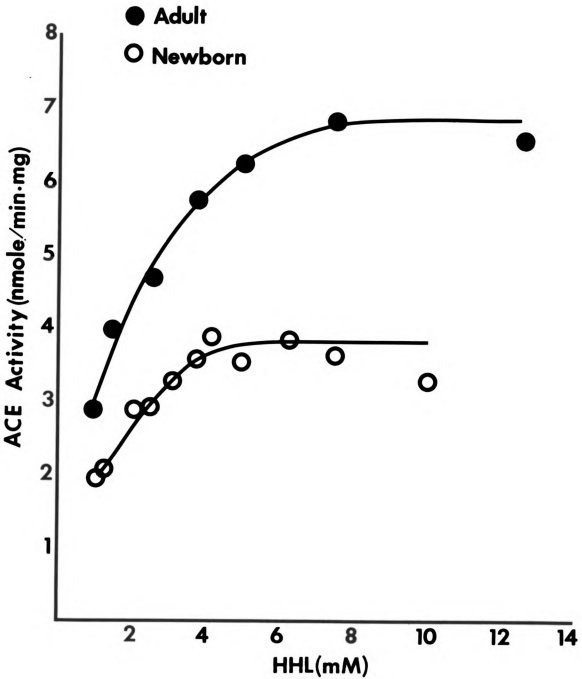
Adult
Newborn
ACE Activity (nmole/min·mg)
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
HHL(mM)

Figure 8

Figure 9. Eadie-Hofstee plot of converting enzyme activity of newborn (7 days) and adult mouse kidney. Each point represents mean of 3 determinations. Converting enzyme activity (V) is expressed as nmoles/min·mg protein and S depicts the concentration of HHL (mM) present in the incubation medium. The best fit line was determined by linear regression, the method of least squares.

