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CENTRAL ANGIOTENSIN-INDUCED WATER INTAKE AND SALT APPETITE IN THE PIG

by

Jeff Mutter

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

Master of Science
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to

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I'm not sure it is a function of the size of my animal model or the size of my project, that so many individuals made significant contributions to my research. It is probably a little of both. I have learned that when one works with pigs one does things in a big way, and thus the two supervisors, two working labs, two offices, a four member advisory committee and fourteen pigs.

When one has two supervisor's it can either be advantageous or disastrous. In my case the former was true. The generosity and supportive nature of Ben Tsang was appreciated. John Kucharczyk's gentle prodding (usually), financial assistance and guidance enabled me to complete my research and thesis, while attempting to further my education in other fields.

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The only reason this thesis is in a presentable form and not my illegible script is because of Elizabeth M. McNally's ability to read hieroglyphics and translate them into English. To type incomplete tables and text on little or no notice, which was often the case, is worthy of great praise.

Finally, it takes a loving supportive family and close friends to help one over the rough spots in life. I am blessed to have both.

If the purpose of research at the Master's level is, as I believe it is, to take a problem, analyse it, develop a suitable means for examining it, test it and derive some conclusions, while keeping in mind technical, spatial and financial restraints, then I feel that I have succeeded. It is the above mentioned people and many others who have made it possible and I thank them.

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ABSTRACT

Previous work has shown that drinking behaviour is an important regulatory response to depletion of the cellular or extracellular body fluid compartments. The purpose of the present study was to investigate the location and nature of the receptors which mediate water intake and salt appetite induced by activation of the renin-angiotensin system (RAS) in the pig.

Prepubertal female pigs fed a commercially-prepared sodium-free diet were tested for drinking of water and 1.8% NaCl for 60 min. following central or peripheral administration of the peptide components of the RAS and the enzyme renin. Infusions of angiotensin II (AII) (a hormonal mediator of extracellular thirst) into the saphenous vein at 125 or 250 mole min^{-1} produced inconsistent and weak drinking responses. In contrast, intracranial injections into the preoptic area and lateral cerebral ventricles of much smaller doses of AII (10^{-12} to 10^{-9} mole) elicited copious drinking of water and 1.8% NaCl in animals in normal fluid-electrolyte balance.

As in other species examined, AII was the most dipsogenic RAS component, followed by angiotensin I (AI), angiotensin III (AIII) and renin substrate (RS), in that order. Pretreatment of central injection sites with saralasin, a specific AII antagonist, had no effect on the drinking response induced by AIII, which indicates that AIII acts on thirst receptors distinct from those for AII. Renin was also dipsogenic at the doses tested (1 and 10 mU), but the response latencies and durations were typically much longer than for the peptide components of the RAS.

The influence of central administration of components of the RAS on salt appetite was also studied. Dose-dependent intakes of 1.8% NaCl solution were induced by AI, AII, AIII and RS; however, only AII and AIII were consistently effective. At higher doses (10^{-9} moles) of AIII, some of the animals drank salt solution before the water, which suggests that this peptide may play a role in stimulating primary salt appetite.

The results of this study are in general agreement with similar studies in other species and suggest that there are multiple sites of receptors for AII-induced thirst in the brain. The data also indicate, however, that AIII is itself dipsogenically active in the pig. Considered together, these findings suggest that more research should be directed towards understanding the role of AII metabolites in the central control of water intake and salt appetite.

1.0 INTRODUCTION

1.1 General Background

Fluid-electrolyte homeostasis is carefully regulated in all mammalian species by a complex set of neural, hormonal and behavioural mechanisms. Water loss can occur in variable amounts as a result of respiration, sweating, defecation, urination and vomiting. Only urinary fluid losses can, however, be controlled to any great extent.

The kidney is the primary regulator of existing body fluids and is able to respond to a changing internal milieu through sympathetic innervation of the glomerular arterioles, as well as through hormones such as vasopressin and aldosterone, which influence the reabsorption of water and electrolytes. By filtering more than thirty times the blood volume in a day, the kidney is able to concentrate urine to many times greater than blood concentration, whereas in times of overhydration the kidney can produce copious amounts of dilute urine.

While renal mechanisms are entirely competent in conserving body fluids, they cannot replenish actual water deficits. This function depends on the ingestion of fluids via drinking. The water content of foodstuffs comprizes approximately 20% of the total water intake in humans. Although water via foodstuffs may be important in some animals, especially desert rodents, normally most water is acquired through drinking.

Thirst, which has been described as the sensory signal of inadequate water stores (Fitzsimons, 1979) was not studied scientifically until the late nineteenth century. By the first world war, three major theories had developed. These were: a) that thirst is a sensation of local

origin related to dryness of the mouth and throat b) that thirst is a generalized sensation arising from loss of body water and that c) thirst arises from stimulation of a thirst center in the brain (cf. Fitzsimons, 1973).

The "dry-mouth" theory of thirst was first described by Haller in 1779 and continued to attract interest as late as 1919 (Cannon). Thirst was viewed to be the consequence of a lowering of the water-content of the body resulting in a decrease of watery salivary secretions. Inadequate salivary production, in turn, failed to keep the mouth and pharynx moist, and this was thought to be the direct stimulus for thirst (cf. Fitzsimons, 1979). Support for this theory was derived from experiments which showed a close relationship between salivary flow and the degree of thirst. Further experimentation has shown, however, that a 'dry-mouth' is not the principal stimulus for initiating drinking, although it may be important for the satiety from thirst. Epstein (1960) found that when a rat is only able to obtain water by injecting it directly into its stomach, there is a tendency for excessive intakes. Likewise, Nicolaidis & Rowland (1975) found that when large amounts of water were given into the stomach of a rat, drinking was still not totally suppressed. Thus it appears that while oropharyngeal cues are important to the overall monitoring of fluid intake they are not essential to the initiation of drinking.

A second theory of thirst was proposed by Schiff in the mid-19th century (cf. Fitzsimons, 1979). He stated that thirst arose not because of a localized feeling of dryness, but rather as a result of a "general sensation" arising from lack of water in the blood. He

reviewed work by other investigators on dehydrated animals that could be totally rehydrated by intravenous injections of water, milk, whey and other fluids.

Further evidence favouring this view over the "dry-mouth" theory was provided by Bernard in 1855, who inserted either gastric or esophageal fistulae into horses and dogs. He found that with the fistulae the animals could not satisfy their thirst, even though the upper part of their alimentary canal was wet. The animals drank until fatigued, rested, and then drank again.

The "general sensation" theory of thirst has developed from a single stimulus theory into what is now accepted as the "double depletion" hypothesis. This theory proposes that a deficit in either the intracellular or extracellular fluid compartments initiates drinking, and that simultaneous deficits in both compartments have an additive effect on thirst.

1.2. Cellular Dehydration as a Thirst Stimulus

Since the turn of the century, there has been speculation that decreased water content inside the cell could lead to thirst and drinking. Experimentally, dehydration of the cellular fluid compartment is an effective method for inducing thirst and can occur under the following circumstances: 1) water deprivation; 2) administration of membrane impermeable hypertonic solutions; 3) potassium depletion (Fitzsimons, 1979).

Elevated osmotic pressure as a correlate of drinking was first reported by Mayer in 1900. Wettendorff (1901), working independently, pointed out that thirst was aroused in dogs shortly after water deprivation, although blood osmotic pressure remained constant for

almost two days. He speculated that "blood preserves its properties as long as possible at the expense of the tissue water" (cf. Fitzsimons. 1979, p. 407).

Leschke (1918) noted that when treating patients for hemopysis by intravenous injections of hypertonic solution, there was a sudden, severe thirst which arose following injections of sodium chloride, but this was not as great following calcium chloride or urea infusions. He concluded that certain crystalloids produced thirst while others did not. However, he was unaware that the critical factor was whether a solute was able to penetrate the cell membrane or not.

Gilman's classical experiment with dogs in 1937 provided an explanation for why some hypertonic solutions stimulate drinking better than others. He measured water intake in dogs one hour after injections of either 40% urea or 20% sodium chloride (2.5 ml/kg). Dogs infused with hypertonic NaCl drank twice as much as those infused with urea (42 and 20 ml/kg, respectively). Gilman concluded that "cellular dehydration rather than an increase in cellular osmotic pressure per se is the stimulus of true thirst" (cf. Blass, 1973). Sodium chloride, by virtue of its rigorous exclusion from the cellular phase, gives rise to an increase in effective osmotic pressure causing an osmotic shift of cellular fluid into the extracellular phase. Urea diffuses freely into most cells, simultaneously increasing cellular and extracellular concentration.

Gilman's key experiment has since been replicated by a number of other investigators in different mammals, including man (Bellows, 1939; Holmes & Giegersen, 1950; Wolf, 1950). In addition, a number

of other hypertonic solutions have been tested, some of which are osmotically active (sodium acetate, sodium sulphate), while others (sorbitol, sucrose) are not. In general, these other solutions produced drinking similar to what one would have expected from Gilman's experiment.

In order to determine the threshold for thirst resulting from intracellular stimulation, a slow intravenous infusion of hypertonic solution was required that would more accurately parallel the slow rise in plasma osmolality observed under normal physiological conditions (Wolf, 1950). Wolf (1950) slowly infused hypertonic NaCl in men and in dogs, noting the point at which drinking occured. He found that a change of 1-2% in blood osmolality would lead to thirst and drinking in both species. Earlier, Verney (1947) had shown in dogs that a rise in blood osmolality of 2% will cause the release of vasopressin. Subsequently, Fitzsimons (1963) tested rats with a number of different hypertonic solutions infused intravenously at different rates of infusion. The threshold for drinking was found to be similar to that reported by Wolf (1950).

There does not appear to be any adaptation in terms of drinking thresholds to increased cellular dehydration. Fitzsimons (1963) showed that the rate of the infusion was unimportant in initiating drinking in rats. As well, when nephrectomized rats were deprived of water for 12 or 24 hours after a hypertonic NaCl injection, there was no difference in the magnitude of the drinking response over one hour between those groups and the rats given access to water immediately following the injection (Fitzsimons, 1971).

Cellular dehydration is detected by osmoreceptors which are probably located centrally. Injections of 3% saline by Andersson & McCann (1955) into different parts of the goat brain were consistently effective only when made into the anterior hypothalamus. However, because of diffusion of injected solutions, it is not known exactly what areas were affected by the injections. Furthermore, other investigators (ie. Epstein, 1960; Grossman, 1962; Seifter, 1962) were unable to replicate the Andersson & McCann (1955) findings in the goat. Thus, anterior hypothalamic 'osmoreceptors' remain unconfirmed.

It is also possible that osmoreceptors may be in the third ventricle, since injections of potassium chloride into the ventricle cause strong drinking responses (Olson, 1969). Another potential site for osmosensitive thirst cells is the lateral preoptic area (LPOA). Injections of 2.0 ml of 1.15 osm. sodium chloride or 1.15 osm. sucrose into the LPOA of the rabbit produced substantial drinking responses, whereas almost 300 other brain sites tested were negative (Peck & Novin, 1971). When the anteromedial portion of the LPOA was bilaterally ablated, the drinking response to cellular dehydration (2 ml 2M NaCl; IP) was abolished (Blass & Epstein, 1971). As well, drinking responses have been observed using more physiological injections (0.33 osm) of sodium chloride or sucrose in to the POA (cf. Blass, 1973).

1.3 Extracellular Dehydration as a Thirst Stimulus

A decrease in extracellular fluid volume induced by hemorrhage or other means has long been known to stimulate thirst (cf. Fitzsimons, 1972). However, drinking is but one means by which the body tries to compensate for hypovolemia. Other mechanisms, such as diversion of available cardiac output, utilization of general interstitial and blood reserves, and reduction of urine production, may all contribute to fluid conservation (Fitzsimons, 1972). In the final instance, however, an actual body fluid deficit can only be corrected by the ingestion of new fluid. As a result, hypovolemia, either naturally occurring or experimentally induced, is a powerful stimulus for thirst.

Wolf (1958) examined the effect of hemorrhage on thirst in horses after removal of 6 L of blood. On the days following hemorrhage water intakes were significantly greater than on control days. Szczepanska-Sadowska (1973) observed that removal of 15% of a dog's blood volume produced the same results.

Experimental procedures have been devised to simulate transient hemorrhage. When a hyperoncotic solution (polyethylene glycol, PG) is injected intraperitoneally in rats, and the animals are given a choice between 1.8% saline and water to drink, the rats always select water. The colloid draws fluid from the plasma and thereby mimics hemorrhage (Fitzsimons, 1961). Hypovolemia may also be simulated by physically impeding the circulation in specific areas. Ligation of the abdominal inferior vena cava was an effective stimulus for drinking in the rat (Fitzsimons, 1964, 1969). As well, constriction of the renal arteries leads to drinking (Fitzsimons, 1972).

The receptors for sensing a loss of ECF are baroreceptors in large vessels entering the heart and in the walls of the atria. Impulses are produced and carried in the vagus nerves and exert an inhibitory effect on thirst neurons. Arterial baroreceptors in the kidney may also play a part (Fitzsimons, 1979).

Hypovolemia can also be induced by removing sodium from the ECF. Dogs depleted of body sodium by diuretics were observed to remain hypovolemic and polydipsic for weeks while maintained on a sodium deficient diet, but plasma volumes and daily water intakes returned to normal with restoration of sodium to the animals (Holmes & Cizek, 1951). The sodium loss decreases the effective osmolality of ECF, thereby disrupting the flux of water across the cellular membrane; the accumulation of water within cells reestablishes osmotic equilibrium at the expense of the extracellular fluid volume.

