The effects of sex and of dietary protein levels on plasma renin activity and angiotensinogen levels in mature beagles

by

Edwin Lincoln Minard, Jr.

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
MASTER OF SCIENCE

Department: Veterinary Anatomy, Pharmacology and Physiology
Major: Physiology

Iowa State University
Ames, Iowa

1975

TABLE OF CONTENTS

INTRODUCTION

The renin-angiotensin system has been shown to be an important control mechanism in the regulation of aldosterone secretion by Laragh et al. (1960), and in the regulation of blood pressure in certain hypertensive diseases, as excellently reviewed by Stein and Ferris (1973). There is increasing evidence that this system may also aid in the regulation of thirst and drinking by Simpson and Routtenburg (1973), and that it may be the final common pathway through which volume homeostasis is maintained, as suggested by Anderson and Olsson (1973).

Recently, it has become apparent that the normal function of this system may become disturbed by the administration of exogenous steroids as shown by Skinner et al. (1969) and by Menard et al. (1974); and, in certain species, by the endogenous steroids released during the estrous cycle, as reported by Nasjletti et al. (1971). The effects upon plasma renin activity and angiotensinogen concentrations by endogenous steroids has been reported in only a few species, however.

Little attention seems to have been paid to the influence of diet, other than mineral intake, on the renin-angiotensin system. Crump et al. (1973, unpublished research) have noted changes in plasma renin activity in dogs fed different levels of dietary protein, and the effects of starvation on various endocrine glands and on plasma renin activity have been noted by Rivero-Fontan et al. (1952) and Wilke (1974) respectively.

The purpose of this study was to note whether dietary protein intake, in diets otherwise identical, influenced plasma renin activity and the circulating concentrations of angiotensinogen in beagles, and to note

any differences in these factors which would be attributable to sex, and presumably therefore, to hormonal differences.

LITERATURE REVIEW

In 1898, Tigerstadt and Bergman announced the discovery of a substance in water extracts of kidney which affected the circulatory system. They named this substance renin for convenience, and noted that intravenous infusions elevated blood pressure and heart rate, and that solutions lost activity after boiling. Further investigations of renal influence on circulation languished until Goldblatt et al. (1934) noted that partial clamping of the renal artery led to persistent hypertension, whereas a full clamp of the renal artery produced uremia and death. In their experiment, they noted that no necrosis of kidney tissue resulted from this partial clamping. In 1938, Kohlstaedt et al. noted that renin, per se, appeared to have no pressor effect, but that it reacted with plasma to produce an active substance.

In 1940, Braun-Menendez et al. thought that renin was an enzyme, as suggested by its specificity of substrate and dependence on substrate to produce its vasoactive effect. The source of the substrate was in the pseudoglobulin (α globulin) fraction of plasma. They concluded that renin's substrate was formed in the liver, and that the active pressor agent was a polypeptide. Their results were confirmed by Page et al. (1941) who noted that extirpation of the liver in dogs removed the source of "renin activator" (angiotensinogen) and eliminated the pressor response to injections of renin. The dogs always remained responsive to doses of the polypeptide "angiotonin" (angiotensin) long after the response to renin had been abolished.

The hepatic source of angiotensinogen has been confirmed by Helmer and Griffith (1952) and recently by Nasjletti and Masson (1972). The latter workers perfused rat livers with labeled amino acids, and measured their incorporation into liver tissue and into secretions appearing in the hepatic veins. They noted that there was little storage of angiotensinogen in the liver, but that it was newly synthesized and released upon proper stimulus.

The nature of the active principle, the peptide, remained obscure until 1954, when Skeggs et al. noted that it existed in two forms, a decapeptide and an octapeptide. The decapeptide, Angiotensin I, was the product of the reaction of renin and angiotensinogen, but seemed to have no biological activity. Angiotensin II, the octapeptide, was formed in the plasma from Angiotensin I by enzymic action and was highly active. A major source of enzyme for the conversion of Angiotensin I is found in the lung (Ng and Vane, 1967).

The exact location of renin in the kidney has been questioned until quite recently. Goormaghtigh (1939) suggested that it was located in juxtaglomerular cells noting that the number of granules in these cells waxed and waned with increasing and decreasing ischemia, and correctly concluded that these cells produced and contained renin. In 1958, Cook and Pickering demonstrated the proximity of the source of renin to the glomerulus. They infused the glomerular tuft with finely divided magnetic material, gently ground the kidney and passed the material over a magnet, thereby retrieving the glomeruli free of tubular material. They demonstrated that the renin content of the magnetic fraction was far higher than that of the remaining material. Hartroft (1963) used immunofluorescence to locate

renin in thin sections of kidney tissue. She found that granules of the juxtaglomerular cells were labeled, but that the macula densa, glomeruli and tubules were not. She noted that sodium depletion and repletion increased and decreased the granularity of the juxtaglomerular cells, and the thickness of the adrenal cortex as well. Further evidence was provided by Thurau et al. (1972). Using an isolated rat juxtaglomerular apparatus, they noted that if the distal tubule was perfused retrograde with isotonic fluid containing 140 mEq/l of sodium, the renin activity of the fluid was three times greater than if the perfusate was normal tubular fluid containing only 40 mEq/l of sodium. The increase in renin release was rapid with changes in sodium concentration, indicating a release of stored renin, rather than a synthesis followed by release.

Until 1960, the pressor effects of the renin angiotensin system occupied the attention of investigators. In that year, however, Laragh et al. announced that angiotensin apparently exerted a major control over aldosterone secretion in man. They noted that, although other pressor substances such as epinepherine, norepinepherine and ephedrin were variable in their effect on aldosterone secretion, Angiotensin II invariably elevated circulating aldosterone, regardless of the state of sodium balance. Laragh and his coworkers noted, however, that in certain edematous states, high aldosterone concentrations were achieved without high levels of angiotensin, suggesting that other control mechanisms existed. In 1972, Brown et al. monitored Angiotensin II and aldosterone levels simultaneously in normal humans. They found a high positive correlation between the two sets of values during periods of sodium depletion and replacement. Aldosterone and angiotensin concentrations increased

during sodium depletion, and fell as sodium was replaced. Laragh (1973) concluded that aldosterone was controlled primarily by a dual mechanism involving potassium and Angiotensin II. If plasma potassium concentration rose, aldosterone also increased, thereby promoting kaliuresis. The effects of Angiotensin II, he thought, were the primary means whereby aldosterone was controlled.

Other effects of the renin-angiotensin system have been noted recently by several investigators. Using segments of rat colon, Hornych et al. (1973) found that low physiological doses of Angiotensin II (100 pg/ml) rapidly increased water flux from the mucosal to the serosal side of the segment. They noted that, like ADH, it increased sodium flux in the ascending colon, and decreased it in the descending colon. They thought the effect of Angiotensin II occurred directly on the mucosal cells, and was apparently mediated by adenyl cyclase.

Waugh (1972) found that solutions containing Angiotensin II in concentrations of 80 pg/ml caused increased sodium retention in denervated kidneys when infused along with isotonic saline. He concluded that this direct effect did not require aldosterone or renal nerve function, and that Angiotensin II may directly facilitate tubular sodium resorption.

Ferrario et al. (1972) reviewed articles connecting the central nervous system and the renin angiotensin system. They noted that Angiotensin II had a direct neural effect, entering the brain by way of the area postrema from the basilar artery, to affect connections between the area postrema and the nucleus of the solitary tract and dorsal vagal nerve. This would effect an increase in arterial pressure, mediated by increased sympathetic tone and, to some extent, by decreased parasympathetic discharge.

Simpson and Routtenburg (1973) found that doses of Angiotensin II amounting to 100 pg elicited drinking behavior in water sated rats, if applied to the subfornical organ. They noted that this organ lies outside the blood-brain barrier, and had previously been found dipsogenic when carbachol was injected. If lesioned, the subfornical area was insensitive to Angiotensin II. As a result of this experiment, these investigators hypothesized that elevated Angiotensin II levels might elicit drinking behavior, noting that this was one of only two known areas of dipsogenesis; the other lay in the lateral preoptic nucleus. Anderson and Olsson (1973) noted that recent studies indicate that an alternative to hypothalamic regulation of fluid balance by osmoreceptors may exist. There is evidence, they felt, that receptors near the third ventricle sensitive to angiotensin levels and sodium concentration may be involved in renal sodium excretion and maintenance of blood pressure.

The mechanism of control over renal renin release is still unknown. At present there are two major theories; control by blood pressure, with or without mediation via renal nerves, and control by fluid composition at the juxtaglomerular cell and/or the macula densa.

Recent supporters of the renal blood pressure theory are Coote et al. (1972) who used unilaterally nephrectomized, denervated cats in their study. They found that stimulation of the renal nerve caused a decrease in renal blood flow and a subsequent rise in plasma renin activity. This effect could be eliminated by pretreating the animal with an adrenergic receptor blocking agent. Their conclusion was that decreased renal blood flow, mediated by activity in the renal nerves, stimulated renin release. Esler and Nestel (1973) noted an increase in plasma renin activity in

hyper-expanded patients when they were tilted. This increase corresponded to an increase in norepinepherine under the same conditions, indicating, they thought, an effect of the sympathetic nervous system.

Evidence for control of renin release by fluid composition at the juxtaglomerular apparatus was provided by Thurau et al. (1972) and by Shade et al. (1973). Shade and his coworkers noted that renal renin release could be controlled by antidiuretic hormone and by Angiotensin II. In their experiment, they noted that there was no net change in renal blood flow, and concluded that the juxtaglomerular apparatus was stimulated directly or by changes in hemodynamics; the afferent arterioles constricting and the efferent arterioles dilating in compensation. Churchill and McDonald (1974) studied the effects of ouabain and partial aortic clamping on renin secretion from the kidney. They found that clamping the aorta decreased afferent arteriole transmural pressure but increased renin release, while ouabain, which also decreased afferent arteriole transmural pressure, did not increase renin release. They concluded that renin secretion was controlled by fluid flow or composition near the macula densa, and not by afferent arteriole transmural pressure.

Persons interested in pursuing the areas covered briefly in the foregoing review will find excellent bibliographies in the appropriate chapters of Kidney Hormones, J. W. Fisher, ed. (1971) which includes research up through 1969, and in Stein and Ferris (1973), a review of the physiology of renin, with particular emphasis on control of effective blood volume. The bibliography of Stein and Ferris must be used with caution, since some of the references are incorrectly cited.

The Effects of Steroid Hormones on Plasma Angiotensinogen Concentrations and on Plasma Renin Activity

Effects on angiotensinogen levels by ACTH and glucocorticoids

In one of the earliest investigations in this area, Haynes et al. (1949) found that injections of porcine derived anterior pituitary extract produced a significant increase in plasma angiotensinogen levels in dogs which lasted for several days. Following this rise, plasma angiotensinogen concentrations returned to normal levels. Activation of the adrenal cortex was assumed because of significant decreases in circulating eosinophils.

Helmer and Griffith (1951) found that ACTH raised plasma angiotensinogen levels in normal and hypophysectomized rats. In untreated, hypophysectomized rats, they found extremely low substrate levels. They also noted that desoxycorticosterone acetate (DOCA) greatly increased circulating substrate concentrations in adrenalectomized rats, thereby suggesting that substrate production by the liver required some adrenal function.

In 1972, Bing noted that angiotensinogen concentrations were about 50% of normal in adrenalectomized, salt substituted rats, and 75% of normal in rats treated with 0.5 mg/day DOCA plus salt. He found that normal levels of angiotensinogen could not be obtained by increasing the amount of DOCA administered from 0.5 mg/day to 1.5 mg/day. He noted that substrate concentrations rose proportionately in normal and adrenalectomized rats when both groups were bilaterally nephrectomized, but was unable to assign a reason for his findings.