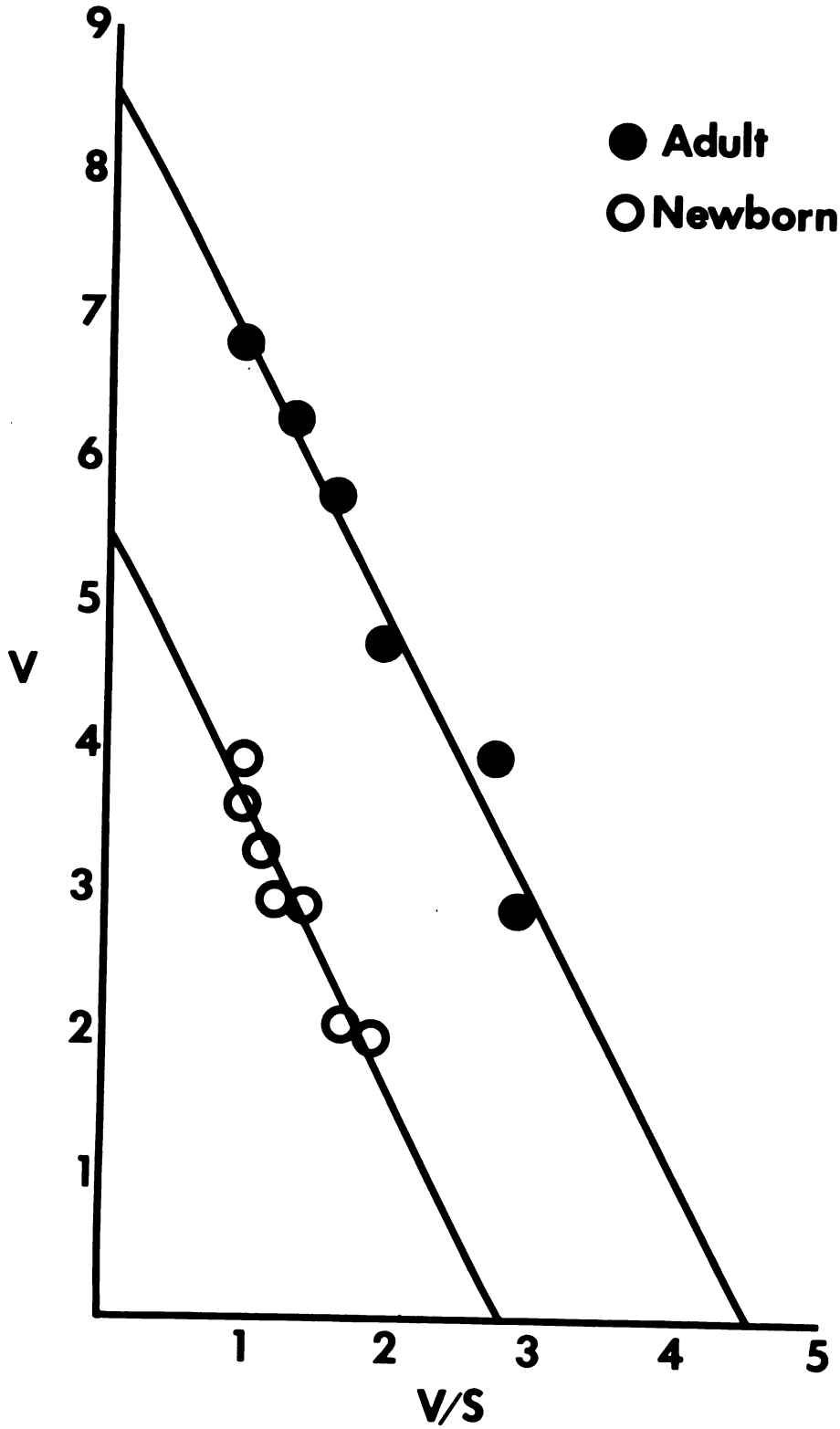

Figure 9

TABLE 4

Kinetic Parameters of the Angiotensin-Converting Enzyme

	RAT LUNG[a]		MOUSE LUNG[a]		MOUSE KIDNEY[a]	
	Newborn (5 days)	Adult	Newborn (7 days)	Adult	Newborn (7 days)	Adult
Vmax[b] (nmoles/min. mg protein)	6.27	19.87	6.65	13.33	5.47	8.58
Km[b] (mM HHL)	1.91	1.80	1.51	1.88	1.95	1.91

[a]Animals were of mixed sexes (n=3).

[b]Calculated from mean of 3 enzyme preparations.

TABLE 5

Age-Dependent Changes in the Renin-Angiotensin System of Rats[a]

	Newborn[b]	Adult
Plasma Renin Substrate Concentration (ng AI/ml/24 hr)	163.3 ±34.1[c]	445.9 ±47.6
Plasma Renin Concentration (ng AI/ml/hr)	52.34± 7.26[c]	18.15± 3.04
Plasma AI (ng/ml)	5.65± 3.14	1.11± .05
Lung ACE Activity (nmoles/min·mg protein)	4.08± .15[c]	11.26± 1.35

[a]Values represent mean ± S.E. of 3 determinations.

[b]Rats were 4 days old.

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

TABLE 6

Effect of Maternal Administration of Aminophylline on Fetal Lung Angiotensin-Converting Enzyme[a]

	Control Fetuses	Aminophylline-Treated Fetuses
Lung Wt. (gm)	0.07± .01	0.126 ± .01[b]
Lung Protein (mg/gm)	43.45±3.09	46.47 ±1.21
Lung ACE Activity (nmoles/min·mg protein)	0.72± .14	1.29 ± .13[b]

[a]Values represent mean ± S.E. (n = 3 litters of prenatal rats at 19 days of gestation).

[b]Significantly different from saline injected controls ($p<.05$).