1.4. Renin-Angiotensin System

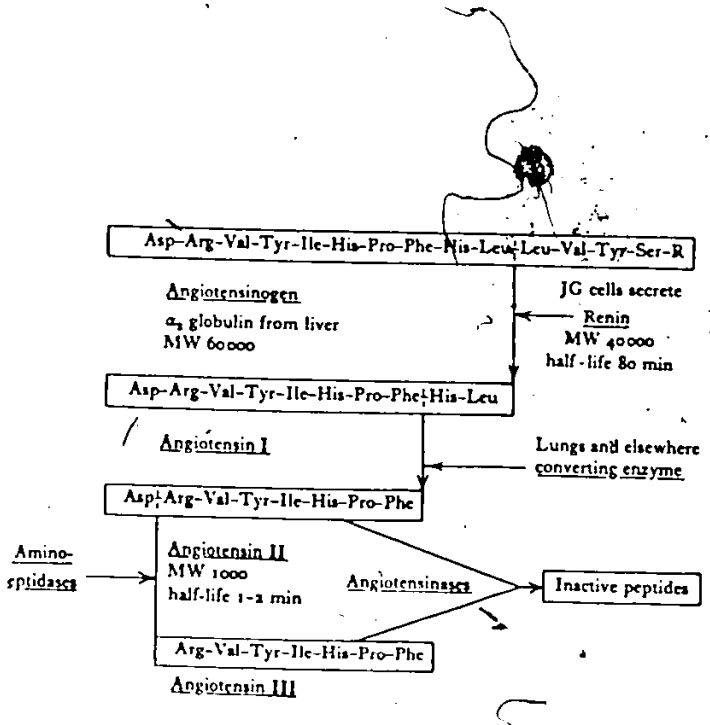
In addition to direct nervous activity from baroreceptors, there are hormonal responses to ECF hypovolemia. The most important hormonal regulatory system is the renin-angiotensin system (RAS).

(i) In vivo biosynthesis. Renin release from the juxtaglomerular cells in the kidneys is the rate limiting step in the biosynthesis of AII. There appear to be three main inputs controlling renal renin production: a) renal baroreceptors respond to a drop in renal perfusion pressure by releasing renin, b) renin release varies inversely as the total load of sodium delivery to the macula densa, and c) volume receptors outside the kidney transmit impulses via sympathetic nerves to the kidney to cause release (cf. Fitzsimons, 1972). Renin has a half-life in

the systemic circulation of 15-20 min in rat, 45-79 min in dog, and 42-120 min in man (Laragh & Sedes, 1973). Renin splits a hepatic α-2 globulin, angiotensinogen (RS), at the leucyl-leucyl bond between amino acid positions 10 and 11 (see Fig. 1). The newly formed decapeptide, angiotensin I (AI), which has relatively few biological properties (Ng & Vane, 1968), is then converted to the most biologically active compound, the octapeptide angiotensin II (AII), by a converting enzyme (CE). The cleaving of the two-terminal amino acids takes place primarily in the lungs. The half-life of AII is only 1-2 minutes, as most degradation takes place in the vascular beds. Des-aspartyl-AII or angiotensin III (AIII) is the only known degradation product to retain any of AII's biological properties (Fitzsimons, 1979).

In order to assess the potency of each RAS component for the various biological activities, a number of enzyme blockers, peptide antagonists, and antisera have been developed (see Fig. 2). Specific AII antiserum infused at a rate of (0.3 ml/kg/h) has been shown to cause a significant reduction in drinking induced by hyperoncotic dialysis (10 ml/kg of 30% 20,000 polyethylene glycol, s.c.) (Abdelaal et al., 1974). Tang & Falk (1974), using an AII competitive receptor antagonist, sarcosine^{1}-alanine^{8}-AII (saralasin, or P113), were able to attenuate drinking in the rat in response to i.v. renin (4 Goldblatt U.) or AII (80 µg). The converting enzyme blocker, SQ20881, has produced inconsistent results. In some cases SQ20881 pretreatment has actually enhanced AII-induced drinking (Goldman & Casner, 1973; 1975), while in other studies (cf. Fitzsimons, 1979) there was an inhibitory effect. One possible explanation for the stimulatory

Figure 1. The renin-angiotensin system cascade mechanism initiated by the release of renin. The half-lives of renin and angiotensin II given on the figure are approximate and vary between species. The molecular weights are also only approximate (adapted from Fitzsimons, 1979, p. 238).

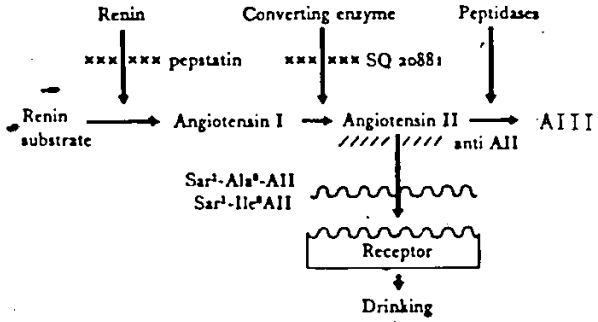

Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser–R
Angiotensinogen
α_2 globulin from liver
MW 60000
JG cells secrete
Renin
MW 40000
half-life 80 min
Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu
Angiotensin I
Lungs and elsewhere
converting enzyme
Asp–Arg–Val–Tyr–Ile–His–Pro–Phe
Amino-
sptidases
Angiotensin II
MW 1000
half-life 1–2 min
Angiotensinases
Inactive peptides
Arg–Val–Tyr–Ile–His–Pro–Phe
Angiotensin III

action of SQ20881 on drinking is that it enhances renal renin release (Lehr et al., 1973). Some of the more commonly used antagonists and inhibitors which have been used to characterize RAS receptors in different tissues are shown in Fig. 2.

(ii) Role of RAS in ECF Homeostasis. The RAS maintains blood volume and pressure via a number of direct and indirect actions. Angiotensin is one of the most potent naturally occurring vasoconstrictors. Its direct action on vascular smooth muscle, along with its stimulating action on the peripheral sympathetic system, help to restore arterial blood pressure. Peripheral infusions of AII into the dog carotid artery increase systemic arterial pressure, after pretreatment with an α-adrenergic blocker (Bickerton & Buckley, 1961), which suggests that AII must act at some central level to increase blood pressure. Further support for this view was provided by Dickinson & Lawrence (1963), who found that non-pressor systemic doses of AII given into the vertebral artery cause a rise in blood pressure. The hypertensive effect of AII could also be demonstrated by injections into the cerebral ventricular system (Severs et al., 1966). Responses to intracerebroventricular injections were slow in onset, whereas the intravertebral injections produced an immediate rise in blood pressure, which lasted as long as the infusion (Scroop & Lowe, 1969).

Of the RAS components, AII is the most potent pressor agent (Fitzsimons, 1979). AI has relatively little pressor activity. AIII is approximately 1/40 as potent as AII in sheep at raising arterial pressure (Blair-West et al., 1981), whereas in man AIII has only 20% the pressor activity of AII (Kono et al., 1983).

Figure 2. The sites of action of angiotensin II antiserum (anti-AII) and of the peptide antagonists of the renin-angiotensin system (eptstain, SQ20881, sar'-Ala^8-AII and Sar^1-Ile^8-AII). The enzyme cascade from left to right leads from renin substrate to degradation of angiotensin II (adapted from Fitzsimons, 1979, p. 314).


Renin
Converting enzyme
Peptidases
pepstatin
SQ 20881
Renin substrate
Angiotensin I
Angiotensin II
AIII
anti AII
Sar¹-Ala⁸-AII
Sar¹-Ile⁸AII
Receptor
Drinking

The RAS also helps to maintain fluid electrolyte homeostasis by stimulating the release of vasopressin through a direct action on neurosecretory cells in the supraoptic nucleus (SON) (Mouw et al., 1971). Angiotensin II and III are also important stimuli for aldosterone secretion (Gross, 1960). Laragh et al. (1960) first found that subpressor doses of AII increased aldosterone secretion in man. It is now known that AII and AIII are approximately equipotent in stimulating adrenal aldosterone synthesis. Blair-West et al. (1980) have demonstrated that in sodium replete sheep, AII and AIII are almost equipotent at various dose levels (1.25, 5, 20 and 80 pmol/dl). Kono et al. (1978) have obtained similar results in man. Recent work by Khairallah et al. (1978), among others, have shown that AIII can stimulate aldosterone biosynthesis in vitro.

These findings suggest there is more than one type of angiotensin receptor. There is a myotropic receptor which is specific for AII. There also appears to be either a less specific steroidogenic receptor able to accommodate AII and AIII, or there are two types of steroidogenic receptors in the adrenal.

In addition to its role in helping to maintain sodium balance via renal mechanisms, the RAS has been associated with stimulating salt appetite.

Increased sodium appetite has been reported following intracranial injections of AII, but not after peripheral AII administration (Avrith & Fitzsimons, 1980). Fischer & Buggy (1978) observed in one hour tests that rats with continuous access to water and isotonic saline shifted in taste preference from water to saline following AII

injections (500 p-mole) into the POA. POA injections of AII also increased sodium intake following dietary sodium deprivation (Fisher & Buggy, 1975). Pharmacological doses of AII (500 ng/8 min for 8 hours) into the lateral cerebral ventricle induced rats to drink substantial amounts of 1.8% or 2.7% saline when both saline and water were offered (Buggy & Wade, 1978). Chiaraviglio (1975) found that AII injected into the third ventricle in low doses restored sodium appetite in the sodium-depelted rat in which the appetite had been abolished by nephrectomy.

Injection of the components of the RAS into the brain of adrenalectomized rats induced a significant increase in 2.7% NaCl as well as water, within the first hour of injection (Fitzsimons & Worth, 1978). The sodium intakes were less reliable and smaller than spontaneous daily intakes of saline intake of sodium-depleted adrenalectomized rats. However, the effect on NaCl ingestion was a real one and was too rapid to be secondary to natriuresis (Fitzsimons, 1979).

AII's most profound effects were observed in long term infusion experiments. Avrith & Fitzsimons (1978) have observed striking increases in intakes of 2.7% saline in rats offered the choice of water and saline to drink following i.c.v. injections of 1 mole AII or RS for 14 days.

The reason for central angiotensin's effectiveness in stimulating salt appetite is not known. However, it may be due to receptor accessibility or obtaining the necessary local concentration needed to produce the response (Fitzsimons, 1979).

The last major mechanism by which the RAS assists in fluid-electrolyte homeostasis is by stimulating thirst. Early evidence for a role of the RAS in drinking was obtained by Linazazoro (1954) who

found that body-weight loss in nephrectomized rats could be prevented by pig kidney extract injections. Linazazoro proposed that the rats may have had an impaired thirst mechanism which prevented sufficient water intake to maintain fluid homeostasis.

Since that time a number of experiments have been done to show that it is AII via renin that stimulates thirst. Almost all of the stimuli known to cause renin release have also been shown to be dipsogenic as well. Vasoconstriction, hemorrhage, exercise, upright posture, low sodium or potassium diets, catecholamines, stimulation of renal nerves and stimulation of sympathetic areas in the brain, are all known stimuli for renin release as well as for drinking (c.f. Fitzsimons, 1979).

Peripheral injections of components of the RAS are a good tool by which one cah mimic those physiological changes which normally cause renin release and thereby stimulate drinking.
Use of the components and various blockers has established that the primary drinking responses are mediated via angiotensin II (Peart, 1969; 1979). Rats, dogs and most other mammalian species tested are sensitive to infusions of RAS components, given i.v. or by intra-carotid (i.c.) injection (Fitzsimons, 1969; 1978). It has been demonstrated, moreover, that i.v. injections of AI have greater potency than i.c. infusions. This may be due to the fact that when AI is given i.v., it is converted to AII as it passes through the lungs before reaching the brain. When AI is infused i.c. there is less time for it to be converted to AII before reaching target tissues in the brain (Fitzsimons, Kucharczyk & Richards, 1978).

It is not known for certain if AII acts on peripheral and/or central receptors to evoke drinking. It has been suggested by Fitzsimons (1977) that AII may induce drinking, a) by a direct action on peripheral receptors, b) by sensitizing the central mechanisms to thirst stimuli arising from receptors elsewhere in the body, and c) by direct action on a central thirst receptor. Overall, this last possibility is considered to be the most likely explanation for AII-induced thirst (Fitzsimons, 1979), and will be considered in detail next.

(iii) Central "thirst receptors". The view that there is a 'thirst center' in the brain has attracted a lot of attention over the last 25 years. Early results were obtained from permanent central cannulation in 1952 by Andersson, who induced drinking in a water-replete goat via a hypertonic saline injection into the hypothalamus. Interest in finding a 'thirst center' has steadily increased, but there is as yet no precise anatomical definition of its location. Indeed, most current reviews (i.e. Fitzsimons, 1979; Kucharczyk, 1984) suggest that there may be multiple central receptor sites for AII, including the anterior hypothalamus and forebrain, the cerebral ventricles, and several circumventricular structures.

Historically, the first area of the brain implicated as a 'thirst center' was the forebrain. A number of investigators in the 1950's found that partial lesions or ablations of parts of the hypothalamus resulted in a reduction or a complete disruption of thirst. Early electrical stimulation work by Andersson et al., (1955) showed that stimulation near the fornix would cause polydipsia in the goat. More recently, Mogenson & Stevenson (1966) observed drinking with electrical

stimulation of the medial forebrain bundle, while Robertson, Kucharczyk & Mogenson (1983) were able to induce drinking by electrically stimulating the subfornical organ.