Reid et al. (1973) compared the effects of dexamethasone and aldosterone on plasma angiotensinogen concentrations. They found that administration of dexamethasone increased plasma angiotensinogen levels, as had earlier workers, but that administration of aldosterone alone would not restore plasma angiotensinogen concentration in adrenalectomized animals. This, they felt, demonstrated that circulating glucocorticoids, and not mineralocorticoids, stimulate angiotensinogen production.

Reid et al. (1974) confirmed their findings of the previous year, noting that spironolactone administered to dogs in mineralocorticoid blocking dosage failed to prevent a return to normal in plasma angiotensinogen levels following a plasma dilution of 57% by exchange transfusion.

Freeman and Rostorfer (1972) noted that bilateral nephrectomy greatly increased plasma angiotensinogen eluted from rat liver slices. They concluded that this response was in part mediated by glucocorticoids, because the effect was minimal if adrenalectomy preceded the nephrectomy. Nasjletti and Masson (1972) also found that bilateral nephrectomy immediately accelerated angiotensinogen formation, but concluded that their findings suggested the possibility of a kidney angiotensinogen inhibitor, because the rise in production of angiotensinogen occurred so rapidly following nephrectomy.

Hasegawa et al. (1973a) have provided some evidence for a stimulator of angiotensinogen formation other than glucocorticoids. They found that rat liver slices taken from normal rats and incubated in 15 hour nephrectomized rat plasma produced angiotensinogen four times faster than did similar slices incubated with normal rat plasma. These investigators noted that the stimulating substance migrated with α globulins on electro-

phoresis was found in greater concentration in plasma than in serum, and was inactivated by boiling or by heating to 60°C for 30 minutes. There were apparently two components to the stimulator, since they claimed that one component disappeared following clotting of the plasma.

Effects of estrogens, progesterone and testosterone

In 1952, Helmer and Griffith noted that the administration of 0.05 mg diethylstilbestrol caused a twofold increase in plasma angiotensinogen concentration in male rats within 3 days of administration. Ethinylestradiol was even more potent; 0.02 mg produced elevations similar to the larger dose of stilbestrol. The effect was direct, these investigators thought, because adrenalectomized rats responded equally well, although hypophysectomized rats did not. Testosterone in 4 mg doses offset 69% of the effect of 0.02 mg of diethylstilbestrol, but progesterone had no discernible effect on angiotensinogen levels. The findings of Helmer and Griffith were confirmed in part by Menard et al. (1970) who administered DES to normal, water deprived, and adrenalectomized rats, and noted that plasma angiotensinogen levels were elevated in all three groups.

When Menard and Catt (1973) administered high doses of estrogens to male rats over a 5 day period, they found that the rise in plasma angiotensinogen levels declined following the second day of treatment, eventually falling below untreated levels by the end of the fifth day. They could offer no explanation for their findings, however.

Nasjletti and Masson (1972) investigated the incorporation of amino acids into rat livers stimulated by diethylstilbestrol and in livers pretreated with mercuric bichloride. They found that stilbestrol increased angiotensinogen production without incorporation of labeled amino acids,

whereas mercuric bichloride treated livers increased amino acid incorporation while increasing angiotensinogen levels. They concluded that the effects of stilbestrol were direct, because angiotensinogen production increased both under pretreatment with stilbestrol and when the stilbestrol was administered only with the perfusion fluid.

Menard et al. (1974) studied the physicochemical properties of angiotensinogen in plasma taken from normal, estrogen treated, and nephrectomized male rats. Using polyacrylamide-gel electrophoresis and isoelectric focusing in polyacrylamide-gel, they could detect no differences in molecular size, weight, or relative mobility in the angiotensinogen taken from the three different groups of rats. They concluded that these results ings were consistent with previous findings that increased substrate concentrations increased plasma renin activity, but did not rule out subtle molecular differences, or the disappearance of a renal inhibitor, to explain the increased plasma renin activity.

The great increases in plasma angiotensinogen under estrogenic stimulation have received great attention from investigators studying the effects of oral contraceptive preparations in women. Laragh et al. (1967) were among the first to note these striking increases. They found a two to eightfold increase in circulating angiotensinogen levels in women on oral contraceptive agents. Beckerhoff et al. (1972) confirmed Laragh's results, and noted that there was evidence that, in humans, substrate levels determined plasma renin activity, because plasma renin activity was elevated in these cases, while plasma renin concentration was significantly reduced below normal levels.

The great sensitivity of the hepatic synthesis to estrogens was demonstrated by Katz and Beck (1974) who noted that, in human females, as little as 100 ug mestranol per day increased angiotensinogen concentrations three to fourfold above the values found in normal, untreated women.

Contrary to the findings of Menard and Catt (1973) in the rat, Cain et al. (1971) noted that oral contraceptives increased angiotensinogen concentrations and that the increase rapidly followed the administration of the contraceptive agents, but that upon cessation of therapy, plasma angiotensinogen concentrations did not return to normal from their elevated state for 2 or 3 months.

The Effects of Steroid Hormones on Plasma Renin Activity

Hasegawa et al. (1973b) studied the effect of hypophysectomy and adrenalectomy on plasma renin activity, plasma angiotensinogen, and plasma renin concentration in perfused rat liver in situ, and with isolated rat liver slices. They found that hypophysectomy had no effect on plasma renin, but decreased angiotensinogen formation both in perfused livers and in isolated liver tissue. Adrenalectomy elevated plasma renin concentration and angiotensin formation, while substrate production decreased, confirming that glucocorticoids increase, but are not essential for, angiotensinogen formation.

Crane et al. (1966) noted that there was a large increase in plasma renin activity in normotensive males and females who were administered ethinylestradiol at 0.5 mg/day. Weinberger et al. (1968) confirmed the results of Crane et al. noting that, in women, the plasma renin activity remained elevated 1 or 2 months following cessation of treatment.

In their investigation of the renin-angiotensin effects of various natural and synthetic estragenic substances in the rat, Menard et al. (1973) noted a high positive correlation between plasma renin activity and plasma renin concentration. They concluded that substrate levels did not determine plasma renin activity in the rat.

Landau and Lugibihl (1958) studied the natriuretic effects of progesterone, noting that they required a functional adrenal cortex. If patients were treated with cortisone and desoxycorticosterone acetate, subsequent progesterone administrations caused pronounced diuresis. In normal patients, cessation of progesterone therapy caused sodium retention, but this was not so in those treated with DOCA. Landau and Lugibihl found that a low dose (20 ug) of aldosterone was overridden by 150 mg of progesterone, but that 50 ug injections of aldosterone were not. They concluded that progesterone could, in high concentrations, competitively displace aldosterone from receptor sites.

Katz and Romfh (1971) measured plasma renin activity and aldosterone concentrations through the menstrual cycle, noting that plasma renin activity and aldosterone peaks coincided, or that plasma renin activity preceded aldosterone by a maximum of 24 hours. They suggested that progesterone may trigger increases in plasma renin activity via progesterone's naturietic effect, with a consequent increase in aldosterone following the increase in Angiotensin II.

Sundsfjord and Aakvaag (1973) found a preovulatory aldosterone peak with unchanged levels of plasma renin activity and plasma angiotensinogen levels. They further noted normal plasma renin activity and aldosterone levels in women with luteal failure, who supposedly had adequate estrogen

levels in urine to warrant an estrogen mediated plasma renin activity increase. These findings, they felt, made estrogen mediation of the increased aldosterone levels unlikely. They concluded that evidence indicated a causal relationship between the rise in plasma renin activity and aldosterone secretion on the one hand and progesterone levels on the other hand.

Species and Sex Differences in the Renin Angiotensin System

Several recent investigations have noted important species and sex differences in plasma renin activity and angiotensinogen levels. In their study on anephric women, Capelli et al. (1968) examined extracts of various internal organs for renin or renin-like activity. They noted that uterine extracts from these patients exhibited an activity toward angiotensinogen and produced a product from this reaction identical to that produced by renal renin. They concluded that the uterus was a source of renin or a renin-like activity in women.

Skinner et al. (1969) studied the effects of estrogens on plasma renin activity in men and women. They found that plasma renin activity depended primarily upon substrate levels, which varied between the sexes. In normal men and women, these levels were approximately the same, but both values were greatly elevated in women on oral contraceptives.

Musa et al. (1965) noted that estrogens increased the hepatic production of circulating plasma proteins, an effect which was dose and duration dependent. They found that the female was more sensitive to a given estrogen dose than were males.

Krakoff (1973) also noted a sex difference, albeit insignificant, in angiotensinogen concentrations and plasma renin activity in human males and females. Females, he found, exhibited greater values for both plasma renin activity and circulating angiotensinogen than did males.

Conradie et al. (1969) studied the renin content of the Sprague-Dawley and Wistar strains of rats. They found large sex and strain differences between rats as measured on a per gram kidney basis, and concluded that age, strain and sex must be considered whenever reporting renin content of kidney tissue.

In contrast to the studies in the human, Nasjletti et al. (1971) noted a significant increase in angiotensinogen levels in rats in estrus, which fell during diestrus. This elevation was eliminated by ovariectomy. Male rats had no such variation, nor were their angiotensinogen concentrations altered by castration. These investigators found angiotensinogen levels in male rats higher than in female rats at all times, although the difference was insignificant when the females were in estrus.

Bing and Jørgensen (1972) also found angiotensinogen concentrations higher in male than in female rats, but could find no alteration in substrate levels following ovariectomy. They further noted wide and significant variations in substrate concentrations over time in groups of rats presumably identical, but were unable to account for these variations.

Effects of Diet on Angiotensinogen Levels and Plasma Renin Activity

In sharp contrast to the voluminous literature available on other aspects of the renin angiotensin system, the effects of diet on plasma renin activity and angiotensinogen levels have been largely overlooked,

with the exception of studies involving the various ions of sodium, potassium and lithium. Beckerhoff et al. (1972) loaded their subjects with 300 mEq of sodium per day to eliminate the effect of variations in dietary sodium intake. Bing (1972) noted the manufacturer of the rat chow used to feed his experimental animals. Most investigators failed to mention any aspect of diet, or noted that they fed a "commercial chow and tap water" (Nasjletti et al., 1971). Reid et al. (1973) fed their dogs "commercial diet plus tap water" and the subjects comprising Landau and Lugibihl's study (1958) ate a "constant adequate diet".

The effect of starvation in rats on adrenal, ovarian, and pituitary weight was studied by Rivero-Fontan et al. (1952) These investigators noted a relative increase in adrenal weight during acute starvation and that adrenal atrophy occurred during chronic starvation. Ovarian atrophy was seen on low protein diets, and uterine weights were significantly lower in rats with a protein intake one-fourth that of rats on a control diet. Rivero-Fontan and his coworkers noted that pituitary weight decreased proportionately faster than whole body weights in female, but not in male, rats.

Robertson (1967) in his study of the effects of oral contraceptives on plasma proteins, found a significant decrease in plasma protein concentrations, primarily in albumin levels, and in the albumin/globulin ratio. He detected no decrease in α_2 globulins, and found a significant increase in α_1 and β globulins.

R. Feher (Department of Veterinary Anatomy, Pharmacology and Physiology, Iowa State University) in a personal communication, has noted a significant decrease in plasma proteins and hematocrit values in dogs on a

17% protein diet, compared to dogs being fed a 38% protein diet. He noted that the decrease was primarily at the expense of the albumin fraction, and detected no decrease in the α_2 globulin fraction in plasma.