DISCUSSION

ACE activity has been identified in both soluble and particulate subcellular fractions (4,9,53,133,165,166,191), the majority of the pulmonary enzyme sedimenting with the 107,000 x g pellet of most species (50). Converting enzyme of rabbit lung has been shown to sediment between 1,000 and 25,000 x g (165). The difference in the fraction in which this enzyme appears can be attributed to differences in the extent to which the enzyme-membrane complex is disrupted by homogenization. Assuming the intact enzyme-membrane complex sediments with the 20,000 x g pellet leaving the unbound enzyme in the supernate, differences in ease of membrane disruption would lead to an erroneous comparison of converting enzyme activity in the 20,000 x g supernatant. In an attempt to identify differences in ease of ACE dispersion from pulmonary endothelial membranes, converting enzyme activity was determined in both the 20,000 x g supernate and pellet of newborn and adult rat lung homogenates. The ratio of enzyme activity in supernate (s) to pellet (p) was no different for newborn than adult (s/p = 0.160±.011 and 0.190±.021 (n=3) respectively) indicating that measurement of converting enzyme in the 20,000 x g supernate provides a valid estimate of total ACE content.

These experiments were carried out in 500 mM potassium phosphate-300 mM sodium chloride buffer (pH 8.3) in contrast to the more

dilute buffer employed by Cushman and Cheung (41). In characterization of this method for measuring ACE, these investigators used a 100 mM potassium phosphate buffer (pH 8.3) and observed converting enzyme to be 7.5 times more active in the presence of 300 mM sodium chloride than in the absence of chloride ion. We found our crude converting enzyme preparation when incubated in 100 mM potassium phosphate buffer to be 20 times more active in the presence of 300 mM sodium chloride than in the absence of chloride. However, when the enzyme was incubated in 500 mM phosphate buffer, 50% of the HHL hydrolyzing activity remained in the chloride-free buffer system, suggesting that the more concentrated buffer prevented complete loss of ACE activity following the removal of chloride from the incubation medium. Several investigators have confirmed the chloride ion requirement of ACE (2,52,58,102,133,166,169), and have adopted this anion-dependence in the definition of true converting enzyme. This anion requirement is best met with halide (12,83,150,170) and varies with the substrate employed (34,53). Huggins et al. (98) showed both acetate and bicarbonate to restore at least 50% of the ACE activity in a chloride-free medium. Chloride has been described as an allosteric effector of converting enzyme (34). Study of the ultraviolet spectrum of purified hog kidney ACE demonstrated that chloride ions induce a spectral shift toward the red, presumably reflecting a conformational change in the enzyme molecule (145). It is possible that the various halides satisfy the anion requirement for AI hydrolysis via induction of a similar conformational change in converting enzyme structure. However, in the presence of 300 mM

sodium chloride, changing the concentration of potassium phosphate in the buffer system had no effect on converting enzyme activity. This might be interpreted to suggest a maximal, common allosteric effect on ACE induced by the various anions, chloride being the most efficacious allosteric effector.

An inhibitor of ACE activity has been identified in plasma (145,191). Such an inhibitor, if present in the crude enzyme preparation, would lead to an erroneous kinetic characterization of ACE. However, since the rate of hippuric acid formation was a linear function of enzyme concentration, it was concluded that the enzyme preparation was devoid of any converting enzyme inhibitors which would act to suppress HHL hydrolysis at high protein concentrations (168). The dipeptide hydrolytic product of ACE activity has been shown to competitively inhibit substrate hydrolysis (83,189,191). Since we were unable to identify converting enzyme inhibition following a 30 minute incubation, we concluded that the dipeptide His-Leu did not elicit product inhibition under our assay conditions.