The controversy over the site of the central 'thirst center' still exists. The results of the more important studies will be reviewed next.

Epstein et al. (1970) reported that sites throughout much of the septum, preoptic area, and hypothalamus were sensitive to AII. Further mapping studies with smaller injection volumes (0.1 µl) delineated the AII-responsive zone to the ventromedial parts of the septum, medial parts of the preoptic area and the hypothalamus; to a zone in the periacqueductal gray; and to a zone in the ventrolateral part of the thalamus (Swanson & Sharpe, 1973).

However, by the mid-1970's, there was uncertainty that AII could in fact cross the blood-brain barrier (BBB) from the systemic circulation and gain access to the postulated receptors in the forebrain (Gatt et al., 1975). This was consistent with the finding of a long latency between i.v. infusion of AII and the onset of drinking (Fitzsimons & Simons, 1969).

One proposed explanation for AII dipsogenic effects intracranially was that there are other receptors outside the BBB, but which have neural connections. Evidence that these so-called "circumventricular organs"(CVO's) are involved in fluid-electrolyte regulation will be dealt with in a subsequent section.

Currently there is considerable research into providing evidence that an iso-RAS exists in the brain. Support for this hypothesis is derived from two sources a) identification of the RAS components in the brain, and, b) observations of intracranial infusions of the components. A cerebral RAS would enable conversion of an inactive protein to the active peptide to take place totally in neural tissue.

While all of the RAS components have been found in the brain, a major remaining problem is to accurately localize and quantify the components. Another problem is that there appear to be species differences for particular components and sites.

Angiotensinogen (RS), for example, has been identified in many areas of the brain. In the dog, RS concentration in CSF of the third ventricle has been reported to be 210 ± 24 ng/ml, as compared to 1002 ± 59 ng/ml in plasma (Reid & Day, 1977). However, when one compares the RS values with total proteins in the fluid, the ratio of RS in the CSF is 15 times greater than in plasma. The higher concentrations in the CSF suggest that the central RS is not some form of contamination in the CSF.

Reid & Day (1977) have shown that peripheral and central RS have similar kinetic, and gel filtration properties. Although the molecules appear to be similar, it has been shown that changes in RS concentration in the periphery do not affect RS in the CSF and vice versa. Reid & Day (1977) have shown that while plasma RS concentration doubled following bilateral nephrectomy, CSF RS concentration remained unchanged.

RS concentration in brain tissue is much less than that of CSF. In the dog, brain extracts contain 9.0 ± 1.6 ng/g protein. A recent study by Hawkins & Pintz (1982), in which they measured RS in 46 brain regions of the rat brain, found the anterior, medial and lateral hypothalamus, thalamus, SON, SN, RF and AP to have the highest concentrations. The concentration of RS in these areas was approximately 2 pg/ng protein.

Renin has been demonstrated in brain with bioassay or radio-immunoassay techniques (Ganten et al., 1971). However, the finding by Day & Reid (1976) that the optimum pH for brain renin activity is 4.5 to 5.5, and that there was little or no activity at a CSF pH of 7.3 makes doubtful that a physiological renin-like substance exists. It was thought that perhaps there is no real cerebral renin, but rather that the cleaving of the RS is done by a somewhat similar non-specific enzyme, cathepsin D, which is found in large amounts in the brain (Reid, 1977). However, use of non-specific antibodies (Hirose et al., 1979) and different separation techniques (gel filtration and affinity chromatography) (Osman et al., 1979) have in more recent work demonstrated the existence of a true brain renin. Hirose et al. (1976) have shown that in hog brain, there is a wide distribution of renin activity in all regions examined. The pineal, adenohypophysis and choroid plexus have the highest activities, while moderate levels are observed in the hypothalamus, cerebellum and amygdala. It was also found that the brain renin was similar in weight and optimum pH characteristics to renal renin, which suggests that cerebral renin may play a physiological role.

Converting enzyme is found in many tissues in the body. The levels in the brain are lower than in many other tissues, but are adequate to sustain rapid conversion of AI to AII (Ramsay, 1979). Specific activity measurements of converting enzyme (CE) have been made in both rat and human brains. Very high levels of activity in the rat brain were found in the pituitary, cerebellum, and striatum, while moderate levels were measured in the hypothalamus, midbrain and hippocampus (Yang & Neff, 1972). Human brain distribution of CE activity is slightly different. High levels are found in the caudate and septum, moderate levels in the hypothalamus and prefrontal cortex, and low levels of activity in the cerebral cortex and hippocampus (Poth et al., 1975). The different levels in activity of CE in the same brain area in different species may partially explain the differential potencies of AII precursor in different species.

Ganten et al. (1971) first reported the presence of angiotensin-like activity in brain extracts. Reid & Day (1977) found concentrations of 8 pg/g tissue of AI in dog brain. Using immunofluorescence, nerve fibers containing AII have been located in several areas of the brain (Fuxe et al., 1976), with particularly high concentrations in the

periventricular regions (Changaris et al., 1977). CSF concentrations of AII have been reported to be low (c.f. Ramsay, 1979).

The body of evidence just presented, showing the presence within brain and cerebral ventricles of all the components of the RAS, adds support to the idea that an endogenous cerebral RAS may play a role in body fluid-electrolyte balance. This idea is further supported by the results of experiments in which AII was administered centrally. Injections of small volumes (0.1 µl) of AII into the septum, preoptic region or anterior hypothalamus produced strong drinking responses in the cat (Swanson & Sharp, 1973) and several other mammalian species (Fitzsimons, 1979). The other components of the RAS are not as potent dipsogens as AII (Fitzsimons & Kucharczyk, 1978).

Fitzsimons et al. (1977) described the interspecies differences in water intake following intracranial injections of RAS components. In the species tested (rat, dog, pigeon), AII was the most dipsogenic particularly at low doses (10^{-12} mole). However, in both the rat and pigeon, both RS and AI were as dipsogenic as AII at higher doses (10^{-11} and 10^{-10} mole), indicating rapid conversion to the biologically active AII. In the dog, AII is more dipsogenic than both AI and RS at intracranial doses of 10^{-12} - 10^{-9} mole (Fitzsimons & Kucharczyk, 1978). The results suggest that in this species there is less efficient conversion of precursors to AII (Fitzsimons et al., 1977). In rat, dog and pigeon, AIII is relatively ineffective in inducing thirst (Fitzsimons et al., 1977). The only species tested thus far to show a strong response to intracranial AIII is the gerbil (Wright et al., 1984)

Thus, while AIII is known to be potent in promoting aldosterone release in the periphery (Blair-West et al., 1975), its effect in the brain has yet to be determined.

Support for the hypothesis that AII acts within the brain is growing. The long latency to drink following jugular infusion of AII (Fitzsimons & Simons, 1969; Abdelaal et al., 1974) and the relatively short latency when AII is directly injected into the forebrain (Kucharczyk & Mogenson, 1975) suggests that receptors must be located in the forebrain.

However by the mid-1970's, there was some uncertainty that AII could cross the blood-brain barrier from the systemic circulation and thereby gain access to the postulated receptors in the forebrain (Ganten et al., 1975). This was consistent with the finding of a long latency between i.v. infusion of AII and the onset of drinking (Fitzsimons & Simons, 1969).

In 1973, Simpson and Routtenberg reported that very low (1 pmole) doses of AII administered directly into a circumventricular structure, the subfornical organ (SFO), of the rat reliably induced drinking. They suggested that the receptors for AII are in the SFO, and that AII administered through cannulae into the anterior forebrain, in fact, acts on the SFO after diffusing through the cerebral ventricles. The "ventricular hypothesis" is an attractive one, mainly because it is known that the SFO lies outside the BBB (Akert, 1969) and could therefore be readily acted upon by blood-borne substances.

The SFO is now known to mediate a number of other AII effects. Vasopressin release has been reported following SFO injections of AII (Keil et al., 1975). As well, the pressor effects of AII have been shown to be significantly reduced in rats after SFO lesions without affecting resting arterial pressure (Mangiapane & Simpson, 1980).

Femtamole doses of AII injected directly into the SFO promote drinking (Simpson & Routtenberg, 1973). SFO lesions prevent drinking induced by i.v. AII (Abdelaal et al., 1974) or after POA injections of the peptide (Simpson & Routtenberg, 1975). SFO lesions also have been shown to reduce or eliminate drinking responses with low doses of AII injected directly into the SFO of the rat (Abdelaal et al., 1974; Simpson et al., 1978), dog (Reed et al., 1980) and oppossum (El'front et al., 1980).

The use of specific AII competive antagonists has shown that the SFO is indeed sensitive to AII, SFO pretreatment with saralasin attenuated the dipsogenic effect of equimolar doses of AII injected into the SFO (Mangiapane & Simpson, 1980), as well as after intrajugularly infused AII (Simpson et al., 1978).

However, some recent research strongly suggests that the SFO is not the exclusive site of AII thirst receptors. The long latency to drink following jugular infusions of AII (Fitzsimons & Simons, 1969; Abdelaal et al., 1974) and the relatively short latency when AII is injected directly into the forebrain (Kucharczyk & Mogenson, 1975) suggests that some of the receptors must also be located in this area of the brain. Kucharczyk & Mogenson (1976) produced drinking in rats with POA cannulae angled to avoid penetrating the cerebral ventricles. They were able to evoke strong drinking responses with 25 p-mole of AII in a volume

of 0.2 µl. It is unlikely,that with this small volume,reflux of AII up the cannulae shaft to the ventricles would have occured.

Swanson, Kucharczyk & Mogenson (1978) induced reliable short-latency drinking in rats after POA injections of 6.6×10^{-12} mole AII in volumes as low as 0.01 µl, and then confirmed by autoradiography that the spread of the injected solution was restricted to the immediate area of the POA. In the dog, Fitzsimons & Kucharczyk (1978) found that the latency to the onset of drinking was shorter after injections of AII into the POA than into the LCVs, which would not be expected if AII receptors were located only in a periventricular structure such as the SFO.

The findings reviewed above suggest that there are multiple sites of receptors for AII-induced thirst in the brain including the SFO and POA. There are also reciprocal neural connections between the SFO and the forebrain. One of the two major sets of efferents from the SFO project into the hypothalamus, particularly the SON, PVN and lateral POA (Miselis, 1981). SFO projections to the SON suggest a possible functional relationship with SON functions such as vasopressin secretion. One proposal (Kucharczyk, Assaf & Mogenson, 1976) is that projections to the POA may be important in initiating the drinking behaviour from peripheral stimuli, whereas the POA receptors respond to AII generated by the cerebral RAS system. It is not yet known whether the stimuli which activate the cerebral RAS system are the same as those which initiate peripheral AII biosynthesis.

1.5. Rationale

The general objective of this study was to compare the efficacy of RAS components in inducing drinking after direct intracranial injection, in order to characterize thirst receptors in the pig.

The specific objectives were:

i) to compare the potency of RAS components injected into the POA and into the LCV, in order to study the possibility of multiple receptor sites for angiotensin-induced drinking. The injection effects were evaluated in terms of the latencies and magnitude of the drinking responses in the hour following the administration of drugs;

ii) to compare AII-induced drinking after central and peripheral injections to determine the sensitivity of the response, as compared to other species

iii) to better quantify the type of receptors in the brain that initiate drinking. The intracranial sites were pretreated with an AII competitive antagonist, saralasin, to establish whether the receptors were only AII-sensitive, or whether they responded to both AII and AIII;

iv) to study RAS injection effects on Na^+ appetite in order to determine if this is a primary effect, or secondary to prior water drinking. 1.8% NaCl intakes in animals fed a Na^+-free diet were measured during the hour after the intracranial injection of the RAS components. This allowed a careful examination of the relationship between salt appetite and ingestion of water.

The prepubertal female pig was selected as the animal model primarily because of its brain size and demonstrated ingestive behaviours. The large brain facilitated the localization of injections and surgical placement of cannulae.

It has been demonstrated that the female reproductive cycle affects fluid and electrolyte homeostasis. The precise nature and extent of this influence has not been elucidated. In an attempt to help clarify this problem an additional study was initiated. The effects of the ovarian female reproductive hormones, estradiol and progesterone, on ad libitum and induced fluid intakes were evaluated. The post-pubertal gilt was a good animal model, as it has a relatively long reproductive cycle (21-23 days), and its hormonal profile is similar to the human. Since the study is preliminary, it has been placed in Appendix I.

2.0. GENERAL METHODS

2.1. Animals; Housing and Diets

Fourteen female prepubertal pigs from nulliparous litters were obtained from the Health Protection Branch of Health & Welfare Canada. They were housed individually in 2 by 3 meter pens with four pigs to a room. The pigs were maintained on a 12 hour light/dark cycle with a controlled temperature of 20°C. The layout of the room allowed the pigs adequate social contact, which is necessary to ensure normal growth and development (Jensen et al., 1970). A 60 centimeter wide, 2 cm thick plywood board was placed between the adjoining pens to prevent chewing of the surgical implants by neighbouring animals.

The floors of the pen were covered with rubber mats on top of which was a thick layer of woodchips to enable the animal care handlers to keep the pens clean. Twice a week the pen was completely cleaned and new wood chips and washed mats were returned.