In his study of the effects of dietary stress on beagles, Wilke (1974) noted that arterial blood pressure declined and plasma renin activity rose after 8 weeks on a 14% protein diet, and noted that this diet eliminated sex differences in plasma renin activity between males and females under barbiturate anesthesia.

Hormone Levels During the Canine Estrous Cycle

There are several recent studies on hormone levels during the canine estrous cycle. Christie et al. (1971) noted that progesterone levels in the beagle were considerably higher than values found in other, polyestrous, species. In this study they found that plasma progesterone concentrations rose from a proestrus value of about 1 ng/ml to an amount exceeding 20 ng/ml 20 days after the onset of estrus. Following this peak, progesterone concentration fell below 5 mg/ml 50 days after the start of estrus. They noted that there was a sharp rise in estrogen levels just before estrus, which fell rapidly prior to the surge of progesterone.

Nett et al. (1975), Edqvist et al. (1975), and Holst and Phemister (1975a,b) all noted an increase in plasma estradiol concentrations in canines just prior to the onset of estrus, increasing from a proestrus low of 7 pg/ml (Edqvist et al., 1975) to reach peak values of 30 pg/ml (Edqvist et al., 1975) or 60 pg/ml (Nett et al., 1975; Holst and Phemister, 1975b). Following the initial surge these high levels of estradiol decreased to

about 30% of peak values within 5 to 7 days after the onset of estrus, where they remained for the duration of the estrous cycle.

Nett et al. (1975) found that plasma progesterone concentrations were less than 2 ng/ml during proestrus, but rose rapidly to a peak of 24 ng/ml 7 days after the onset of estrus. At day 28 following estrus, they noted that progesterone was still elevated (18 ng/ml) in nonpregnant bitches. Edqvist et al. (1975) noted similar findings with respect to the proestrous and early estrous period, but found great variability in progesterone concentrations during metestrus; some dogs attained high, 30 ng/ml to 50 ng/ml, stable values for progesterone concentrations 3 to 4 weeks following estrus, while others showed great variability in their week to week levels of progesterone.

The Assay of Plasma Renin Activity and Angiotensinogen

There are two commonly used techniques for the measurement of plasma renin activity; the bioassay and the immunoassay. In the bioassay, the pressor effect of Angiotensin II is measured in a conscious or unconscious animal or by the contraction of a smooth muscle preparation induced by Angiotensin II. In the immunoassay, a known amount of labeled Angiotensin I or Angiotensin II competes with the unlabeled angiotensin of the sample for binding sites on a limited amount of antiangiotensin or antibody. The amount of antibody present is kept low insuring that only a fraction of the angiotensin of both types will be bound. The ratio of antibody bound to unbound angiotensin in the labeled fraction can then be used to derive the fraction of total angiotensin present which was contributed by the sample under test.

Bioassay techniques

Tigerstedt and Bergman (1898) were the first to note and make use of a bioassay technique for renin activity, but their test was only qualitative; it measured the presence or absence of renin in extracts made from kidney tissue.

Page and Helmer (1940) noted that a combination of plasma plus renin extracted from kidneys would cause constriction of a segment of rabbit intestine, and used this technique to study the renin angiotensinogen reaction in normal and hypertensive subjects. They noted that only the combination had the smooth muscle stimulatory property; neither renin nor plasma alone would cause constriction.

In 1943, Goldblatt et al. quantified the pressor effects of the renin-angiotensinogen reaction. His unit, now known as the "Goldblatt unit", was that amount of renin which, when injected into a conscious, trained, 10-25 Kg dog, raised its mean femoral arterial blood pressure "at least 30 but not more than 35 mm Hg in 3 minutes" in three separate dogs.

Helmer and Judson (1963) described techniques suitable for the measurement of low as well as high concentrations of Angiotensin II. Their techniques made use of a spirally cut segment of rabbit aorta in a muscle chamber filled with Krebs salt solution. With this preparation, they were able to measure amounts of Angiotensin II as low as 0.7 ng. Their technique for higher concentrations utilized a 2 day nephrectomized, pithed cat; the pressor response to the injected test material was compared with the response following injection of a known amount of pure Angiotensin II. Helmer and Judson used this technique to convert the "Goldblatt unit"

to weight measure; one Goldblatt unit was equal to 0.42 µg of pure Angiotensin II; while their own "Angiotensin unit" was equivalent to 0.07 µg Angiotensin II. They noted that pH 5.4 was optimal for the renin-angiotensinogen reaction.

Regoli and Vane (1964) introduced the rat colon preparation for the bioassay of Angiotensin II. They noted that it was a sensitive and relatively selective preparation, reacting to the same angiotensin analogs yielding pressor responses in the rat. They noted that this preparation had some drawbacks, however, including spontaneous activity, which could be diminished only at the cost of greatly reduced sensitivity.

Haas and Goldblatt (1972) after a review of some current literature noted that the bioassay yielded consistently higher values for plasma renin activity than did the currently used immunoassay procedures, and recommended that all results be reported, not in terms of plasma renin activity, but as equivalent to Goldblatt units of renin in plasma measured against purified homologous renin.

Radioimmunoassay techniques

The first practical radioimmunoassay for the detection of small protein-like molecules and peptides was developed by Yalow and Berson (1960), who used it to detect insulin in small (10 µl and 20 µl) plasma samples. Their procedure used antibody to insulin derived from guinea pigs injected with beef insulin emulsified in mannide mono-oleate. Because of inefficient coupling methods used in their technique, only a small percent of labeled product could be obtained from each preparation of labeled reagent, and great care had to be used to insure that minimal damage to the insulin component of the reagent occurred during the labeling process.

Boyd et al. (1967) and Catt et al. (1967) developed radioimmunoassays for the detection of Angiotensin II. In the procedure developed by Boyd and his coworkers, Angiotensin II was extracted from a large amount of blood by a cumbersome and inefficient procedure, although the limit of sensitivity of their assay was 30 pg. The assay was highly specific for Angiotensin II and relatively unaffected by Angiotensin I. In the procedure developed by Catt et al. (1967) a double isotope was used. A known amount of ^{125}I labeled Angiotensin II was added to plasma before the extraction. This was used as an internal standard to correct for extraction losses, while ^{131}I labeled Angiotensin II was used for the radioimmunoassay itself.

The tedious, cumbersome and inefficient extraction procedure was eliminated in the technique developed by Haber et al. (1969) with the result that analysis could be made on smaller samples, and with greater speed. Haber's technique used ^{125}I labeled Angiotensin I, with the unknown Angiotensin I generated in the presence of EDTA, dimercaprol and 8-hydroxyquinoline. These inhibitors prevented degradation of the peptide, and its conversion to Angiotensin II during incubation, and allowed 90% of the generated angiotensin to be recovered intact after 24 hours. To minimize the risk of degradation and conversion, Haber and his associates chose an incubation time of 3 hours.

Assay procedures for angiotensinogen

Helmer and Griffith (1951) measured the changes in angiotensinogen levels in normal and hypophysectomized rats treated with ACTH. In their technique, angiotensinogen was exhausted by the addition of a large amount of hog renin. Their angiotonin unit was equal to 30 µg of lyophilized

material from the 5 minute reaction of 1 ml of plasma plus three Goldblatt units of renin. These levels were measured by their pressor response in pithed cats.

Schaffenburg et al. (1960) measured angiotensinogen concentrations in several species of animals, using homologous renin to exhaust the angiotensinogen present. In general, they found the angiotensinogen present in 1 ml of plasma capable of generating the angiotensin equivalent of 0.2 to 0.5 Goldblatt unit of renin.

Arakawa et al. (1967) measured the species specificity of the renin-angiotensinogen reaction, and noted that renin from rat, bovine, and hog kidneys would liberate angiotensin I from human substrate, but at a slower rate than human renin would.

In 1973, Menard et al. used semi-purified hog renin to exhaust substrate in rat plasma. They noted that this heterologous renin gave results "identical" to those that they obtained using homologous (rat) renin. Their immunoassay for the detection of the Angiotensin I had a sensitivity of 25 pg.

In general, bioassay procedures are suitable for measurement of angiotensin in relatively high concentrations. Procedures for the detection of small amounts (less than 5 ng Angiotensin II) are difficult because of system "noise" (Regoli and Vane, 1964). The radioimmunoassay is better suited for detection of lesser quantities of angiotensin; the difficulty in using this procedure for the detection of large quantities of angiotensin lies in the extreme sensitivity of the test. This requires great dilutions of the test material with consequent magnification of errors. Felber (1966) noted other drawbacks; problems associated with

equilibrium in a multielement system, the problem of immunologic "identity" (or nonidentity) between the labeled and unlabeled species, and the difficulty in preparing pure forms of the peptide for immunizing animals for antibody production. This purity, he noted, is important only if the unknown or standard may contain impurities similar to those in the immunogenic material.

MATERIALS AND METHODS

The 19 mature male and female beagles used in this experiment were part of the inbred beagle colony maintained for research purposes at Iowa State University College of Veterinary Medicine. All dogs were between 48 and 72 months of age at the start of the experimental period. All dogs had eaten their respective diets since weaning. Protein levels were the major difference in the diets, constituting 38% of the high protein diet and 17% of the low protein diet. The two test groups on high protein diets contained seven male and four female dogs, of which one female died 4 weeks into the experiment from peritonitis, a sequella to an acute metritis. The low protein groups contained three males and five females. All dogs lived in outdoor runs, separated by dietary group and sex. They were examined daily and routinely for injuries, parasites and ill health, and were treated accordingly. All female dogs were given a weekly examination for signs of estrus, which consisted of an examination for external signs of estrus (vaginal discharge, swelling of the vulva) and a microscopic examination of vaginal smears. At the start of the sampling period, all but one of the female dogs was in estrus.

Beginning 24 April 73 and at weekly intervals thereafter during the course of the experiment, 10 ml blood samples were drawn from the external jugular vein into Vaccutainer[1] tubes containing 7.5 mg disodium EDTA. The tubes were gently inverted several times, then chilled in an ice and water bath until all 19 dogs were sampled. The tubes were then centrifuged at

---

[1]Vaccutainer #4770, Becton, Dickinson and Co., Rutherford, N.J.

200 x g in a 4°C cold room, and the plasma carefully removed from the cells, frozen, and stored at -20°C for future analysis of plasma renin activity and angiotensinogen levels. All analyses were made following the conclusion of the 14 week sampling period.

Each sample was assigned a four digit code number at the time of collection with the numbers assigned in sequence. Samples were selected for analysis subsequently by appearance of the code number in a random number table, and were analyzed in batches of 20 or 30, depending upon availability of space on the scintillation counter.

Plasma renin activity and angiotensinogen levels were determined by a radioimmunoassay using ^{125}I labeled Angiotensin I, unlabeled Angiotensin I and Angiotensin I antiserum procured from a commercial source.[1] The analysis of angiotensinogen levels utilized, in addition, purified hog renin, available from ICN Nutritional Biochemicals.[2] Each sample was analyzed simultaneously for plasma renin activity using techniques described by Haber et al. (1969) and for angiotensinogen concentration by incubation with hog renin. A detailed description of these procedures is found in Appendix B. Preliminary data analysis was performed on a Wang 600 programmable calculator.[3] The analysis of variance used the regression procedure of SAS. Appendix B contains a description of data analysis.

---

[1]New England Nuclear, 575 Albany St., Boston, Mass.

[2]ICN Nutritional Biochemicals, Cleveland, Ohio.

[3]Wang Laboratories, Inc., Tewkesbury, Mass.