A number of experimental observations leave little doubt that rat lung enzyme assayed by conversion of HHL to hippuric acid is identical with the angiotensin-converting enzyme. The rat lung enzyme required chloride ion for activity and was inhibited markedly by the more tightly bound substrates, angiotensin I and bradykinin. Bradykinin and AI, both being substrates for converting enzyme, function as competitive inhibitors of HHL hydrolysis (4,101,166, 191). Bradykinin has been shown to be more tightly bound by converting enzyme than AI (52), as indicated by the lower Km value

determined for bradykinin hydrolysis. Within the sensitivity of our determinations, however, we were unable to demonstrate a greater inhibition of HHL hydrolysis by bradykinin than AI. Angiotensin-converting enzyme requires a divalent cation cofactor for activity (4,12,41,54,169,175,191). Purification of the enzyme demonstrated ACE to contain one molar equivalent of bound zinc (43), however the metal requirement for activity can be fulfilled by other divalent metals (41,42,43,54,58). One-half of the HHL hydrolyzing activity was inhibited by addition of 35 μM EDTA to the incubation medium. Other investigators have observed similar inhibition of ACE activity by EDTA (169) and have ascribed inhibition to sequestering of the enzyme-bound zinc cofactor (4,41,43,105,166). Cupric sulfate also inhibited the enzyme activity, supposedly by an exchange reaction for the bound zinc (41,189). The most potent inhibitor of HHL hydrolysis was the nonapeptide, SQ20,881. This peptide is one of the many peptides originally isolated from the venom of Bothrops jararaca (72,105) and later shown to be inhibitors of ACE activity (9). The Bothrops peptides have been shown to be non-metabolizable substrates of the converting enzyme (34,101) and exhibit competitive inhibitory characteristics.

A similarity in converting enzyme isolated from various sources has been postulated. Lanzillo and Fanburg observed comparable molecular weight and subunit structure of ACE from rat, rabbit and hog lung (116). These investigators also found lung and serum converting enzyme of guinea pig to possess similar substrate affinity and inhibition characteristics (117). Immunologic studies have

demonstrated cross-reactivity between hog kidney, lung and plasma converting enzyme (64,145), hog and rat lung ACE (160), and between the pulmonary converting enzyme of rat, rabbit, guinea pig and dog (37). The molecular weight of human plasma converting enzyme was estimated by gradient centrifugation to be 150,000 (120), which is similar to the values obtained for rat, rabbit, guinea pig and hog lung ACE (116,119). The comparable substrate affinity observed for adult and newborn converting enzyme was interpreted to indicate that the similarity in ACE from lung and kidney persisted throughout development. The Km values obtained in these experiments agree with those found for ACE from other sources (41,43,117). The increasing converting enzyme activity with age without a concomitant change in substrate affinity implies that the increase in activity was due to a greater amount of active ACE rather than further activation of pre-existing enzyme. The increased amount of active enzyme can be attributed to increased enzyme synthesis, dilution of a non-competitive inhibitor, or increased availability of the enzyme to the substrate. However, inasmuch as homogenization of the tissue tends to mask any affect of enzyme availability, and since we were not able to demonstrate the presence of an inhibitor, we concluded that the developmental increase in ACE activity of both lung and kidney was due to increased enzyme content.

A striking maturation-dependent increase in ACE activity has also been observed in rat testicular fluid (40) and lungs of sheep (91). Friedli, Kent and Olley (75) demonstrated pulmonary bradykininase activity, as measured by the systemic pressor response to bradykinin infusion into the right atrium, to be undetectable in

prenatal sheep, low in term fetuses, and significantly higher in newborn lambs, but still far less active than in lungs of adult ewes. Lieberman measured ACE activity in normal human subjects of different ages (123). Analysis of the data indicates a statistically significant decrease in serum converting enzyme activity with advancing age. Lieberman also observed serum ACE activity to be greater in males than females. However, eventhough the differences were statistically significant, the changes were so small and the sample numbers so large that the physiological relevance is questionable. Furthermore, the development of ACE activity in serum was not quantitated for subjects less than 13 years of age, the time during which the largest changes would most likely occur.