The gilts were fed a commercially prepared balanced diet consisting of at least 15% protein (Ralston-Purina) ad libitum. In order to assess sodium appetite, the feed was specially made to exclude the normal 0.5% sodium chloride. The other components that made up the diet are reported in Appendix B. In order to replenish lost sodium and assess so-ium appetite, a 1.8% sodium chloride solution containing food dye, was available ad libitum, as was distilled water. The fluids were contained in 20 liter containers that gravity fed through hoses to pig nozzles which were firmly attached to the pen fencing (Fig. 3).

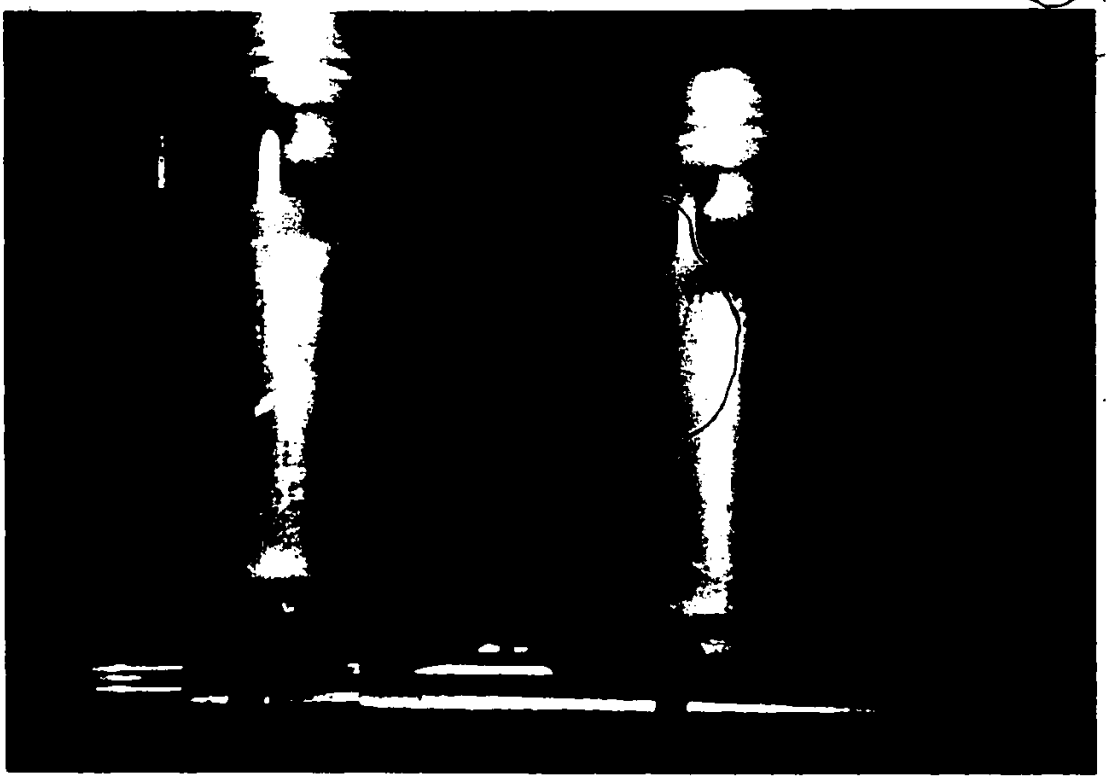
Figure 3. Gravity-feed drinking system.

a) 20 l containers of 1.8% NaCl and water.

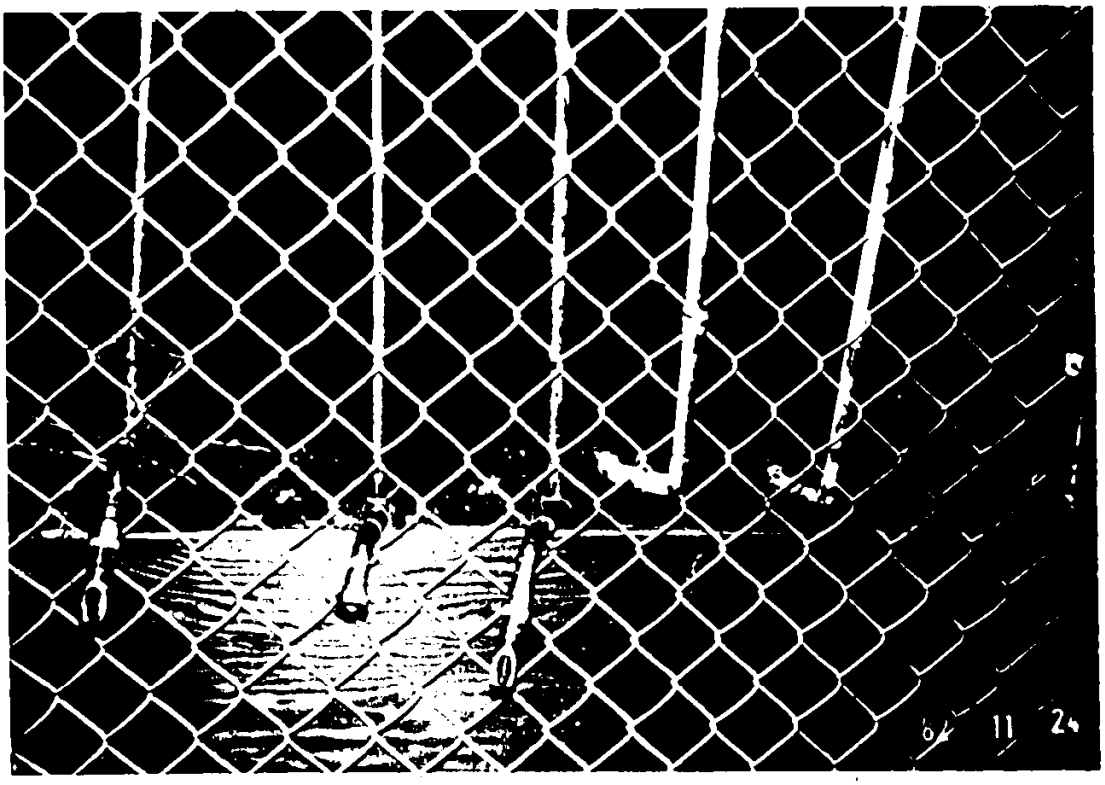
b) separate drinking nozzles attached to the fencing.

Fig. 3

(a)



(b)



2.2. Measurements of Fluid Intake

During an experimental period 24 h intakes of water and 1.8% NaCl were recorded at 9 a.m. daily by refilling the 20 liter containers with the appropriate fluid. Measurements were made to the nearest 10 ml.

2.3. Intracranial Cannulation (Fig. 4)

When the pigs weighed approximately 50 kg, intracranial guide cannulae were stereotaxically implanted into the forebrain and lateral cerebral ventricles using surgical procedures previously employed by Fitzsimons & Kucharczyk (1978).

Prior to the surgery, the pigs were pre-treated with atropine sulphate (0.04 mg/kg) to reduce salivary and bronchial secretions and sodium pentobarbitol (50 mg/kg). The drugs were administered intramuscularly using a 5 ml syringe and a 23 gauge butterfly needle.

Thirty minutes after the drug injections, the animal was transported to the preoperative preparation room, where it was masked down with nitrous oxide (1000 ml/min) and oxygen. Anesthesia was maintained using a closed system of halothane (4-9%, Ayerst Laboratories, New York, NY) and O_2 (500-1000 ml/min) (see Figure 5a).

The head was shaved with animal hair clippers and cleaned with an antiseptic solution. The pig was then transferred to the sterile operating and its head was placed into a stereotaxic device (Kopf Type 500; David Kopf Instruments, Tajunga, Calf.). Estimates were made for the positioning of two preoptic area (POA) and two lateral ventricle (LV) cannulae by referring to a dog stereotaxic atlas (Dua-Sharm et al., 1970) and from skull surface landmarks

such as bregma and lambda. Subsequent histological examination confirmed that the placements were correctly made.

Using a dentist's drill, four small holes were drilled through the skull at predetermined locations. Four additional holes were drilled partially into the skull, approximately 7 mm from the completely bored holes. A blunted stainless-steel needle, 30 mm in length and 0.7 mm in outer diameter (21 gauge) was then lowered into the bored hole. In order to ensure that the cannula was well anchored, stainless steel support screws, about 8 mm in length, were inserted into the partially drilled holes and joined to the cannula by means of dental acrylic. Cannulae were separated from each other by inserting a piece of parafilm between them. This was necessary since the pig's skull was still growing, and the positions of the cannulae may otherwise have been displaced by mechanical pressure. A stainless steel obdurator and easily removable rubber diaphragm were placed in each cannula to prevent infection or blockage of the guide cannula.

Postoperatively, the pigs were injected daily with 0.5 g chloramphenicol (Roger) for seven days. The area of the intracranial implant was cleaned every day with alcohol and hydrogen peroxide until it had healed. The animals were eating and drinking normally within two days after surgery and appeared to suffer no ill-effects from the surgery.

2.4. Intracranial Injections of Test Solutions (Fig. 4)

Intracranial injections were carried out in the pigs at 13:00 h, twice each week, after food depriving them for at least one hour. The injection involved inserting a 30 gauge injector needle, 35 mm in length, into the lumen of the cannula, after first removing the indwelling obdurator. The injector was attached by polyethylene tubing (PE 10, Clay Adams) to a 20 µl capacity syringe (Hamilton, Number 710). A polyethylene collar (PE 50, Clay Adams) was placed around the injector to ensure that the injector would be inserted the proper distance down the guide cannula. The injector and tubing were filled with the solution to be injected. The microsyringe injected 1 µl of solution by oil displacement from the syringe.

A valid injection consisted of: a) the insertion of the injector cleanly to the appropriate depth; b) an infusion of 1 µl of solution; c) an allowance of 15 to 20 sec for the solution to naturally displace; so that the solution did not travel up the injector tract; and d) the removal of a straight injector needle. Problems occasionally occured if the animal quickly moved its head and either bent or shook out the injector. As the pigs became accustomed to being handled, this became less of a problem.

In order to assess the appropriate positioning of the cannula, the first component of the RAS tested was AII at a dose of 10^{-10} mole. This component and dose was selected first since this has been shown to be the most dipsogenic component in other species (Fitzsimons, 1972; see Introduction).

Figure 4. Intracranial injection of test solutions.

a) shows the injector needle being lowered into the intracranial cannula.

b) shows the injector needle after it has been positioned and the test solution injected.

c) shows a closeup of b). Note the four cannula sites with rubber diaphragms.

d) shows the drinking response observed shortly after injection of the test solution.

Figure 4a) Insertion of injector needle

b) Injector needle placement

Fig. 4

(a)

(b)

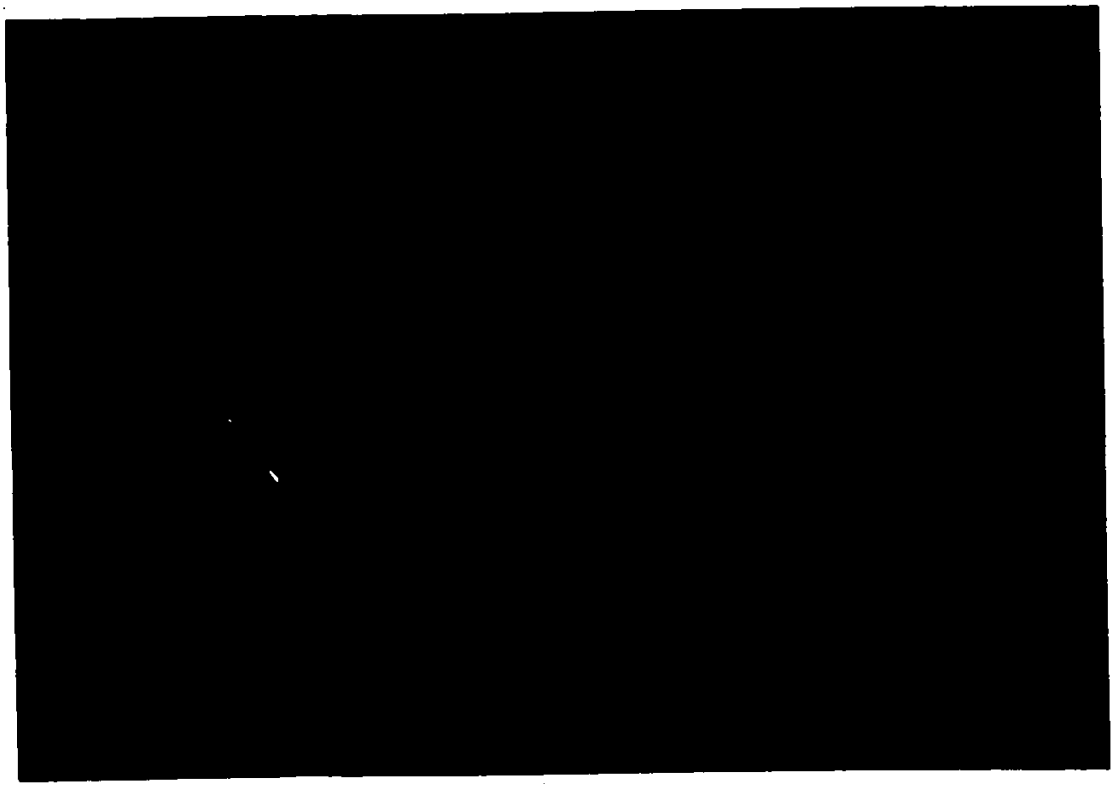
Fig. 4c) Close-up of 4b)

c) Behavioural response

Fig. 4

(c)

(d)



The dipsogenic response of each of the peptide components of the RAS (RS, AI, AII and AIII) (all obtained from Beckman) were examined at four doses from 10^{-12} to 10^{-9} mole, while hog renin (Nutritional Biochemicals) was tested at 1 and 10 mIU. The tests were made in a random manner over a period of 6-8 weeks. Appropriate controls were routinely carried out in every active site to control for possible behavioural conditioning. This consisted of sham injections of the vehicle alone (1 ml of 0.9% NaCl) following the same test procedures. The parameters used to determine the dipsogenic response were latency period and volume of fluid ingested within 60 min after completion of an injection. The latency period was determined as the time taken from the completion of the injection to the first ingestion of either water or 1.8% saline.