RESULTS

Analysis of variance on plasma renin activity indicated that there was a diet by sex interaction ($p<0.1$), but no sex or diet effect alone (Table A-1). There was also a significant ($p<0.0001$) effect attributable to the week of sample (Table A-2). The data were then divided into two groups, and the analysis repeated on the supposition that physiologic changes had occurred in the dogs during the course of sampling. This was suggested by the rapid decline in levels of plasma renin activity in the female dogs on the high protein diet during the first 4 or 5 weeks of the sampling period. During this time, all but one of the female dogs, including those on the high and the low protein diet, were in estrus and diestrus. This might represent a sufficient stress to elevate both plasma renin activity and angiotensinogen levels sufficiently above the group mean in the high protein females such that the week of sampling would become an important variable (see Figure 2).

Analysis of the data from the first 4 weeks of the experiment yielded a diet by sex interaction ($p<0.1$) (Figure 1). There was a significant ($p<0.003$) difference in plasma renin activity based upon week of sampling. A great decline in activity during this period, particularly among those female dogs, is depicted by a plot of plasma renin activity for the female dogs (Figure 2). A slight decline was portrayed on similar plots of data from the male dogs (Figure 3).

Analysis of the last 10 weeks demonstrated that the effects noted earlier were a result of the changes noted in the first 4 week period. The analysis of variance demonstrated no significant differences in any of

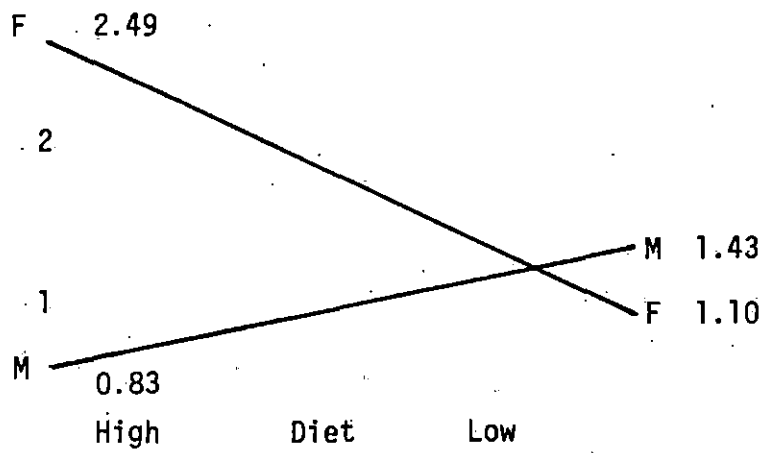

Figure 1. The sex by diet interaction in plasma renin activity during the first 4 weeks of the sample period

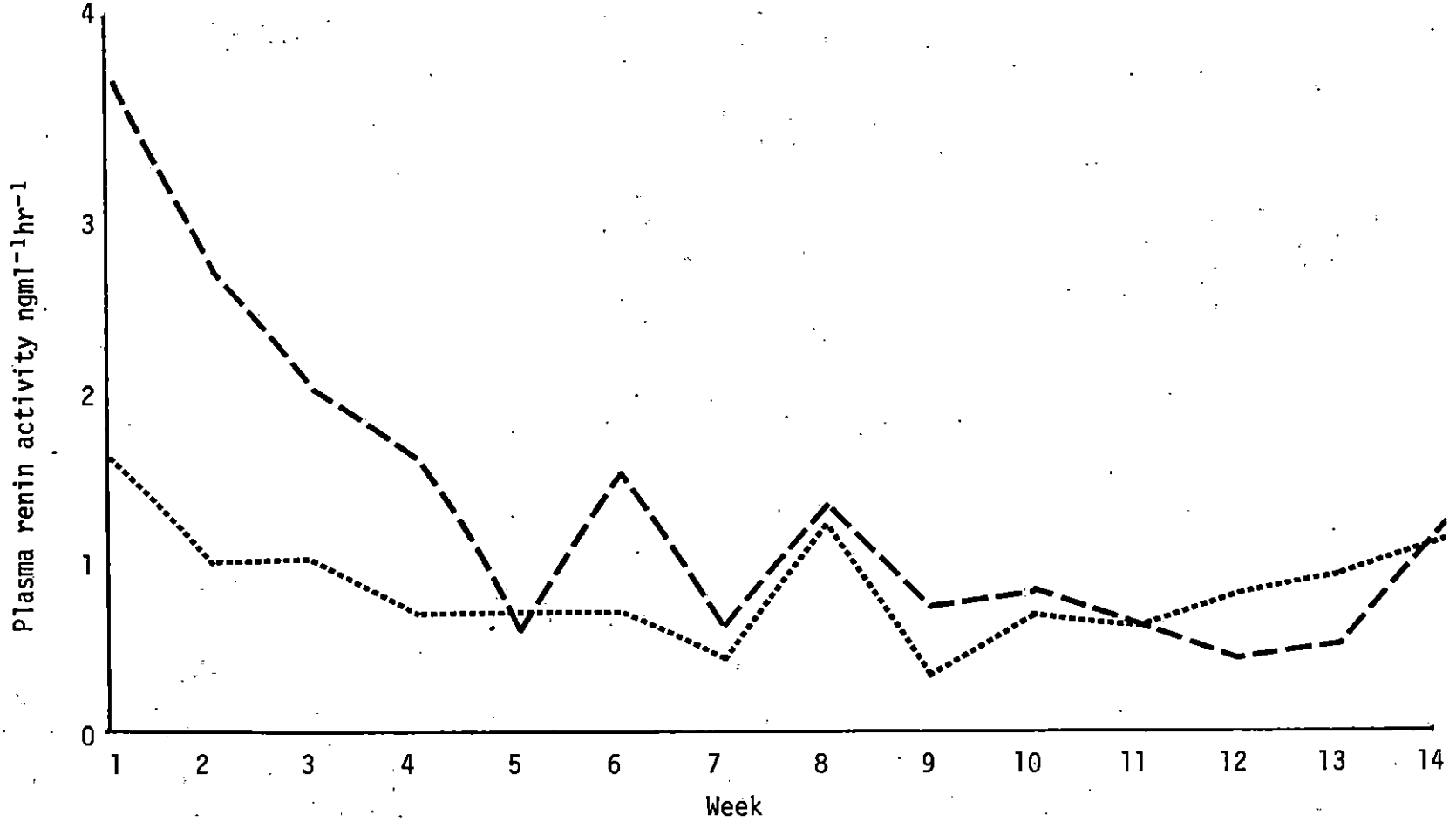

Figure 2. Plasma renin activity in female beagles on high protein diet (---) and low protein diet (...) during the 14 week sample period

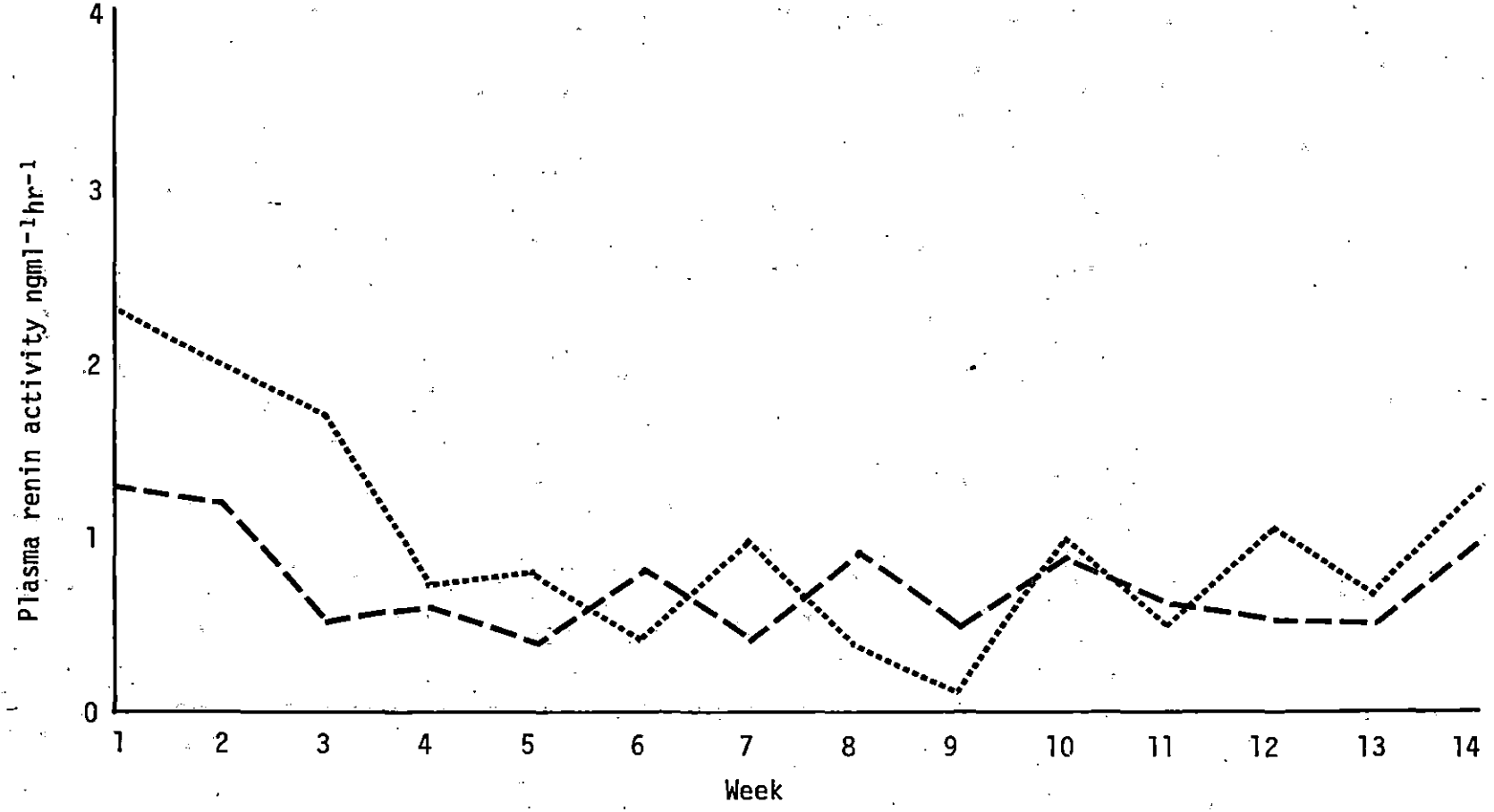

Figure 3. Plasma renin activity in male beagles on high protein diet (---) and low protein diet (...) during the 14 week sample period

the measured variables, nor were any of the interactions significant (Tables A-5 and A-6). All four groups of dogs demonstrated approximately equal plasma renin activities during this period.

Angiotensinogen Concentration

Differences between dietary groups demonstrated significant ($p<0.025$) differences in plasma angiotensinogen concentration over the 14 week sampling period (Table A-7).

Group	Mean
High protein diet	1324
Low protein diet	1734

Angiotensinogen means over the 14 week sampling period were also different ($p<0.1$) when sex was considered (Table A-7).

Group	Mean
Males	1309
Females	1723

A significant ($p<0.003$) diet by week interaction was noted in angiotensinogen concentrations (Figure 4 and Table A-8).

When the data were divided into the 4 and 10 week intervals as had been done for plasma renin activity, only dietary means of angiotensinogen concentrations remained significantly different ($p<0.01$) during the first 4 weeks of the sample period (Tables A-9 and A-10).