Several speculations can be made as to the cause of this increased ACE content during development. Both pulmonary and renal ACE have been shown to be concentrated in the microsomal fraction (9,11,64,65,166,191). Increases in activity of various other pulmonary microsomal enzymes occur between birth and one month of age (7,74,88,97). It is therefore possible that the increase in converting enzyme activity observed during the first 3 weeks may reflect the postnatal development of microsomal enzyme systems in general. Development of these enzyme systems might reflect either an increased differentiation into or specialization of the pulmonary endothelial cells. Alternatively, since the rat at birth is far less mature than most other species and enters puberty shortly after weaning (180), prepubertal endocrine changes might influence the synthesis and/or availability of new converting enzyme. Elevation of plasma estrogen concentration has been suggested to be responsible

for the increased plasma renin substrate concentration during estrus in females (134) and during the third trimester of pregnancy (94, 149). However, since no sex differences in ACE activity were observed throughout development, it would seem that the gonadal hormones are without effect on converting enzyme. Therefore, if the endocrine system does act to alter the activity of this enzyme, hormones other than androgens and estrogens must be responsible. Finally, this system might be subject to dietary influences. Davis (46) has described the role of the renin-angiotensin system in the regulation of water, salt and circulatory homeostasis. The water and electrolyte intake of suckling, weaning and adult animals is drastically different. In the newborn, when ACE activity is low, electrolyte requirements are largely furnished through maternal milk. However, once the animal develops beyond the weaning stage, water and electrolyte intake become more sporadic. The increased ACE activity during this period might reflect the development of a mechanism by which the animal can regulate body fluid composition in response to a fluctuating intake of salt, water and other nutrients.

Bradykinin inactivation and angiotensin I conversion have been attributed to a single enzyme activity. Pure converting enzyme has been shown to catalyze both angiotensin I and bradykinin hydrolysis (52,145,175), the ratio of these two activities remaining constant throughout purification (182). Angiotensin I and bradykinin have been shown to compete for hydrolysis by ACE (4,101,166,191), and purified venom peptides of Bothrops jararaca have been shown to

diminish the pressor response to AI and potentiate the vasodilator effect of bradykinin in vivo (36,60,85,107,179). Antibodies prepared against pure converting enzyme have also been observed to inhibit the hydrolysis of both AI and bradykinin (29,145). Both activities have been localized in the microsomal fraction of the pulmonary endothelium (161,163) and require transitional metal ions for maximal activity (4,12).

The developmental implications of the bradykinin degrading activity of ACE are most evident during the perinatal period. Bradykinin production is stimulated by raising arterial oxygen tension (95), and has been suggested to play a major role in mediating circulatory changes of the neonate at term (128,157). Bradykinin, in very low concentrations, consistently induces constriction of isolated umbilical arteries (5,45,59), dilates the fetal pulmonary vasculature (6,30) and causes contraction of the ductus arteriosus (114). The low converting enzyme activity at term suggests less bradykinin degradation in these animals and thus increased circulating levels of this important vasoactive peptide during and immediately following parturition.

The physiological implications of the developmental increase in angiotensin I-converting enzyme can best be interpreted when viewed in context of the entire renin-angiotensin system. Plasma renin activity (PRA) has been observed to be elevated in newborn puppies when compared to adult dogs (84). Similarly, several investigators have demonstrated a developmental decrease in PRA in humans (90, 151). An age-related decrease in PRA was substantiated in these