In experiments where a competitive AII-antagonist (Sar(1)-Ala(8)-AII, Beckman) was used, the antagonist was injected at least five minutes before the test solution using the same cannula. The antagonist was injected in isotonic saline at a concentration 10 times greater than that of the test substance.

2.5. Chronic Intravenous Cannulation (Fig. 5).

The catheter used in these studies was similar to the one described in Withey et al. (1973) (see Fig. 5c). The cannula consisted of an inner silastic tube with a diameter of 1.0 mm. A blunted 23 gauge stainless-steel luer-lock needle was inserted into the tubing and tied into place by 30 silk. An outer cannula with a diameter of 3.0 mm was placed over the top as a protective cover. It was held in place by medical grade elastomer. The cannula was then inserted into a dacron weave pad which was shaped like a baseball home plate with a base of 5 cm. The weave pad was later modified into a 10 x 4 cm oval. The

dacron pad was anchored to the cannula with elastomer (Fig. 5b & c)

Once the cannula was appropriately situated, dental acrylic was added around the exteriorized tip for added protection. As well, a plastic luer-lock syringe tip (plus stopper) was attached to the end to seal the cannula closed. The lumen of the cannula was filled with a isotonic NaCl and 1% heparin solution except when test

A silastic catheter approximately .05 m in length was surgically implanted into the saphenous vein and exteriorized on the midline of the back as described by Knipfel et al. (1975) and Withey et al. (1973) with modifications as suggested by Robert Young, Marilyn Keaney and Janet Lemoine (Fig. 5d).

The protocol for the implantation was as follows:
Prior to the operation the pigs were pre-treated i.m. with atropine sulphate (Atravet 0.04mg/kg and a short acting barbituate, sodium pentobarbitol (50 mg/kg). Thirty minutes after the i.m. injection of the drugs the pig was masked down with nitrous oxide (1000 ml/min) and oxygen. Anaesthesia was maintained using a closed system of halothane (4-9%, Ayerst Laboratories, New York, N.Y.) and O_2 (500-1000 ml/min).

The operation involved making a mid-dorsal incision approximately 10 cm long through the skin, approximately 15 cm anterior to the lumbar region of the spine. The dacron weave pad was then positioned subcutaneously in the 10 cm incision. The large diameter tubing was exteriorized through a 2 cm stab wound incision about 3 cm anterior to the larger incision. The inner tubing was then passed

subcutaneously to the area of the saphenous vein in the rear hock by means of a 400 cm stainless steel trochar (Figs. 5e-h).

The saphenous vein was located by making a 5 cm incision through the skin at the level of the hock. After the vein had been located the cannula was inserted into the lumen and pushed towards the heart. After the cannula was checked for patency, it was anchored to the surrounding tissue with 30 silk.

The animals were given 0-5 g of chloramphenacol and 300 mg of iron i.m. following the operation. I.m. chloramphenacol injections were continued for 5 days following the operation. Daily washings of the incisions with 70% alcohol and warm water were carried out to prevent infection, and in some cases a topical antibiotic salve was administered if required.

Within 1 or 2 days the animals were eating, drinking and behaving normally. In less than a week the incisions were no longer tender to the touch. The sutures were removed when the incisions had adequately closed, which was approximately 10 days after the cannulation. In general, a catheter would remain patent for about ten weeks. Occasionally small clots would form in the lumen, which were removed by flushing the catheter with the heparin solution. In those instances when the catheter became permanently blocked, it was removed and the other saphenous vein was catheterized.

2.6. Intravenous Testing

At 13:00 h, infusions of AII were made through the chronically implanted saphenous catheter at a rate of 125 or 250 p-mole/min for

15 min. The infusions were continued until either the animal began to drink or thirty minutes, whichever came first*. Measurements of the total fluids drunk, both water and 1.8% NaCl, in the following 60 min were measured to the nearest 10 ml.

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*The latency period to drinking was noted, as well as amount drunk in one hour.

Figure 5. Implantation of an indwelling intravenous silastic cannula. a) to h) show the sequence of steps taken in placing an indwelling silastic cannula into the saphenous vein of the prepubertal female pig.

a) shows the closed-system anaesthetic set-up used during both intravenous cannula implantation and intracranial cannulation.

b) shows the intravenous cannula, with the scapel as a reference for size.

c) shows the cannula in more detail. The inner and outer cannulae are in cross-section with the felt pad, male adapter and plug (adapted from Withey et al., 1973).

d) shows the route of the cannula from the middle of the back, where the cannula is exteriorized, under the skin to the hock where it is inserted into the saphenous vein (adapted from Young, 1978).

e) shows the mid-dorsal incision into which the dacron-weave pad was inserted. The dacron pad was placed subcutaneously under the large incision and the end of the cannula is exteriorized via the small incision just dorsal to the larger one.

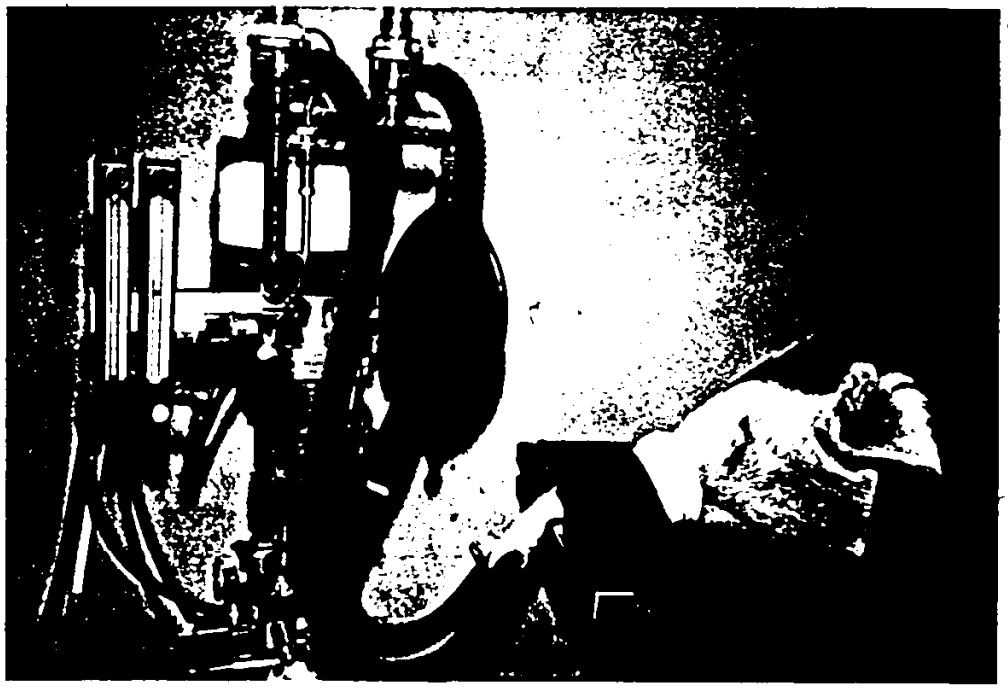
f) displays the upper end of the cannula in position.

g) shows the end of the 400 cm long trachar, which has been pushed subcutaneously towards the hock.

h) displays the saphenous vein at the level of the hock, after it has been isolated and prepared for cannulation. Note the silastic cannula emerging from beneath the skin.

Fig. 5

(a)



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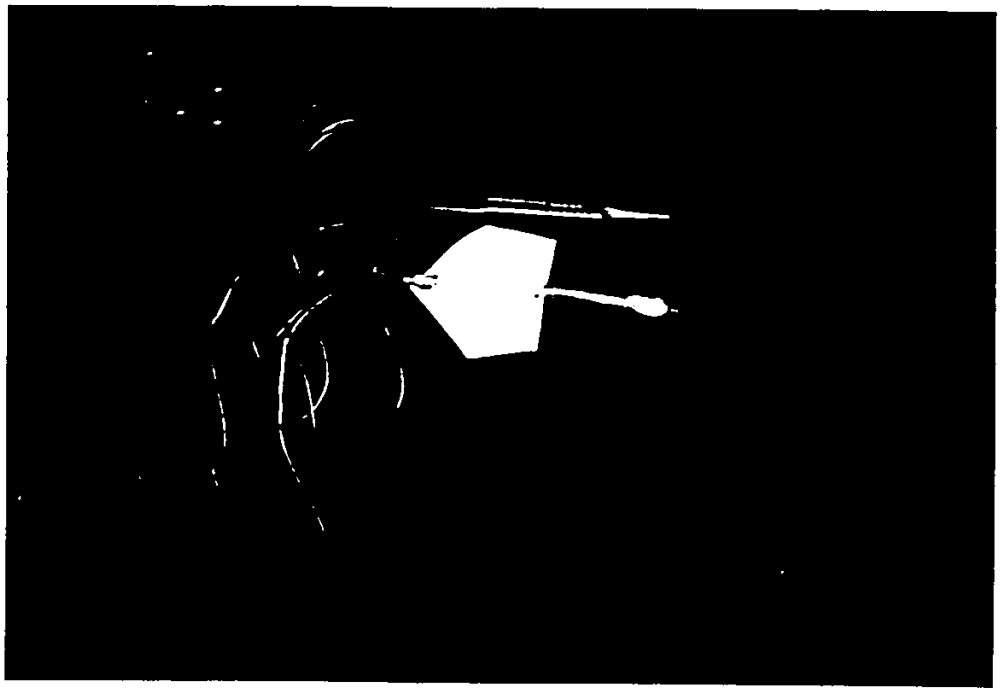


Fig. 5c

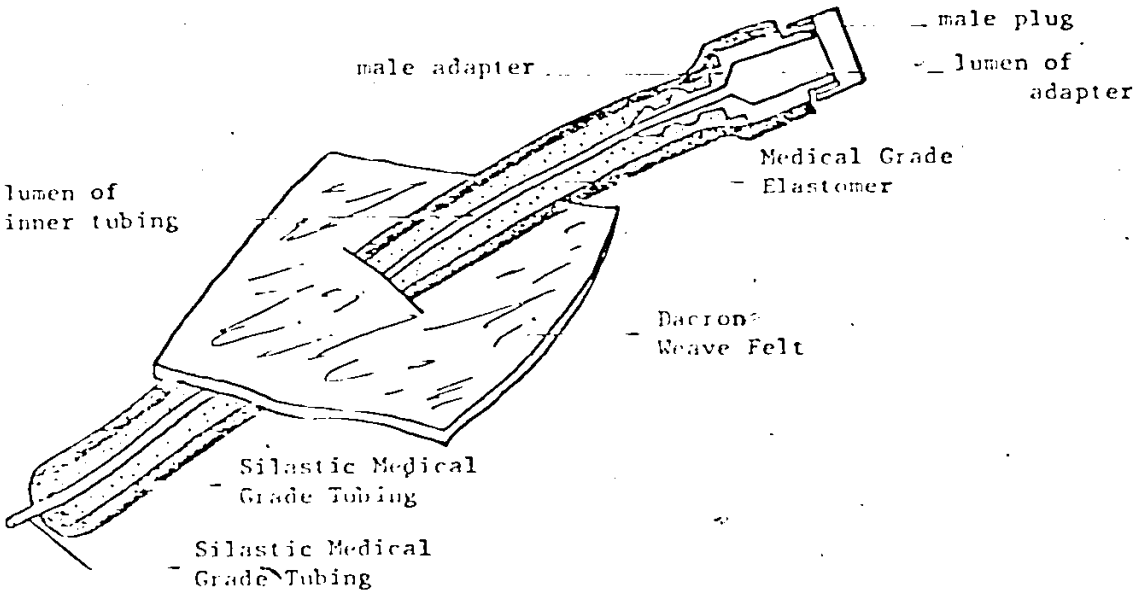

male plug
male adapter
lumen of adapter
Medical Grade Elastomer
lumen of inner tubing
Dacron Weave Felt
Silastic Medical Grade Tubing
Silastic Medical Grade Tubing