Group	Mean
High protein diet	1393
Low protein diet	2113

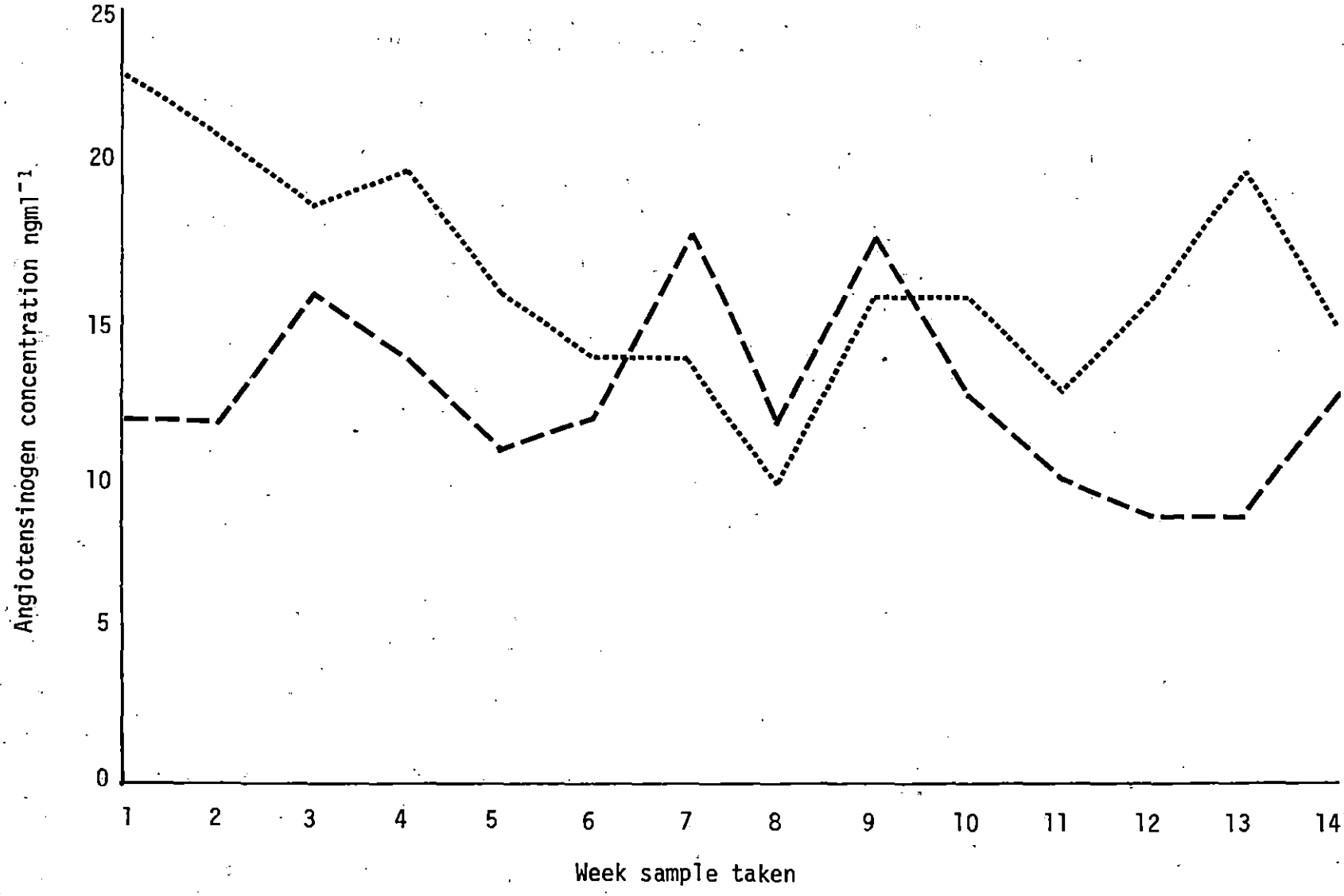

Figure 4. Plasma angiotensinogen concentrations of beagles on high protein diet (---) and low protein diet (...) during the 14 week sample period

During the 10 remaining weeks of the study, the significant ($p<0.05$) difference in angiotensinogen concentrations remained between the sex means (Table A-11).

Group	Mean
Males	1237
Females	1628

The significant diet by week interaction ($p<0.05$) was again demonstrated (Table A-12).

DISCUSSION

With respect to plasma renin activity, the sex by diet interaction demonstrated that sex and diet were important influences on plasma renin activity in the dogs used in this experiment. This effect was explained by the high plasma renin activity in the female dogs near estrus, because the significant difference disappeared during the last 10 weeks of the sample period. None of the tested variables was important during this portion of the sampling period. The slight elevation of plasma renin activity and its subsequent decrease in male dogs during the first weeks of this study may possibly be a result of the stress of fighting, which occurred among these dogs while the females, in nearby pens, were in estrus. This lack of sex difference tends to agree with the findings of Skinner et al. (1969) and Krakoff (1973) in humans.

Plasma angiotensinogen concentrations varied with sex and diet independently during the 14 weeks of sampling, demonstrating that female dogs maintain higher plasma angiotensinogen concentrations than do males. This might be expected in light of the findings of Edqvist et al. (1975) who noted that estrogen concentrations remained low, but elevated above anestrus values during the 10 week period following estrus, and the findings of Nasjletti et al. (1971), who noted the increase in angiotensinogen concentrations in rats in estrus, but not in diestrus. There is also evidence that dogs on low protein diets maintain higher plasma angiotensinogen concentrations than do dogs on high protein diets. Feher (personal communication) found the α_2 globulin fraction of dogs on the low protein diet was higher than the α_2 globulin level in dogs fed the high

protein diet in this experiment. The higher angiotensinogen concentrations in dogs on low protein diet confirm Feher's findings for a particular α_2 globulin. The elevation of these proteins may indicate dietary stress, as suggested by Wilke (1974) although the diet can be considered nutritionally adequate utilizing the criteria that it will support both growth and maintenance in dogs. The influence of diet was manifested early in the sample period; during the last 10 weeks, the effects of diet were not significant. The reverse of this condition obtained with respect to sex differences, which were insignificant during the first 4 weeks, but became apparent and important later in the sampling period.

A consistent explanation for the findings of this study is difficult to achieve. If the presumption is made that the sharp increase in estrogens in the pre-ovulatory period elevated plasma angiotensinogen concentration, then sex differences should have been manifested in the first 4 weeks, or, near the time of ovulation and maximal estrogen concentrations, and not later in the cycle, when both estrogen (and progesterone) concentrations had fallen from their peak values. If circulating progesterone concentrations were the major cause of the elevated angiotensinogen levels, then there should have been an increase in substrate concentrations during the first few weeks followed by a long, elevated plateau of values, with a slight decrease toward the end of the 14 week period.

A possible explanation fitting the observations of this study may be as follows: The true difference in angiotensinogen concentrations is a sex difference, as observed during the last 10 weeks of the study, which is brought about by low circulating estrogen concentrations in the female, and which are presumably not present in the male. These estrogens result

in higher plasma angiotensinogen concentrations in female dogs. However, if the low protein diet is considered somewhat stressful, as suggested by the results of Wilke's study (1974) and by the preliminary results of Feher (unpublished), the effect might be increased circulating angiotensinogen concentrations mediated by increased glucocorticoid concentrations in the dogs on the low protein diet. Wilke (1974) noted that dogs on a 24% protein diet had lower plasma renin activity levels under stress than did dogs on a 14% diet, and suggested that the dogs on the higher protein diet had means other than activation of the renin-angiotensin system or the sympathetic nervous system to elevate blood pressure under stress. If it is assumed that the period near estrus was stressful to both sexes, as was suggested previously, then it is possible to explain the dietary difference in angiotensinogen concentrations during the first 4 weeks of the study. It may be that the sex difference was masked by the greater glucocorticoid release of the dogs on low protein diets under the stresses of estrus. The elevated corticoid concentrations, in turn, activated mechanisms for the hepatic synthesis of angiotensinogen, which then increased sufficiently in the plasma to override the sex effect in dogs on the low protein diet during those first few weeks of the study.

The sharp week to week variations in substrate concentrations seen in all groups are probably a result of environmental stress, as suggested by Bing and Jørgensen (1972).

This pilot study, in light of the recent literature, suggests that a study of canine plasma renin activity and angiotensinogen concentrations, simultaneously with determinations of circulating estradiol, progesterone, and glucocorticoid levels, would be rewarding.

BIBLIOGRAPHY

Anderson, B. and K. Olsson. 1973. On central control of body fluid homeostasis. Conditional Reflex 8:147-159.

Arakawa, K., M. Nakatani and M. Nakamura. 1967. Species specificity in reaction between renin and angiotensinogen. Nature 207:636.

Beckerhoff, R., J. A. Luetscher, R. Wilkinson, C. Gonzales and G. W. Nokes. 1972. Plasma renin concentration activity and substrate in hypertension induced by oral contraceptives. J. Clin. Endocrinol. Metab. 34:1067-1073.

Bing, J. 1972. Relation between renin substrate and acute phase proteins. Acta Path. Microbiol. Scand., Section A 80:646-650.

Bing, J. and J. Jørgensen. 1972. Influence of age and sex and of castration on renin substrate of normal and nephrectomized rats. Acta Path. Microbiol. Scand., Section A 80:641-645.

Boyd, G. W., J. Landon and W. S. Peart. 1967. Radioimmunoassay for determining plasma-levels of angiotensin II in man. Lancet 2:1002-1005.

Braun-Menendez, E., J. C. Fasciolo, L. F. Leloir and T. M. Munoz. 1940. The substance causing renal hypertension. J. Physiol. 98:283-298.

Brown, J. J., R. Frazer, A. F. Lever, D. R. Love, J. J. Morton and J. I. S. Robertson. 1972. Raised plasma angiotensin II and aldosterone during dietary sodium restriction in man. Lancet :1106-1107.

Cain, M. D., W. A. Walters and K. J. Catt. 1971. Effects of oral contraceptive therapy on the renin-angiotensin system. J. Clin. Endocrinol. Metab. 33:671-676.

Capelli, J. P., L. G. Wesson, Jr., G. E. Aponte, C. Feraldo and E. Jaffe. 1968. Characterization and source of a renin like enzyme in anephric humans. J. Clin. Endocrinol. Metab. 28:221-230.

Catt, K. J., M. C. Cain and J. P. Coghlan. 1967. Measurement of angiotensin II in blood. Lancet 2:1005-1007.

Christie, D. W., E. T. Bell, C. E. Horth and R. F. Palmer. 1971. Peripheral plasma progesterone levels during the canine oestrus cycle. Acta Endocrinologica 68:543-550.

Churchill, P. C. and F. D. McDonald. 1974. Effect of ouabain on renin secretion in anesthetized dogs. J. Physiol. (Lond.) 242:635-646.

Conradi, K., J. Jelinek and F. Gross. 1969. Strain and sex differences in renin content of rat kidneys (34350). Proc. Soc. Exptl. Biol. Med. 132:984-986.

Cook, W. F. and G. W. Pickering. 1958. The location of renin within the kidney. J. Physiol. (Lond.) 143:78P-79P.

Coote, J. H., E. J. Johns, U. H. Macleod and B. Singer. 1972. Effect of renal nerve stimulation, renal blood flow and adrenergic blockade on plasma renin activity in the cat. J. Physiol. 226:15-36.

Crane, M. G., J. Heitsch, J. J. Harris and V. J. Johns. 1966. Effect of ethinyl estradiol (estinyl) on plasma renin activity. J. Clin. Endocrinol. Metab. 26:1403-1406.

Crump, M. H., F. B. Hembrough, L. Wilke and R. C. Feher. 1973. The effect of diet, sex and sampling time on plasma renin activity in the canine. Department of Veterinary Anatomy, Pharmacology and Physiology, Iowa State University, Ames, Iowa.

Edqvist, L. E., E. D. B. Johansson, H. Kasström, S. E. Olsson and M. Richkind. 1975. Blood plasma levels of progesterone and oestradiol in the dog during the oestrus cycle and pregnancy. Acta Endocrinologica 78:554-564.