experiments. Along with the decreasing PRA was a concurrent development increase in plasma renin substrate concentration, however plasma AI was not significantly different in the 4 day old rat than adult. Kotchen et al. observed renin substrate concentration to be elevated in newborn human plasma (113). Pohlova and Jelinek described changes in plasma renin, renin substrate and renal angiotensinase in developing rats (152). These investigators noted the inverse relationship between plasma renin concentration (PRC) and the age-related increase in arterial blood pressure observed by Litchfield (126). Pohlova (152) assumed ACE activity either remained relatively constant or was not a limiting factor in the generation of AII. However, both renal and pulmonary ACE activity were low at birth and progressively increased with age. In contrast to the adult animal in which renin is believed to be the rate determining component of the renin-angiotensin system (149,174), the low activity of ACE in the newborn might function to restrict AI conversion, thus limit plasma AII concentration. Broughton Pipkin et al. (23) found that in rabbits, circulating AII concentrations increased during the first two weeks of postnatal life and then declined to adult values. The increasing levels of arterial AII during the immediate postnatal period could be explained by the increasing activity of ACE. Angiotensin II could then depress plasma renin concentration by either increasing renal perfusion pressure (86) or by a direct inhibitory feedback mechanism on renin secretion (16,183). It is therefore possible that during the very early stages of development, ACE activity may limit AII production,

whereas later in development, plasma renin activity becomes the rate determining component of the renin-angiotensin system.

Several investigators have demonstrated accelerated fetal lung maturation following maternal (48,49,124,125) or fetal (112,131) administration of glucocorticoids. The enhanced pulmonary maturation was measured by both increased lung compliance and by elevated concentrations of pulmonary surfactant. The rate-limiting step in the synthesis of surfactant has been observed to be catalyzed by the enzyme choline phosphotransferase (67,80). Farrell and Zachman (71) were able to demonstrate selective stimulation of this epithelial enzyme following corticosteroid treatment. Maternal administration of aminophylline has also been shown to induce fetal lung maturation (104), by mechanisms which appeared to be very similar to that of hydrocortisone (14). Inasmuch as pulmonary converting enzyme activity was elevated in fetal rats following maternal administration of aminophylline, a possible correlate between epithelial and endothelial enzyme systems may exist. Inasmuch as the protein concentration of fetal lungs was not affected by aminophylline treatment, and since induction of the enzyme was apparent when activity was expressed per mg protein, it was concluded that stimulation of ACE activity was a relatively specific effect of the drug treatment. Increased converting enzyme activity in fetal animals could suggest increased bradykinin degradation. Since bradykinin has been implied to play an important role in regulating circulatory changes at term (128,158), such an increased inactivation of this peptide may lead to circulatory anomalies in the neonate. If ACE functions to limit AII production in the newborn, induction of this

enzyme activity of pre-term animals may produce alterations in the normal regulation of the renin-angiotensin system. Enhanced ACE activity following aminophylline treatment may act to increase circulating AII concentrations during the immediate postnatal period, and may result in the accelerated appearance of the time at which peak levels of AII are observed in newborn plasma. Such an effect could reflect an earlier time in development at which renin assumes the rate-determining function in the production of AII.

SUMMARY AND CONCLUSIONS

Angiotensin-converting enzyme was present in fetal rat lungs and progressively increased in activity up to 6 weeks postpartum. Similar increases in converting enzyme activity occurred in rat kidney, mouse kidney and mouse lung. The nature of the difference between newborn and adult was attributed to increased ACE content in the older animals rather than further activation of pre-existing enzyme. Kinetic analyses indicated a similarity in ACE which persisted throughout development of the various tissues. Induction of pulmonary epithelial development was accompanied by a concomitant increase in activity of the endothelium-associated ACE, suggesting a correlation between epithelial and endothelial enzyme systems.

In contrast to the increasing activity of ACE, plasma renin concentration decreased with age. In accordance with the age-dependent changes in circulating AII concentration and the reciprocal relationship between converting enzyme and plasma renin activity, it was speculated that sometime early in development, there is an interchange of the rate-determining process in the production of AII. During the immediate postnatal period, plasma AII concentration increases in parallel with the increasing ACE activity, suggesting the low converting enzyme activity to be limiting AII production. Sometime thereafter, however, plasma AII reaches peak levels then declines to adult values. During this later stage of development plasma renin activity may function to limit circulating AII.

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