Fig. 5d

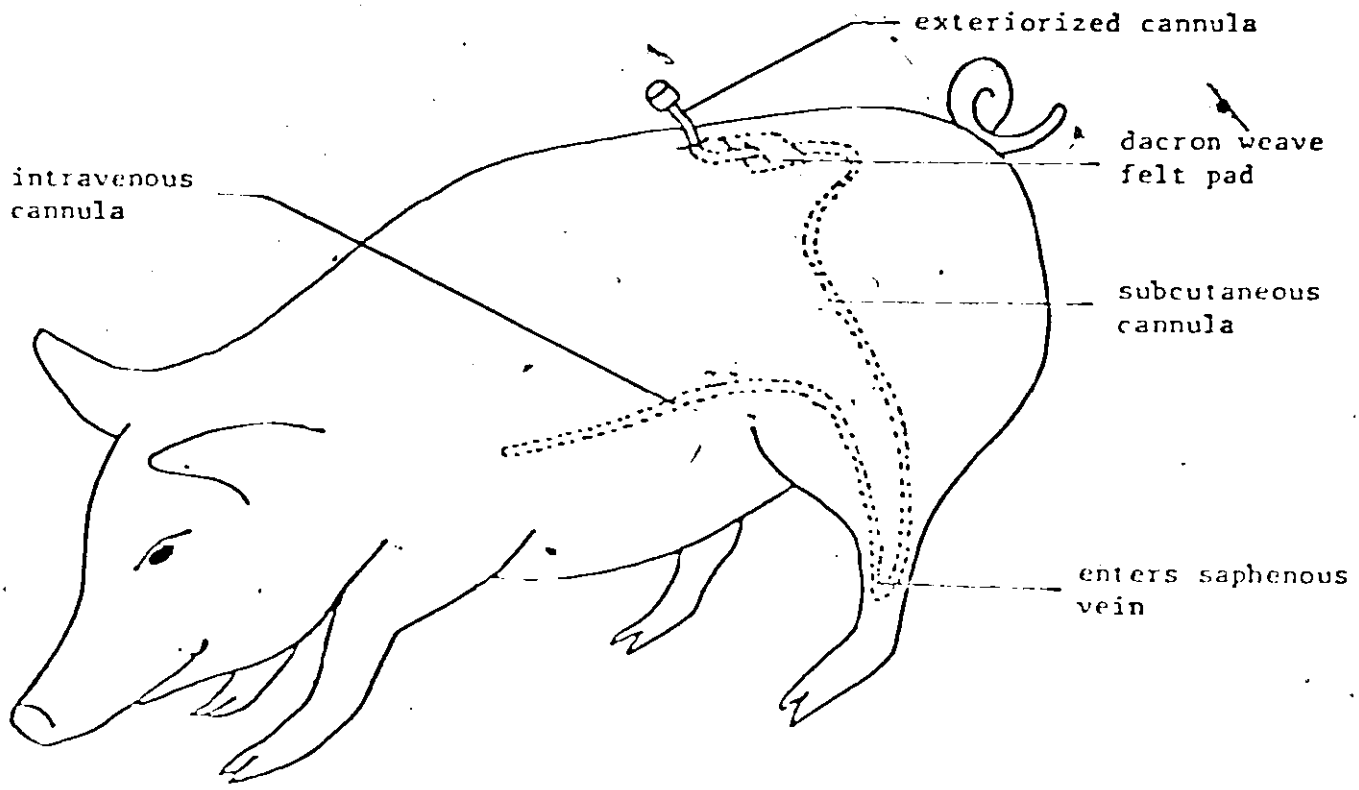
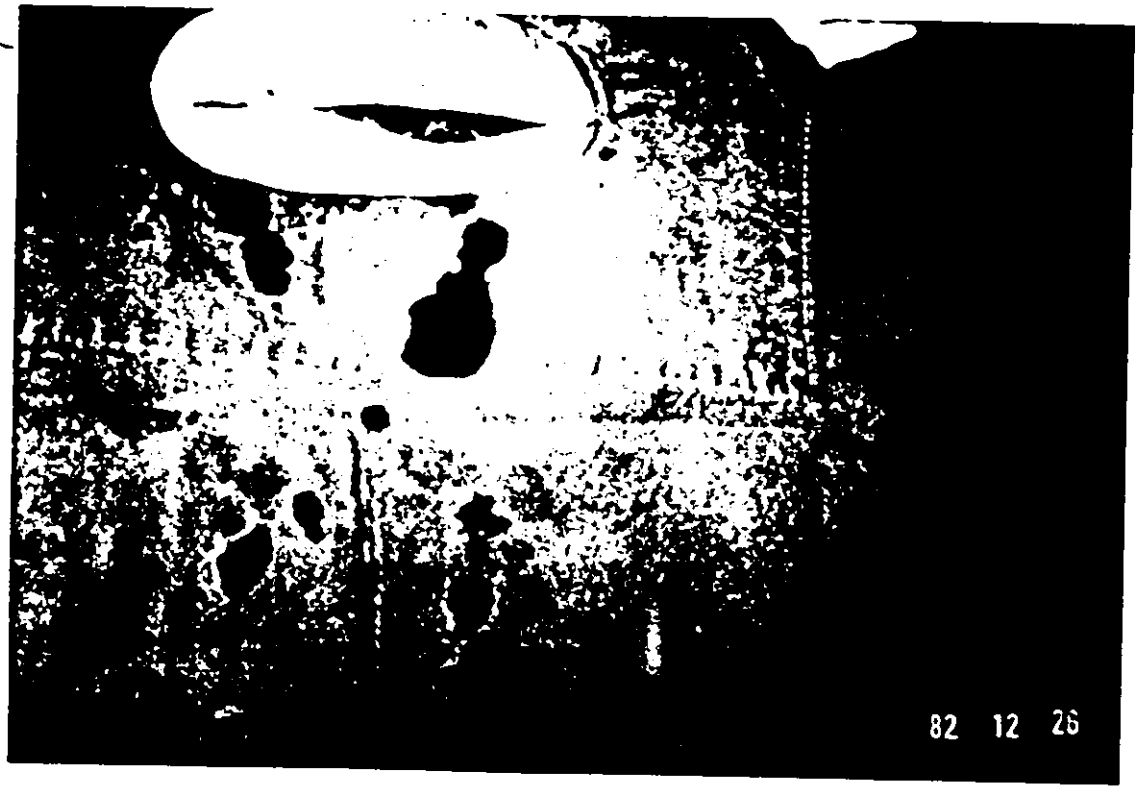

exteriorized cannula
dacron weave
felt pad
intravenous
cannula
subcutaneous
cannula
enters saphenous
vein

Figure 5e) Mid-dorsal incision.

f) Dorsal placement of cannula.

Fig. 5.

(e)



(f)

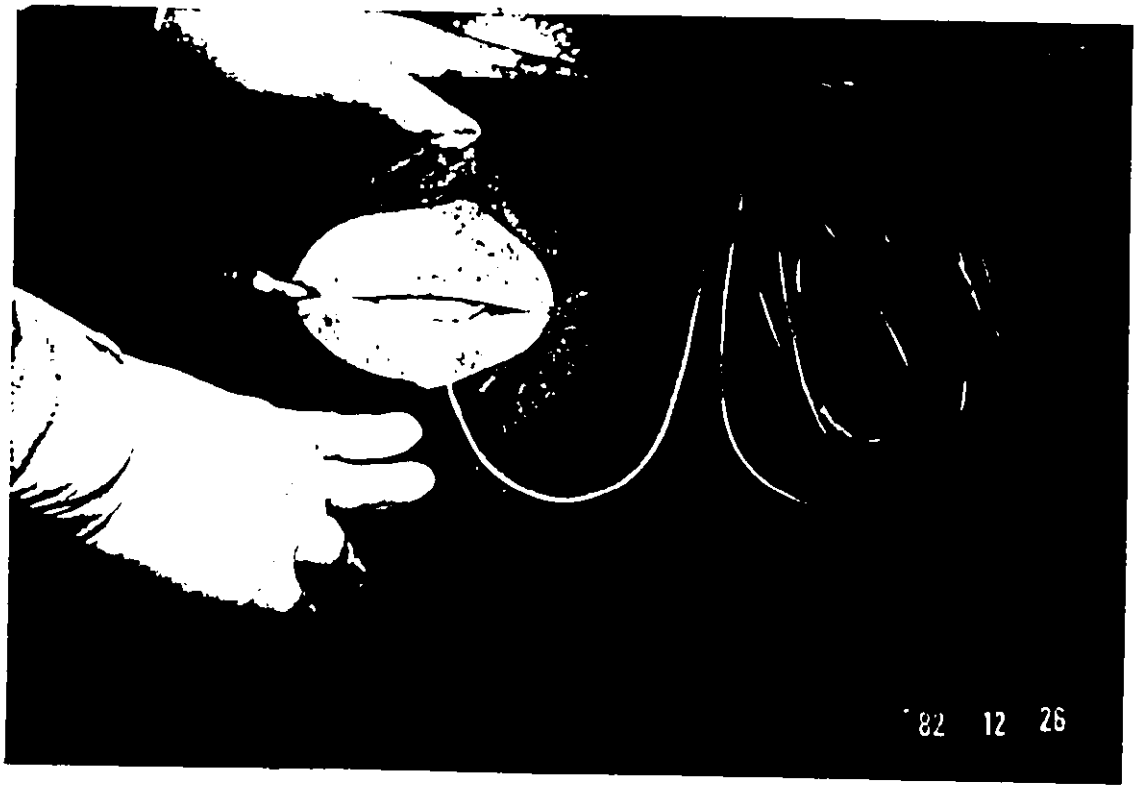


Fig. 5

(g)

(h)

2.7. Histology

When the experiments had been completed, the pig was sacrificed by an overdose of intravenous pentobarbitol. The brain was then perfused in situ by first injecting 500 ml of isotonic saline through each carotid artery, or alternatively into the left ventricle of the heart after clamping off the descending aorta. Following the isotonic saline infusion, up to 1 liter of 10% buffered formalin in 0.9% NaCl was infused via the same route. The brain was removed and fixed in 10% formalin. Sections were cut at a thickness of 60 m using a freezing microtome. Serial sections were stained with thionin or cresyl violet for subsequent histological analysis. Verification of cannula placements was made with the assistance of a stereotaxic atlas for the dog (Dua-Sharma et al., 1970).

2.8. Data Analysis

In order to compare the present findings in the pig with those observed by others in other species, standard statistical methods were used in evaluating the results of the experiments. Quantitative results are expressed throughout as mean + standard error of the mean (SEM) for each group. Results were expressed in this fashion rather than as confidence limits, because the magnitude of the response rather than the ability to respond was being tested. Differences between the means were evaluated using analyses of variance. When a comparison of two means was required, a Neuman-Keuls test or Student's t-test was performed.

2.9. Chemical and Solutions

a) Renin: Lyophilized hog renin (1 Unit/mg; Nutritional Biochemicals, Montreal, Quebec) was dissolved in 0.9% NaCl solution. The solution

was stored at 4°C in the original bottle for a maximum of 72 hours. On the days of testing, the solution was warmed to room temperature and used for intracranial injections.

b) Synthetic RAS Components: Synthetic Ileu(5)-angiotensin I (AII), as well as decapeptide Ileu(5)-angiotensin I (AI), des-Asp(1) AII (AIII), and renin tetradecapeptide (RS) (all from Beckman) were received in their crystalline form. The amount to be used in each series of experiments was determined and measured out using a lab electrobalance (Sartorius, #2474) and transferred to a 5 ml glass vial. An appropriate volume of sterile isotonic saline was added to achieve a stock solution of 500 g/ml. The solution was kept for no longer thaq four weeks at 4°C. Prior to a test, serial dilutions were performed to achieve the desired dose to be used in the intracranial injections.

c) Angiotensin-II Competitive Antagonist: 1-Sarcosyl-8 Alanyl Angiotensin II (saralasin, P113, Beckman, Montreal) was obtained in its pure crystalline form. Immediately prior to use the appropriate quantity of the drug was weighed and dissolved in sterile saline solution to a concentration of 500 g/ml. The solution was stored at 4°C for a maximum of one week and used for intracranial injections.

d) Sodium Chloride Solutions: i) Drinking solution: A 1.8% sodium chloride solution was prepared by dissolving reagent grade sodium chloride crystals in distilled water. The solution was dispensed into a 20 liter drinking vessel which was stored at room temperature. ii) Isotonic Saline: A 0.9% saline solution was prepared by dissolving reagent grade sodium chloride crystals in double distilled water. The solution was then autoclaved and stored at 4°C. The solution was used for intracranial injections,

making other solutions, and brain perfusions.

e) 10% Buffered Neutral Formalin Solution: Five liters of solution were prepared as follows: 20 g of sodium phosphate monobasic, 32.5 g of sodium phosphate dibasic (anhydrous), and 0.5 liters of 37-40% formaldehyde were added to 4.4 liters of distilled water to bring it up to 5 liters. The buffered solution was stored at room temperature and was used for in situ perfusion of the pig brains.

3.0. RESULTS

3.1. Daily Fluid Intakes.

In order to assess the responses of the prepubertal female pigs to dipsogenic stimuli, ad libitum 24 h intakes of water and a 1.8% NaCl solution were initially measured. Daily fluid intakes were determined for one week prior to the commencement of intracranial or intravenous testing. In the six prepubertal pigs examined, the ad libitum 24 h intakes of water were 3470 ± 240 (mean ± S.E.M.) ml (Table 1). 1.8% NaCl had to be ingested by the pigs, since they were maintained on commercially prepared Na^+-free diet (see Introduction). The average ad libitum NaCl intake for the six pigs was 400 ± 75 (Table 1) which corresponds to an intake of 7.2 ± 1.4 grams of NaCl a day.

3.2. Intravenous AII-Induced Drinking

Preliminary experiments were carried out with pre-pubertal and post-pubertal gilts to determine the dipsogenic response to i.v. infusions of AII. The results from three pigs (Table 2) demonstrate that the prepubertal pig is responsive to infusions of low doses (125 p-mol/min) of the peptide. Two of three pigs responded to the dipsogen with an average latency of 4 min 30 sec ± 2 min 30 sec. The responses of the pigs were restricted to drinking water (200 ml ± 100 ml); no drinking of 1.8% NaCl was observed during the hour following the infusion.