Esler, M. D. and P. J. Nestel. 1973. Renin and sympathetic nervous system responsiveness to adrenergic simuli in essential hypertension. Amer. J. Cardiol. 32:643-649.

Fagard, R., E. Fossion, M. Carupforts, R. Deruyck and A. Amery. 1974. Extraction and production of renin and AngII across several vascular beds in dogs. Experientia 30/6:598-600.

Felber, J. P. 1966. Radioimmunoassays, general principles and methods. Helvetica Medica Acta 33:367-377.

Ferrario, C. M., P. L. Gildenberg and J. W. McCubbin. 1972. Cardiovascular effects of angiotensin mediated by the central nervous system. Circulation Res. 30:257-262.

Fisher, J. W., ed. 1971. Kidney hormones. Academic Press, New York. 665 pp.

Freeman, R. H. and H. H. Rostorfer. 1972. Hepatic changes in renin substrate biosynthesis and alkaline phosphatase activity in the rat. Am. J. Physiol. 223:364-370.

Goldblatt, H., J. Lynch, R. F. Hanzal and W. W. Summerville. 1934. Studies on experimental hypertension: The production of persistant elevation of systolic blood pressure by means of renal ischemia. J. Exptl. Med. 59:347-379.

Goldblatt, H., Y. J. Katz, H. A. Lewis and E. Richardson. 1943. Studies on experimental hypertension XX: The bioassay of renin. J. Exptl. Med. 77:309-313.

Goormaghtigh, N. 1939. Existance of an endocrine gland in the media of the renal arterioles. Proc. Soc. Exptl. Biol. Med. 42:688-689.

Haas, E. and H. Goldblatt. 1972. Indirect assay of plasma renin. Lancet 1:1330-1332.

Haber, E., T. Koerner, L. B. Page, B. Kliman and A. Purnode. 1969. Application of a radioimmunoassay for angiotensin I to the physiologic measurements of plasma renin activity in normal human subjects. J. Clin. Endocrinol. Metab. 29:1349-1355.

Hartroft, P. M. 1963. Juxtaglomerular cells. Circulation Res. 12:525-530.

Hasegawa, H., A. Nasjletti, K. Rice and G. M. C. Masson. 1973a. Role of pituitary and adrenals in the regulation of plasma angiotensinogen. Am. J. Physiol. 225:1-6.

Hasegawa, H., H. Tateishi and G. M. C. Masson. 1973b. Evidence for an angiotensinogen-stimulating factor after nephrectomy. Can. J. Physiol. Pharmacol. 51:563-566.

Haynes, F. W., P. H. Forsham, G. W. Thorn and L. Dexter. 1949. Effect of pituitary adrenocorticotropin on the plasma hypertensinogen concentration of dogs. Federation Proceedings 8:70.

Helmer, O. M. and R. S. Griffith. 1951. Biological activity of steroids as determined by assay of renin substrate (hypertensinogen). Endocrinology 49:151-161.

Helmer, O. M. and R. S. Griffith. 1952. The effect of the administration of estrogens on the renin-substrate (hypertensinogen) content of rat plasma. Endocrinology 51:421-426.

Helmer, O. M. and W. E. Judson. 1963. The quantitative determination of renin in the plasma of patients with arterial hypertension. Circulation 27:1050-1060.

Holst, P. A. and R. D. Phemister. 1975a. Onset of diestrus in the beagle bitch: Definition and significance. Am. J. Vet. Res. 35:401-406.

Holst, P. A. and R. D. Phemister. 1975b. Temporal sequence of events in the estrous cycle of the bitch. Am. J. Vet. Res. 36:705-706.

Hornych, A., P. Meyer and P. Milliez. 1973. Angiotensin, vasopressin, and cyclic AMP: Effects on sodium and water fluxes in rat colon. Am. J. Physiol. 224:1223-1229.

Katz, F. H. and P. Beck. 1974. Plasma renin activity, renin substrate and aldosterone during treatment with various oral contraceptives. J. Clin. Endocrinol. Metab. 39:1001-1005.

Katz, F. H. and P. Romfh. 1971. Plasma aldosterone and renin activity during the menstrual cycle. J. Clin. Endocrinol. Metab. 34:819-821.

Kohnstaedt, K. G., O. M. Helmer and I. M. Page. 1938. Activation of renin by blood colloids. Proc. Soc. Exptl. Biol. Med. 39:214-215.

Krakoff, R. 1973. Measurement of plasma renin substrate by radioimmunoassay of angiotensin I: Concentration in syndromes associated with steroid excess. J. Clin. Endocrinol. Metab. 37:110-117.

Landau, R. L. and K. Lugibihl. 1958. Inhibition of the sodium retaining influence of aldosterone by progesterone. J. Clin. Endocrinol. Metab. 18:1237-1245.

Laragh, J. H. 1973. Potassium, angiotensin and the dual control of aldosterone secretion. New Engl. J. Med. 289:745-747.

Laragh, J. H., M. Angers, W. G. Kelly and S. Lieberman. 1960. Hypotensive agents and pressor substances. JAMA 174:234-240.

Laragh, J. H., J. E. Sealey, J. G. G. Ledingham and M. A. Newton. 1967. Oral contraceptives. JAMA 201:918-922.

Menard, J. and K. J. Catt. 1973. Effects of estrogen treatment on plasma renin parameters in the rat. Endocrinology 92:1382-1388.

Menard, J., A. Malmejac and P. Milliez. 1970. Influence of diethylstilbestrol on the renin-angiotensin system of male rats. Endocrinology 86:774-780.

Menard, J., P. Corvol, A. Foliot and J. P. Raynaud. 1973. Effects of estrogens on renin substrate and uterine weight in rats. Endocrinology 93:747-751.

Menard, J., J. Breminer and P. Corvol. 1974. Physicochemical properties of plasma renin substrate in normal, binephrectomized, and estrogen-treated rats. Am. J. Physiol. 226:1434-1437.

Musa, B. U., U. S. Seal and R. P. Doe. 1965. Elevation of certain plasma proteins in man following estrogen administration: A dose-response relationship. J. Clin. Endocrinol. 25:1163-1166.

Nasjletti, A. and G. M. C. Masson. 1972. Studies on angiotensinogen formation in a liver perfusion system. Circulation Res. 30,31 (Suppl. 2):187-198.

Nasjletti, A., M. Matsunaga and G. M. C. Masson. 1971. Effects of sex hormones on the renal pressor system. Can. J. Physiol. Pharmacol. 49:292-301.

Nett, T. M., A. M. Akbar, R. D. Phemister, P. A. Holst, L. E. Reichert, Jr. and G. D. Niswender. 1975. Levels of luteinizing hormone, estradiol and progesterone in serum during the estrous cycle and pregnancy in the beagle bitch. Proc. Soc. Exptl. Biol. Med. 148: 134-139.

Ng, K. K. F. and J. R. Vane. 1967. Conversion of angiotensin I to angiotensin II. Nature (Lond.) 216:762-766.

Page, I. H. and O. M. Helmer. 1940. A crystalline pressor substance (angiotonin) resulting from the reaction between renin and renin-activator. J. Exptl. Med. 71:29-42.

Page, I. H., B. McSwain, G. M. Knapp and W. D. Andrus. 1941. The origin of renin-activator. Am. J. Physiol. 135:214-222.

Regoli, D. and J. R. Vane. 1964. A sensitive method for the assay of angiotensin. Br. J. Pharmacol. 23:351-359.

Reid, I. A., W. H. Tu, K. Otsuka, T. A. Assaykeen and W. F. Ganong. 1973. Studies concerning the regulation and importance of plasma angiotensinogen concentration in the dog. Endocrinology 93:107-114.

Reid, I. A., J. R. Blair-West and W. F. Ganong. 1974. Simulation of angiotensinogen release by hemodilution in the dog. Endocrinology 95:1482-1485.

Rivero-Fontan, J., K. E. Paschkis, E. West and A. Cantrow. 1952. Influence of different dietary protein levels in starvation on the endocrine system of male and female rats. Endocrinology 51:100-109.

Robertson, G. S. 1967. Serum protein and cholinesterase changes in association with contraceptive pills. Lancet 1:232-235.

Schaffenburg, C. A., E. Haas and H. Goldblatt. 1960. Concentration of renin in kidneys and angiotensinogen in serum of various species. Am. J. Physiol. 199:788-792.

Shade, R. E., J. O. Davis, J. A. Johnson, R. W. Gotshall and W. S. Spielman. 1973. Mechanism of action of Ang II and antidiuretic hormone on renin secretion. Am. J. Physiol. 224:926-929.

Simpson, J. B. and A. Routtenburg. 1973. Subfornical organ: Site of drinking elicitation by angiotensin II. Science 181:1172-1175.

Skeggs, L. T., Jr., W. H. Marsh, J. R. Kahn and N. P. Shumway. 1954. The existence of two forms of hypertensin. J. Exptl. Med. 99: 275-282.

Skinner, S. L., E. R. Lumbers and E. M. Symonds. 1969. Alteration by oral contraceptives of normal menstrual changes in plasma renin activity, concentrations and substrate. Clinical Science 36:67-76.

Stein, J. H. and T. F. Ferris. 1973. The physiology of renin. Arch. Intern. Med. 131:860-872.

Sundsfjord, J. A. and A. Aakvaag. 1973. Variations in plasma aldosterone and plasma renin activity throughout the menstrual cycle, with special reference to the pre-ovulatory period. Acta Endocrinologica 73:499-508.

Thurau, K. W. C., H. Dahlheim, A. Grüner, J. Mason and P. Granger. 1972. Activation of renin in the single juxtaglomerular apparatus by sodium chloride in the tubular fluid at the macula densa. Circulation Res. 30,31(Suppl. 2):182-186.

Tigerstedt, R. and P. G. Bergman. 1898. Niere und kreislauf. Skandinavisches Archiv fur Physiologie 8:223-271.

Vander, A. J. 1965. Effect of catecholamines and the renal nerves on renin secretion in anesthetized dogs. Am. J. Physiol. 209:659-662.

Waugh, W. H. 1972. Angiotensin II: Local renal effects of physiological increments in concentration. Can. J. Physiol. and Pharmacol. 50: 711-716.

Weinberger, M. H., A. J. Dowdy, G. W. Nokes and J. A. Leutscher. 1968. Plasma renin activity and aldosterone secretion in hypertensive patients during high and low sodium intake and adminstration of diuretic. J. Clin. Endocrinol. 28:359-371.

Wilke, W. L. 1974. The effects of age, sex and a severe dietary stress upon plasma renin activity in the beagle. Unpublished M.S. thesis, Iowa State University, Ames, Iowa.

Yalow, R. S. and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. J. Clin. Invest. 39:1157-1175.

ACKNOWLEDGMENTS

I should like to extend my appreciation to Dr. Malcolm H. Crump, my major professor, for his patience and guidance during the course of this research, and to Dr. Frederick B. Hembrough, whose assistance in finding the necessary funds made this project possible. To the members of my graduate committee, Dr. William C. Wager, Dr. James A. Thomas, and Dr. Franklin A. Ahrens, for their patience and their kindness in reviewing this manuscript, many thanks.

A heartfelt "thank you" to Mr. Robert Feher for some useful material from his current research, to Mr. Art Anderson, for help in programming the Wang, to Dr. Michael Brown, for his data on the spring, 1973 estrus cycle in "my" dogs, and to Ms. Chor-San Heng, whose penetrating questions usually made me see more clearly. My appreciation and admiration to Mrs. Charmian Nickey, who translated my poor typing and illegible scribbles into this manuscript.

My deepest appreciation is reserved for my wife, JoLynne, whose support, encouragement, and prodding aided me in completing this study.

This project was supported in part by General Foods, Battle Creek, Michigan, and in part by Public Health Service Grant HD-00041-15 from the National Institutes of Health, Education and Welfare.

APPENDIX A: TABLES

Table A-1. Results of the analysis of variance for diet or sex effects, or a diet by sex interaction in plasma renin activity over the 14 week sampling period

Source	df	Mean square	F	Probability of >F
Diet	1	1.45	N.S.	
Sex	1	3.65	N.S.	
Diet*sex	1	5.39	3.57	p<.1
Error	15	1.51		

Table A-2. Results of the analysis of variance to test for week of sample effect, or diet*week, sex*week, or diet*sex*week interactions in plasma renin activity over the 14 week sampling period

Source	df	Mean square	F	Probability of >F
Week	13	2.90	4.08	p<0.0001
Diet*week	13	0.58	N.S.	
Sex*week	13	0.45	N.S.	
Diet*sex*week	13	0.88	N.S.	
Error	154	0.71		

Table A-3. Results of the analysis of variance to test for diet or sex effects or a diet*sex interaction in plasma renin activity during the first 4 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Diet	1	4.63	N.S.	
Sex	1	6.55	N.S.	
Sex*diet	1	11.29	3.38	p<.1
Error	15	3.34		

Table A-4. Results of the analysis of variance to test for week of sample effect, or diet by week, sex by week, or diet by sex by week interactions in plasma renin activity during the first 4 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Week	3	5.41	5.76	p<0.005
Diet*week	3	1.27	N.S.	
Sex*week	3	0.20	N.S.	
Diet*sex*week	3	0.93	N.S.	
Error	33	0.94		

Table A-5. Results of the analysis of variance to test for diet or sex effects, or a diet by sex interaction in plasma renin activity during the last 10 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Diet	1	0.01	N.S.	
Sex	1	0.21	N.S.	
Diet*sex	1	0.18	N.S.	
Error	14	0.37		

Table A-6. Results of the analysis of variance to test for week of sample effect, or a diet by week, sex by week, or diet by sex by week interaction in plasma renin activity during the last 10 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Week	9	0.53	N.S.	
Diet*week	9	0.37	N.S.	
Sex*week	9	0.30	N.S.	
Diet*sex*week	9	0.09	N.S.	
Error	106	0.40		

Table A-7. Results of the analysis of variance to test for diet or sex effects, or a diet by sex interaction in plasma angiotensinogen levels during the 14 week sampling period

Source	df	Mean square	F	Probability of >F
Diet	1	3630558	6.74	p<0.025
Sex	1	2252371	4.18	p<0.1
Diet*sex	1	955013	N.S.	
Error	15	538548		

Table A-8. Results of the analysis of variance to test for week of sample effect, or a diet by week, sex by week, or a diet by sex by week interaction in plasma angiotensinogen levels during the 14 week sampling period

Source	df	Mean square	F	Probability of >F
Week	13	831893	2.06	p<0.01
Diet*week	13	1052061	2.61	p<0.005
Sex*week	13	666991	1.65	p<0.1
Diet*sex*week	13	541914	N.S.	
Error	154	402863		

Table A-9. Results of the analysis of variance to test for diet or sex effects, or a diet by sex interaction in plasma angiotensinogen levels during the first 4 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Diet	1	5958282	8.93	p<0.01
Sex	1	1435693	N.S.	
Diet*sex	1	317293	N.S.	
Error	15	667069		

Table A-10. Results of the analysis of variance to test for a week of sample effect, or a diet by week, sex by week or diet by sex by week interaction in plasma angiotensinogen levels during the first 4 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Week	3	82338	N.S.	
Diet*week	3	596016	N.S.	
Sex*week	3	461439	N.S.	
Diet*sex*week	3	619403	N.S.	
Error	33	424603		

Table A-11. Results of the analysis of variance to test for a diet or sex effect, or a diet by sex interaction in plasma angiotensinogen levels during the last 10 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Diet	1	1003203	N.S.	
Sex	1	2024271	5.69	p<0.05
Diet*sex	1	1102451	3.10	p<0.1
Error	14	355642		

Table A-12. Results of the analysis of variance to test for a week of sample effect, or a diet by week, sex by week, or a diet by sex by week interaction in plasma angiotensinogen levels during the last 10 weeks of the sampling period

Source	df	Mean square	F	Probability of >F
Week	9	491084	N.S.	
Diet*week	9	874371	2.26	p<0.05
Sex*week	9	710293	1.84	p<0.1
Diet*sex*week	9	517429	N.S.	
Error	106	386832		

APPENDIX B: THE DETERMINATION OF PLASMA RENIN ACTIVITY AND ANGIOTENSINOGEN CONCENTRATIONS BY THE USE OF A RADIOIMMUNOASSAY

Plasma renin activity was determined by measurement of Angiotensin I formed by the reaction between endogenous renin and angiotensinogen in plasma incubated at 37°C. Angiotensinogen was measured by the Angiotensin I generated in a reaction between endogenous substrate and 0.1 Goldblatt unit of exogenous (hog) renin.[1] The system was buffered to a pH of 6.0 in order to increase the reaction rate, and was incubated at 37°C for 3 hours. The measurements of plasma renin activity and angiotensinogen levels were conducted simultaneously on the same plasma aliquot. Angiotensin I generated by the renin-angiotensinogen reaction in both determinations was measured by a radioimmunoassay based on the procedure developed by Haber et al. (1969).

Plasma Renin Activity and Angiotensinogen Concentrations

Samples to be analyzed were transferred from the freezer to the refrigerator and allowed to thaw at 4°C. When completely fluid, each sample was mixed on a Vortex Genie[2] mixer for 5 seconds, and a 1 ml aliquot was drawn and placed in a 12 x 75 mm capped plastic culture tube.[3] This aliquot was kept cold in an ice and water bath; the sample was

---

[1]ICN Nutritional Biochemicals, Cleveland, Ohio.

[2]American Hospital Supply Corporation, Evanston, Illinois.

[3]Falcon Plastics, Oxnard, California.

returned to the freezer. Ten µl 8-hydroxyquinoline (0.34M) and 2 µl dimercaprol (0.80M) were added to the aliquot to inhibit degradation. The aliquot was mixed thoroughly on the vortex mixer.

From the treated aliquot, 0.1 ml was removed for angiotensinogen determination, 0.4 ml was removed to the refrigerator to be maintained at 4°C as a control to determine preformed Angiotensin I in the sample. The remainder of the aliquot was incubated at 37°C for 3 hours to allow the endogenous renin to react with the substrate.

The 0.1 ml aliquot was transferred to a tube containing 0.9 ml phosphate buffer, pH 6.0 and thoroughly mixed. From this aliquot, 0.1 ml was transferred to a tube containing 1.9 ml phosphate buffer, pH 6.0 containing 0.1 Goldblatt unit of hog renin. These tubes were incubated at 37°C for 3 hours to allow for the renin-angiotensinogen reaction to occur.

While all aliquots were incubating, nine more 12 x 75 mm plastic tubes were prepared for each sample, each tube containing 0.8 ml chilled (4°C) Tris buffer containing 1 mg lysozyme/ml. For each batch of samples analyzed, 30 additional tubes, for the determination of a standard curve, were prepared. The standard curve consisted of six points, determined in triplicate. Tubes 1-21 received 0.1 ml ^{125}I labeled Angiotensin I[1], an amount sufficient to yield about 5000 CPM. Tubes 4-6 received 0.05 ng unlabeled Angiotensin I[1]; tubes 7-9, 0.1 ng; 10-12, 0.20 ng; 13-15, 0.30 ng; 16-18, 0.50 ng; and 19-21, 0.75 ng. Tubes 22-24 received 0.2 ml ^{125}I labeled Angiotensin I and tubes 25-27, 0.3 ml labeled Angiotensin I as a check on binding ability of the Angiotensin I antiserum. Tubes 28-30 were

---

[1]New England Nuclear, 575 Albany St., Boston, Mass.

left blank to determine background radiation. All tubes were mixed on the vortex mixer, then tubes 1-3 were set aside and 0.1 ml angiotensin antiserum was added to tubes 4-30, an amount sufficient to bind about half the Angiotensin I present. At this binding level, the accuracy of the assay should have been maximum. The 30 tubes for the standard curve were placed in the refrigerator overnight in order to allow equilibration of the two species of Angiotensin I to occur.

After incubation for 3 hours, from each aliquot destined for plasma renin activity, a 0.1 ml aliquot was drawn and added to each of three tubes containing 0.8 ml Tris buffer with lysozyme. From the tube incubated for the angiotensinogen determination, three 0.1 ml aliquots were drawn and added to each of three tubes containing Tris buffer with lysozyme as described above. Similarly, 0.1 ml aliquots were drawn from the control samples incubated at 4°C for 3 hours. To each of the nine tubes was added 0.1 ml of the labeled Angiotensin I and 0.1 ml Angiotensin I antiserum as described previously for the standard curve. All tubes were mixed on the vortex mixer and allowed to equilibrate overnight in the refrigerator.

Following equilibration, 1.0 ml of dextran coated charcoal suspension in barbital buffer was squirted into each of the nine sample tubes, and the 30 tubes prepared for the standard curve. The charcoal suspension consisted of 3.125 g Norit A charcoal and 0.3125 g radioimmunoassay grade dextran in 500 ml of barbital buffer containing 3.825 g sodium chloride, 0.7357 g sodium barbital, and 0.4857 g sodium acetate in 500 ml. The pH of the buffer was adjusted to 7.4 with 0.1N hydrochloric acid prior to bringing to the final 500 ml volume.

The tubes were then mixed on the vortex mixer and immediately separated by centrifugation at 1700 x g for 15 minutes, until the charcoal pellet was quite firm. The supernatant was decanted into a second, similarly numbered tube for counting the free ^{125}I labeled Angiotensin I. Counting was performed on a Nuclear Chicago model 4216 well type solid crystal gamma counter. The supernatant tube was counted first, then its charcoal pellet. Each tube was counted for 5 minutes, or to about 25,000 counts per 2 tubes. Each sample, therefore, involved a supernatant and charcoal pellet tube for each of the tubes of the triplicate assay for plasma renin activity, angiotensinogen, and 4°C control, or a total of 18 tubes. The dilutions selected allowed most of the samples to fall in the middle of the standard curve, i.e., they contained about 0.10-0.5 ng of generated Angiotensin I. Any samples whose values fell outside the standard curve were rerun with an appropriately adjusted dilution.

Calculations

Preliminary calculations for samples and the determination of the standard curve were performed on a Wang model 600[1] programmable calculator, using four separate programs entitled Renin I, Renin II, Renin III, and Renin VII.

The first program, Renin I, performed two functions; it calculated a correction factor (described below) and then applied this to all subsequent tubes in a particular standard curve and its associated sample

---

[1]Wang Laboratories, Inc., Tewksbury, Mass.

unknowns. The first three tubes of the standard curve, containing only labeled Angiotensin I, charcoal suspension and Tris buffer, should have demonstrated no CPM in the supernatant, assuming that the charcoal was 100% efficient in sweeping labeled angiotensin from the solution. Because of mechanical losses of CPM into the supernatant, a correction factor was necessary. The CPM of the pellet was divided into the sum of the CPM of the pellet and its supernatant. The average of these three fractions yielded a figure which anticipated losses in subsequent tubes. This average value was then applied to all tubes in a particular batch of samples and its standard curve in the following manner: The CPM of each tube consisted of the supernatant and its pellet. The total (T) CPM was then multiplied by the correction factor to give a corrected total (T_o) from which the CPM of the pellet was subtracted. The remaining CPM constituted the corrected, antibody bound fraction remaining in the supernatant (B_c).