At the higher i.v. dose of 250 p-mole/min, drinking was not observed during the 30 min test period in 2 pigs. Other behavioral effects observed included cessation of activity, lack of motivated behavior, and a change in breathing pattern. These experiments at the higher dose of AII were discontinued as it was felt that the

Table 1. Average daily intakes of water and 1.8% NaCl by prepubertal pigs maintained on an NaCl-free diet. Values shown are means ± S.E.M. Number of animals in parentheses.

Water	3470 ± 240 ml (6)
1.8% NaCl	400 ± 70 ml* (6)

*The NaCl equivalent is 7.2 ± 1.4 g.

Table 2. Intakes of water and drinking latencies in pre- and post-pubertal pigs infused i.v. with AII at 125 or 25 p-mole min^{-1}. Infusions were for 30 min or until drinking began. Values shown are means ± S.E.M.

	Dose (p·mol/min)	Water Intake (ml/30 min)	Latency (min)	# Responded	# Tested
Prepubertal	125	200 ± 100	4.5 ± 2.5	2	3
	250	0	0	0	2
Postpubertal	125	500 ± 260	13.2 ± 8.4	2	5
	250	0	0	0	2

pressor effects of the AII were overriding the effect on thirst.

Of the five post-pubertal female pigs tested at the low dose of AII (125 mole min^{-1}), only two responded to the peptide. Behaviors induced by AII infusions in post-pubertal animals are effected by the reproductive cycle (Kucharczyk, 1984), however, and thus merit separate study.

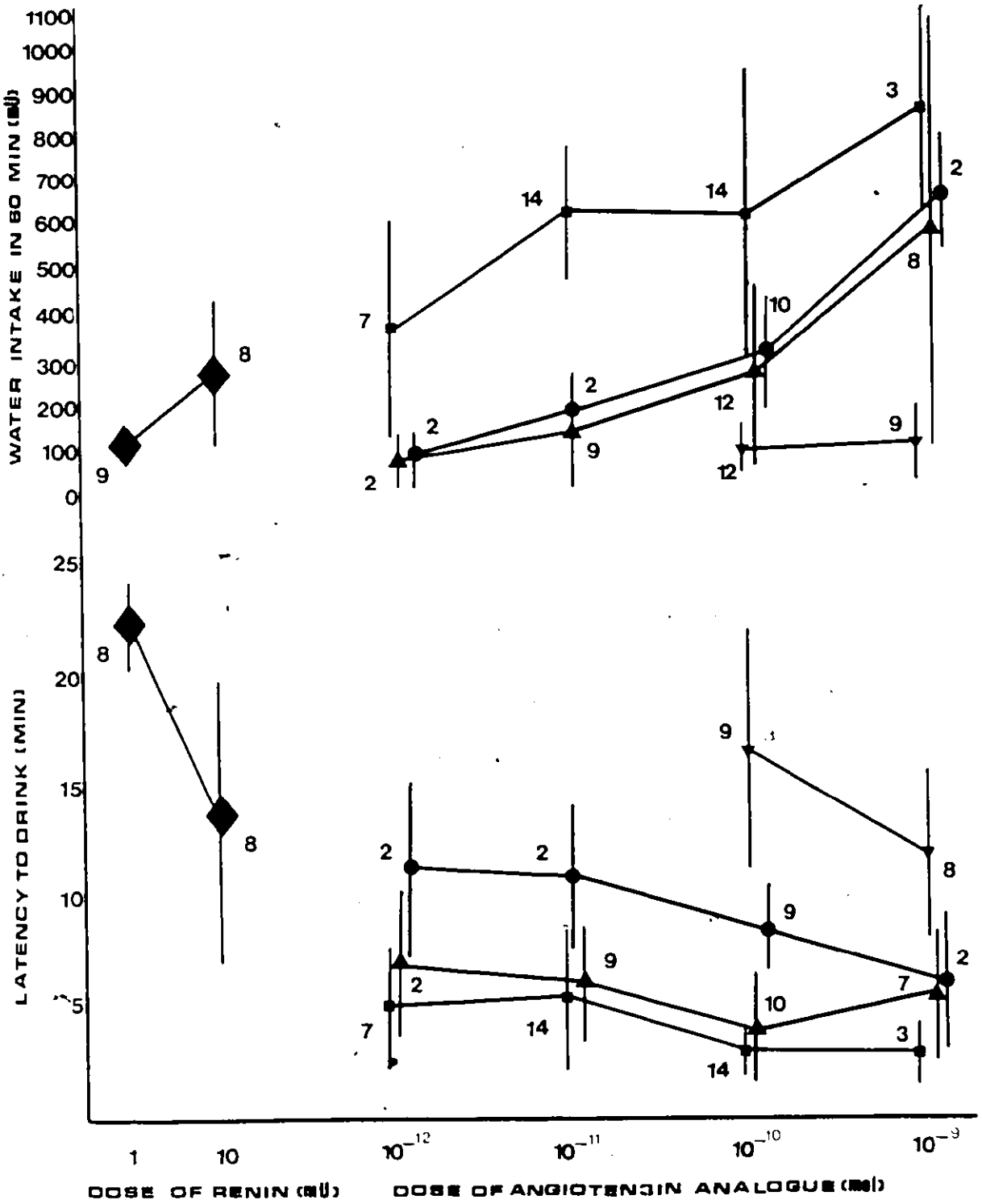
The results which were obtained from these preliminary experiments indicate that the female pig is sensitive to intravenous AII infusions. The precise level has yet to be determined, but is one logical follow-up to this study.

3.3. Drinking Induced by Intracranial Injection of RAS Components

Intracranial injections of the components of the RAS in 14 water-replete animals produced drinking that was dose-dependent, but varied with the intracranial site and RAS component tested (Fig. 6). However the responsiveness did not change over time with a given dose at a given intracranial site. Over the dose range 10^{-12} to 10^{-9} mole, which is similar to that used by other investigators (Fitzsimons, 1979), AII was the most dipsogenic RAS component. At a dose of 10^{-12} mole, it produced a drinking response comparable to that of AI and AIII given at 100 times the dose. AII produced significantly greater drinking (ANOVA) than AI ($p < .01$), AIII ($p < .01$), and RS ($p < .001$) over the dose range used. The drinking response in the one h following the injections increased from 380 ± 220 ml at 10^{-12} mole to 840 ± 200 ml at 10^{-9} mole.

The time to initiate drinking (latency), as has been observed in other species, was short, usually less than 5 min. At higher doses (10^{-9}, 10^{-10} mole), the latency was very short, less than 3 min (Fig. 6).

Figure 6. Water intake during 60 min after intracranial injections of RS (▼), AI (▲), AII (■) and AIII (●) over a dose-range from 10^{-12} to 10^{-9} mole and renin (⬢) at 1 and 10 m I.U. were measured (top panel). The latency to drink was also noted (bottom panel). The results are expressed as mean ± standard error of mean (SEM) with the number of observations adjacent to the point.


WATER INTAKE IN 60 MIN (ml)
1100
1000
900
800
700
600
500
400
300
200
100
0
LATENCY TO DRINK (MIN)
25
20
15
10
5
1
10
10^{-12}
10^{-11}
10^{-10}
10^{-9}
DOSE OF RENIN (mU)
DOSE OF ANGIOTENSIN ANALOGUE (mol)

The change in behavior was very noticeable; shortly after the injection the animal would stop its current activity and move, often very quickly, to the fluid nozzles and begin drinking. The latency to drink after AII injections was significantly less than for RS ($p < .01$) and AIII injections ($p < .05$), but not significantly less than AI injections.

The other components of the RAS also induced drinking following intracranial injection. As in other species examined, the dipsogenic potency depended upon which component was injected (Fitzsimons et al., 1978). AI and AIII were found to be equidipsogenic over the dose range tested. At the lowest dose (10^{-12} mole), only 50% of the pigs responded to either AI or AIII, and drank small volumes of water (80 ± 80, 90 ± 90, respectively). At the highest dose (10^{-9} mole), both peptides produced strong drinking responses. All the pigs tested responded to AIII, while 90% responded to AI. The responses correspond to approximately 20% of the total daily water intakes (AI = 575 ± 500 ml, AIII = 675 ± 125 ml). These RAS components thus produced dose-dependent responses which paralleled each other.

The drinking latency to AI was not significantly different than the latency for AIII. At 10^{-12} mole, AIII had an average latency of 11 ± 4 min, while AI initiated drinking in 6.5 ± 3 min. At higher doses (10^{-9} moles), the delay was almost the same and in some cases more rapid (AI = 5.4 ± 2 min; AIII = 6 ± 3 min.).

RS was a relatively weak and slow acting intracranial dipsogen at the two doses tested (10^{-10} and 10^{-9} mole). It produced significantly less drinking than AII ($p \leq .01$) and AIII ($p \leq .05$). It took longer to initiate drinking following RS injections than AII ($p \leq .01$) and AI ($p \leq .05$).

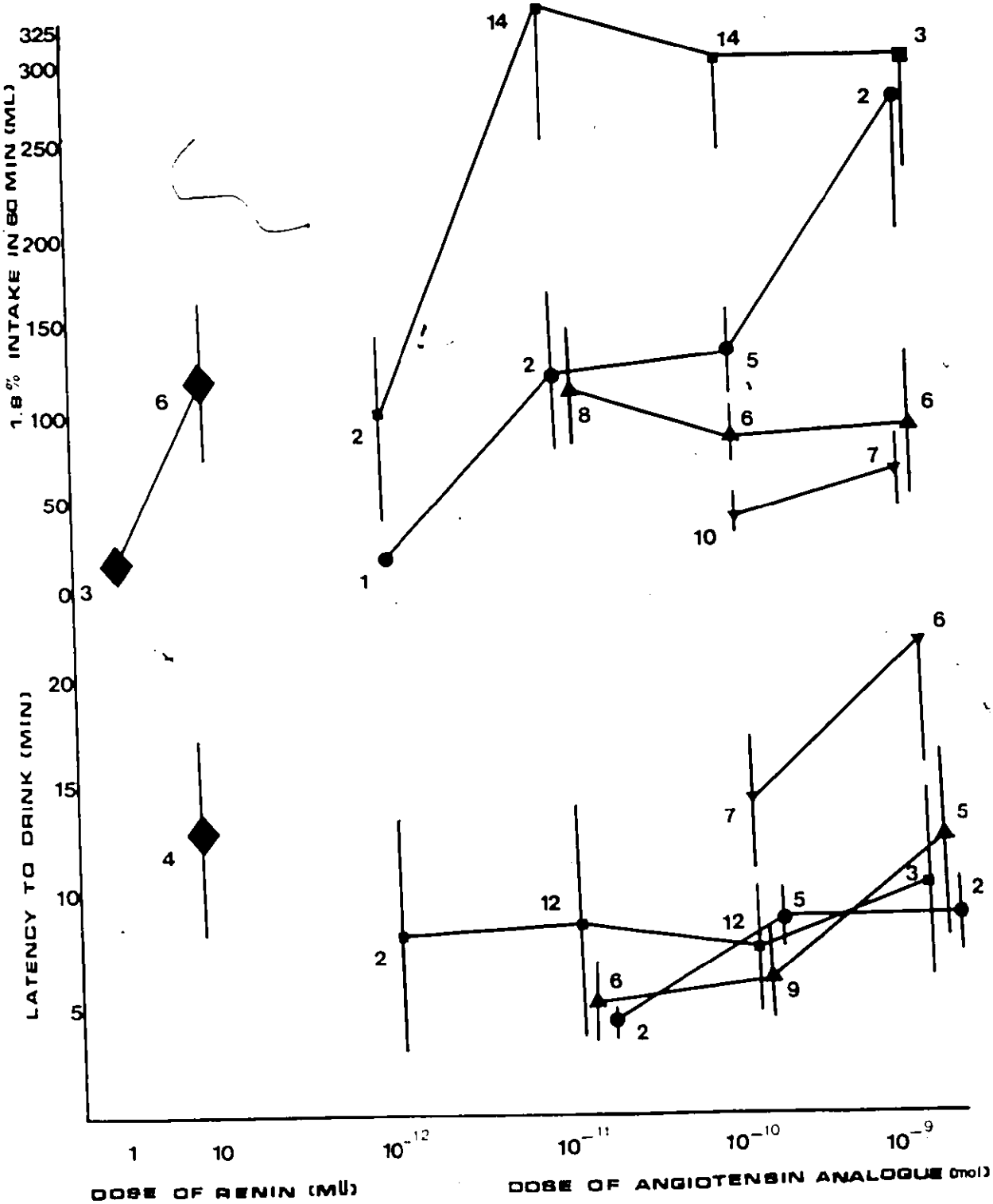
Central injections of 1 and 10 mU of hog renin produced moderate water intakes (150 ± 20 ml and 300 ± 120 ml, respectively). Drinking was initiated after 8 of 9 injections at a dose of 1 mU and after all 8 injections at 10 mU. The latency response following administration was characteristically longer than for the peptide analogues and was much more variable between individual animals.