Each point on the standard curve and each determination of plasma renin activity and plasma angiotensinogen concentration was determined in triplicate. Renin II, a rejection program, compared each of the F/B_c values in the triplicate determination to the mean of the three, and to the mean of the other two values. Tolerance limits for rejection were based upon prior experience with the PRA assay.

Renin III calculated the standard curve and determined values for the samples. It fit the best linear line through the plot of F/B_c values for each of the standard points. Using the slope of this line and the F/B_c ratios of the unknowns, it interpolated the Angiotensin I levels of the samples.

Renin VII calculated angiotensinogen levels and plasma renin activity from values determined by Renin III. It multiplied the Angiotensin I values by the appropriate dilution factor and divided the plasma renin activity value by three, the incubation time, to give a level in ng ml^{-1} hr^{-1}.

The information from Renin VII was treated by a regression procedure in the SAS package to determine the significance of the various means and interactions.

APPENDIX C. DATA SET

Dogno[a]	Sex[b]	Diet[c]	Wkst[d]	Bsa[e]	Detenz[f]	Subenz[g]
1	1	1	3	9	1.451	1357
1	1	1	4	10	1.542	1212
1	1	1	5	7	0.441	760
1	1	1	6	5	0.713	823
1	1	1	7	2	-[h]	1799
1	1	1	8	4	1.136	1113
1	1	1	9	2	1.030	2777
1	1	1	10	6	0.398	814
1	1	1	11	5	0.608	813
1	1	1	12	6	0.559	994
1	1	1	13	9	0.623	1189
1	1	1	14	10	0.828	937
2	1	1	1	3	0.758	1280
2	1	1	2	4	1.539	1828
2	1	1	3	3	0.183	1092
2	1	1	4	3	0.424	803
2	1	1	5	1	0.215	1658
2	1	1	6	4	1.789	595
2	1	1	7	7	0.273	657
2	1	1	8	7	0.323	873
2	1	1	9	2	0.314	2035
2	1	1	10	10	0.086	865
2	1	1	11	3	1.459	874
2	1	1	12	9	-	1472
2	1	1	13	5	0.845	529

[a]Dog identification number.

[b]Sex, coded 1 for male, 2 for female.

[c]Diet, coded 1 for high (38%) protein and 2 for low (17%) protein.

[d]Week of sample within the 14 week sampling period.

[e]Sample analysis number.

[f]Plasma renin activity in ng $ml^{-1}hr^{-1}$.

[g]Angiotensinogen, as ng angiotensin I ml^{-1}.

[h]Sample destroyed during analysis.

Dogno	Sex	Diet	Wkst	Bsa	Detenz	Subenz
2	1	1	14	9	1.166	824
3	1	1	3	10	0.264	1021
3	1	1	8	6	0.585	1037
3	1	1	9	4	0.602	2589
3	1	1	10	10	2.087	943
3	1	1	11	3	0.218	818
3	1	1	12	10	1.673	891
3	1	1	13	6	0.255	899
3	1	1	14	9	0.687	865
4	1	1	1	5	0.647	724
4	1	1	2	8	0.045	816
4	1	1	3	9	0.431	857
4	1	1	4	9	0.265	893
4	1	1	5	7	0.192	739
4	1	1	6	5	0.227	936
4	1	1	7	2	0.521	3061
4	1	1	8	9	0.705	1243
4	1	1	9	5	0.451	752
4	1	1	10	2	0.252	1877
4	1	1	11	4	0.626	1424
4	1	1	12	10	0.221	943
4	1	1	13	1	0.218	810
4	1	1	14	2	0.724	1848
5	1	1	1	6	1.551	990
5	1	1	4	8	0.067	608
5	1	1	5	5	0.179	678
5	1	1	6	8	0.434	698
5	1	1	7	4	0.332	1099
5	1	1	8	9	0.689	955
5	1	1	9	9	0.382	1269
5	1	1	10	3	0.795	1462
5	1	1	11	2	0.185	2295
5	1	1	12	3	0.237	867
5	1	1	13	1	0.173	848
5	1	1	14	2	0.486	3864
6	1	1	1	1	3.057	2189
6	1	1	2	7	1.091	799
6	1	1	3	3	0.114	1629
6	1	1	4	4	1.365	914
6	1	1	5	6	1.212	1033
6	1	1	6	5	0.553	808
6	1	1	7	10	0.476	982
6	1	1	8	9	2.163	1437
6	1	1	9	8	0.417	839
6	1	1	10	6	1.307	1232
6	1	1	11	7	0.871	655

Dogno	Sex	Diet	Wkst	Bsa	Detenz	Subenz
6	1	1	12	4	0.071	1333
6	1	1	13	5	0.994	872
6	1	1	14	9	2.122	1409
7	1	1	1	7	0.492	800
7	1	1	2	4	2.033	1622
7	1	1	3	1	0.216	2599
7	1	1	4	8	0.041	478
7	1	1	5	7	-	930
7	1	1	6	4	1.265	905
7	1	1	7	5	0.152	910
7	1	1	8	6	0.447	834
7	1	1	9	4	0.547	1072
7	1	1	10	9	1.241	1604
7	1	1	11	7	0.280	692
7	1	1	12	6	0.470	1000
7	1	1	13	8	0.282	687
7	1	1	14	8	1.310	336
8	1	2	1	1	0.366	3981
8	1	2	2	9	0.710	2409
8	1	2	3	4	0.760	2313
8	1	2	4	6	1.242	2515
8	1	2	5	3	1.165	1283
8	1	2	6	1	0.294	1047
8	1	2	7	5	0.185	1497
8	1	2	8	8	0.342	1079
8	1	2	9	7	0.057	1521
8	1	2	10	3	1.121	1382
8	1	2	11	7	0.516	1323
8	1	2	12	3	0.718	860
8	1	2	13	2	-	1739
8	1	2	14	3	0.474	1898
9	1	2	1	9	5.569	1624
9	1	2	2	6	0.814	1050
9	1	2	3	9	2.771	2266
9	1	2	4	5	0.833	1377
9	1	2	5	7	0.614	1122
9	1	2	7	4	2.809	1451
9	1	2	8	8	0.520	894
9	1	2	10	7	0.997	3398
9	1	2	11	5	0.508	1145
9	1	2	12	2	0.708	1858
9	1	2	13	4	0.655	2879
9	1	2	14	9	0.742	2180
10	1	2	1	7	0.928	1587
10	1	2	3	3	1.638	2455
10	1	2	4	1	0.067	2201

Dogno	Sex	Diet	Wkst	Bsa	Detenz	Subenz
10	1	2	5	2	0.552	1421
10	1	2	6	6	0.431	1643
10	1	2	7	7	0.156	607
10	1	2	8	3	0.248	1003
10	1	2	10	8	1.051	973
10	1	2	11	10	-	1253
10	1	2	12	9	1.941	1932
10	1	2	13	1	0.146	-
10	1	2	14	4	2.741	948
11	2	1	1	10	6.927	1280
11	2	1	2	8	5.050	1629
11	2	1	3	6	4.521	2159
11	2	1	4	2	3.190	3465
11	2	1	5	6	1.110	2417
11	2	1	6	5	0.886	1927
11	2	1	7	2	1.010	3585
11	2	1	8	10	0.450	1396
11	2	1	9	10	0.036	1209
11	2	1	10	7	0.683	857
11	2	1	11	3	0.642	748
11	2	1	12	2	0.942	-
11	2	1	13	10	0.296	1224
11	2	1	14	5	1.995	902
12	2	1	1	1	3.878	1360
12	2	1	2	8	1.093	748
12	2	1	3	10	01257	991
12	2	1	4	4	0.366	1640
12	2	1	5	7	0.556	1082
12	2	1	6	2	0.308	1711
12	2	1	7	10	0.341	1691
12	2	1	8	9	1.782	2611
12	2	1	9	2	0.296	1814
12	2	1	10	2	1.621	2345
12	2	1	11	5	0.423	674
12	2	1	12	7	0.438	713
12	2	1	13	3	0.340	921
12	2	1	14	1	0.945	-
13	2	1	1	7	0.696	1005
13	2	1	2	3	1.432	1078
13	2	1	3	2	0.430	3634
13	2	1	4	4	1.348	2661
13	2	1	5	1	0.195	647
13	2	1	6	4	3.361	3246
13	2	1	7	2	0.450	2870
13	2	1	8	10	1.789	1178
13	2	1	9	2	1.883	4524

Dogno	Sex	Diet	Wkst	Bsa	Detenz	Subenz
13	2	1	10	4	0.353	1021
13	2	1	11	1	0.733	1191
13	2	1	12	10	0.365	1234
13	2	1	13	10	0.780	1507
13	2	1	14	8	0.394	695
14	2	1	1	8	-	992
14	2	1	2	10	3.036	1222
14	2	1	3	7	2.595	1376
15	2	1	1	2	0.100	2535
15	2	1	2	2	0.656	3237
15	2	1	3	6	0.946	1626
15	2	1	4	9	0.465	1795
15	2	1	5	9	0.915	2189
15	2	1	6	10	0.196	1295
15	2	1	7	2	0.454	1305
15	2	1	8	8	0.650	1165
15	2	1	9	1	0.084	2563
15	2	1	10	5	0.503	1270
15	2	1	11	10	0.371	1105
15	2	1	12	4	0.111	1291
15	2	1	13	8	2.073	965
15	2	1	14	9	0.443	2047
16	2	2	1	9	4.224	2125
16	2	2	2	10	1.490	4597
16	2	2	3	7	0.496	1670
16	2	2	4	9	0.714	2778
16	2	2	5	4	1.220	1711
16	2	2	6	1	0.648	-
16	2	2	7	5	0.405	1951
16	2	2	8	3	1.735	1588
16	2	2	9	6	0.042	1911
16	2	2	10	2	0.091	2084
16	2	2	11	1	0.493	2046
16	2	2	12	9	1.169	1945
16	2	2	13	1	0.787	3632
16	2	2	14	8	2.893	1416
17	2	2	1	10	1.632	1337
17	2	2	2	8	1.035	1192
17	2	2	3	8	1.616	1456
17	2	2	4	9	1.475	1637
17	2	2	5	6	0.808	2010
17	2	2	6	8	1.136	1906
17	2	2	7	9	0.287	2211
17	2	2	8	3	-	662
17	2	2	9	5	0.840	789
17	2	2	10	2	1.449	1676

Dogno	Sex	Diet	Wkst	Bsa	Detenz	Subenz
17	2	2	11	10	0.311	1079
17	2	2	12	10	0.936	1303
17	2	2	13	1	0.331	2126
17	2	2	14	4	0.879	1227
18	2	2	1	9	1.854	2343
18	2	2	2	7	1.176	1475
18	2	2	3	8	1.365	1175
18	2	2	5	1	0.300	3023
18	2	2	6	5	1.190	1568
18	2	2	7	8	1.543	926
18	2	2	10	3	0.110	709
18	2	2	12	1	1.270	2407
18	2	2	13	3	0.562	1449
18	2	2	14	8	1.218	1038
19	2	2	1	2	0.551	3203
19	2	2	2	8	0.472	1297
19	2	2	3	9	0.360	2447
19	2	2	4	6	0.177	1698
19	2	2	5	10	0.176	1195
19	2	2	6	2	0.168	1027
19	2	2	7	6	0.178	1634
19	2	2	8	3	1.284	838
19	2	2	9	5	0.097	1245
19	2	2	10	3	1.432	1557
19	2	2	11	9	0.766	1575
19	2	2	12	9	0.726	1943
19	2	2	13	9	0.511	1344
19	2	2	14	6	0.203	1608