3.4. Intakes of 1.8% NaCl Induced by Intracranial RAS Injections

In prepubertal female pigs maintained ad libitum on a Na^+-free pig chow, the same ranking order of dipsogenic effectiveness was observed for 1.8% NaCl intakes in the 60 min following intracranial administration of the RAS components (Figure 7). AII produced significantly more drinking of the salt solution than RS ($p \leq .01$), AI ($p \leq .05$), AIII ($p \leq .05$) over the doses 10^{-12}, 10^{-11}, 10^{-10} mole. Salt drinking was observed in 60% of the pigs at an AII dose of 10^{-12} mole. At the highest dose used (10^{-9} moles) 75% drank the saline solution. The average response was 300 ± 100 which is 75% of the normal daily 1.8% intake (see 3.1.)

AIII was effective, particularly at higher doses, in stimulating salt drinking. At the highest dose (10^{-9} mole), the amount of salt solution drunk in one hour was not different than that induced by AII (250 ± 80, 270 ± 60 ml, respectively). At the two highest doses tested, AIII elicited significantly greater 1.8% NaCl intakes than AI ($p \leq 0.05$) and RS ($p \leq 0.05$).

Figure 7. Intakes of 1.8% NaCl during 60 min after intracranial injection of RS (▼), AI (▲), AII (■) and AIII (●) over a dose-range from 10^{-12} to 10^{-9} mole and renin (⬢) at 1 and 10 m I.V. were measured (top panel). The latency to drink was also noted (bottom panel). The results are expressed as mean ± standard error of mean (SEM) with the number of observations adjacent to the point.


1.8% INTAKE IN 60 MIN (ML)
325
300
250
200
150
100
50
0
LATENCY TO DRINK (MIN)
20
15
10
5
1
10
10^{-12}
10^{-11}
10^{-10}
10^{-9}
DOSE OF RENIN (MU)
DOSE OF ANGIOTENSIN ANALOGUE (mol)

AI and RS were equipotent in stimulating salt appetite. Although the intakes for AI-induced drinking were greater than for RS, they were not statistically different. The latencies for inducing salt intakes by AI, and RS were not significantly different, although the latency for AI-induced intakes was usually less than RS.

Renin was ineffective in producing salt intakes. At 1 mU, in which 8 of 9 animals drank water, none were observed to drink salt during the one hour test period. However, at the higher dose (10 mU), some salt drinking (20 $\pm$ 30 ml) did occur in 6 of the animals.

The latency period to the initiation of salt ingestion for all peptide components (except RS) was similar. There appeared to be little, if any, dose-dependent relationship with respect to the times elapsed before 1.8% NaCl drinking began. RS took longer than other components, however. It was significantly slower than AII ($p \leq .05$) at both 10^{-10} and 10^{-9} mol.

In about 75% of the tests, the intracranial injections of one of the peptide components or renin caused drinking of water followed within 1-2 minutes by one or several draughts of the salt solution. The animal would then typcially alternate between the water and 1.8% NaCl. In the remaining tests, particularly after the AIII injections, the animal would first drink a large quantity of saline, then water, followed by a period of alternatively drinking the 1.8% NaCl and the water.

In order to control for the possibility of behavioural conditioning, isotonic sterile saline (0.9% injections were routinely made. Injections of control volumes of isotonic saline failed to produce drinking of water or 1.8% NaCl at any intracranial site tested.

3.5. Intracranial Testing in the Cerebral Ventricles and Anterior Hypothalamus

Injections of RAS components were made through cannulae directed at the preoptic area (POA) and lateral cerebral ventricles (LCV) in order to examine differences and similarities of these reported dipsogenic sites.

Injections of various doses of RS, AI and AIII produced no significant differences between sites (Table 3a, 3b, 3d). At both sites tested, dose-dependent drinking responses were observed for each component. Renin was equipotent at both tissue and ventricular sites at the two doses tested (Table 3e).

AII appears to be more dipsogenic at POA injections sites, than in the LCV (Table 3c). It produced greater one hour drinking responses over the dose range at 10^{-12} to 10^{-10} mole ($p \leq .05$). At the lowest dose (10^{-12} mole), there was no significant difference between AII at the other analogues; however, at higher doses the responses were

Table 3a. One hour water intakes following injections of renin substrate (RS) at different doses through cannulae directed at either the preoptic area (POA) or the lateral cerebral ventricle (LCV). Values shown are means ± S.E.M. Numbers of animals in parentheses.

	Dose of RS (mole)	
	10^{-10}	10^{-9}
POA	90 ± 20 (9)	100 ± 40 (4)
LCV	110 ± 20 (7)	110 ± 30 (3)

Table 3b. One hour water intakes following injections of angiotensin I (AI) at different doses into either the POA or LCV. Values shown are means ± S.E.M. Numbers of animals in parentheses.

	Dose AI (mole)		
	10^{-11}	10^{-10}	10^{-9}
POA	130 ± 60 (5)	180 ± 50 (8)	630 ± 200 (4)
LCV	130 ± 20 (4)	440 ± 220 (4)	540 ± 320 (4)

Table 3c. One hour water intakes following injections of angiotensin II AII) at various doses into either the POA or LCV. Values shown are means ± S.E.M. Numbers of animals in parentheses.

	Dose AII (mole)			
	10^{-12}	10^{-11}	10^{-10}	10^{-9}
POA	400 ± 200 (5)	400 ± 100 (16)	890 ± 220 (6)	0 (2)
LCV	420 ± 130 (3)	260 ± 170 (6)	340 ± 50 (7)	650 ± 280 (4)

Table 3d. One hour water intakes following injections of angiotensin III (AIII) at different doses into either the POA or LCV. Values shown are means ± S.E.M. Number of animals in parentheses.

	Dose AIII (mole)			
	10^{-12}	10^{-11}	10^{-10}	10^{-9}
POA	80 ± 80 (2)	175 ± 25 (2)	370 ± 95 (5)	675 ± 25 (2)
LCV	-	-	290 ± 120 (4)	-

Table 3e. One hour water intakes following intra cranial injections of Renin into either the POA or LCV. VAlues shown are means ± S.E.M. Numbers of animals in parentheses.

	Dose renin (mIU)	
	1 m.I.U.	10
POA	135 ± 30 (7)	370 ± 160 (3)
LCV	30 ± 30 (3)	120 ± 50 (3)

markedly greater.

3.6. Peptide Sensitivity of the Angiotensin "Thirst Receptor"

In order to exclude the possibility that the dipsogenic effect of intracranially administered AIII resulted from stimulation of AII receptors (Bradley et al., 1983), two pigs were pretreated with LCV injections of 10^{-9} mole of Sar^1Ala^8-AII, a specific competitive antagonist to AII. Five minutes later a 10^{-10} mole injection of AIII was made down the same cannula. Following pretreatment, AIII induced drinking of 250 ± 110 ml of water and 70 ± 50 ml of 1.8% NaCl within one hour (Table 4). The responses did not differ significantly from those induced by administration of AIII without saralasin pretreatment.

3.7. Histology

The angiotensin-sensitive placements in the anterior hypothalamus (POA) were located in a region bound dorsally by the anterior commissure and centered near the border of the lateral and medial preoptic nuclei. Tissue damage was produced adjacent to the path of the cannula, as well as to structures near the POA. In most of the pig brains there was some enlargement of one or both lateral cerebral ventricles, no doubt the cumulative effect of the numerous injections that each pig received into the POA and LCV over the time course of the investigation. Fig. 8 is a photomicrograph showing the location of the intracranial cannulae in one pig.

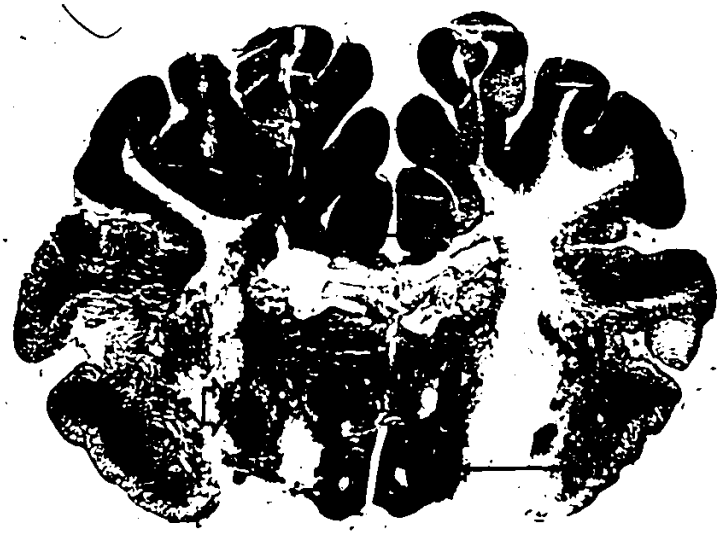
The extent of the spread of the intracranial injections was not directly measured. Earlier work has shown that an injection of a radioactive analogue in a volume of 1 µl into rat neural tissue will only spread one cubic millimeter (Fitzsimons and Kucharczyk, 1978). As the brain of the pig is much larger than the rat, the location of the injectate was probably much better. Therefore only the post-mortem histology was employed.

Table 4. Water and 1.8% NaCl intakes in the hour following AIII intracranial injections with and without pretreatment of the injection site with an AII-competitive antagonist, saralasine. In the pretreatment tests an antagonist to agonist ratio of 10:1 was used (10^{-9}:10^{-10} mole). The values are expressed as means ± S.E.M.

	Water Intake (ml/h)	1.8% NaCl (ml/hr)
Untreated (6)	260 ± 140	30 ± 20
Treated (2)	250 ± 110	70 ± 50

Figure 8. Frontal section of pig brain (actual size) at the level of the anterior hypothalamus showing the location of the tips of cannulae (solid arrows) used for injections into a lateral cerebral ventricle (left side) (A) and anterior hypothalamus adjacent to the mammillothalamic tract (right side) (B). Both sites were highly sensitive to injections of peptides as well as renin. Injections into the far-lateral hypothalamic area (open arrow) failed to produce drinking of water or 1.8% NaCl (C).

Fig. 8



4.0 DISCUSSION

The main findings of this study may be summarized as follows: a) AII is a more effective dipsogen when given intracranially than following peripheral infusions; b) AII responsive sites in the brain include neural tissue as well as periventricular and ventricular sites; c) AII is the most dipsogenic component followed by AI, AIII and RS; d) In contrast to other mammalian species so far tested, AIII is an effective intracranial dipsogen in the pig; e) Finally, in contrast to other mammalian species, the components of the R.A.S., particularly AII and AIII, appear to stimulate salt appetite.

These findings will now be considered in the context of previous research in this area.

4.1. AII as an Intracranial Dipsogen.

Since the first detailed discussions of intracranial angiotensin-induced thirst appeared in 1970 (Epstein et al.; Severs et al.) great strides have been made in identifying the location and nature of the thirst receptor. The first experiments demonstrated that injections of val(5) or isoleu(5) - AII into the anterior diencephalon of rats produced copious drinking, usually after 1-2 min. The drinking was dose-dependent over the dose range 10^{-12} to 10^{-9} mole, reproducible within a given test session, and was often stable for several months in each animal with implanted cannulae (Epstein et al., 1970). The behaviour elicited by central injections was specific to drinking. Feeding and other potentially competing behaviours were not observed. The intracranial injections would rouse a sleeping animal or stop a

recently starved animal from eating and cause it to start drinking (Epstein et al., 1970).

Since this early work, similar experiments have been performed on 7 mammals (rabbit, goat, sheep, cat, dog, monkey, and gerbil), four birds, (chicken, pigeon, sparrow and Japanese quail) and one reptile (iguana) (see Introduction). In all species tested, AII has been shown to initiate drinking when given intracranially. The minimum dose required to elicit a drinking response, also termed the threshold, dose, varied from species to species. For instance, doses as low as 10^{-16} mole will cause drinking in the rat, whereas doses as high as 10^{-9} mole are required in the chicken (Epstein et al., 1970; Everad & Fitzsimons, 1976a).

The results from the present study demonstrate taht the pig, like all species thus far tested, is sensitive to intracranial injection of AII. The lowest dose tested, 10^{-12} mole, consistently initiated drinking in all eight animals when administered into the forebrain or lateral ventricle. The response was rapid ($\leq$ 5 min) and large volumes of water (< 400 ml/60 min) were ingested.

Compared to the effects of intracranial injections, the drinking response of the pig to peripherally infused AII (125 or 250 X 10^{-12} mole min^{-1}) was much more variable. Intravenous injections of 100 to 1000 times the intracranial dose produced less copious drinking responses with a longer latency period in most animals.

In the i.v. infusion experiments, drinking was observed in only 2 of 3 pigs tested at a dose of AII of 125 X 10^{-12} mole/min for 30 min. The drinking responses following i.v. administration were approximately

half as large as those induced by the lowest dose of intracranial AII (10^{-12} mole) (200 ± 100 ml, 380 ± 220 ml) (Table 2, Fig. 6). At the higher i.v. dose (250 X 10^{-12} mole/ml at 2.5 ml/min), no drinking responses were initiated during the 30 min infusion period.

The present findings are in agreement with the results reported for the dog (Fitzsimons & Kucharczyk, 1978), rat (Fitzsimons, 1979) and pigeon (Evered & Fitzsimons, 1976). In all of these studies greater drinking responses were observed following intracranial injections than following i.v. infusion of AII. In some animals, i.v. AII infusions are usually ineffective (goat, sheep), whereas the same species are sensitive to intracranial AII (Adersson & Westbye, 1970; Olsson & Rundgren, 1975). These data have been interpreted to mean that the thirst receptors for AII are located centrally (Fitzsimons, 1979).

It is possible that the relative ineffectiveness of peripheral injections to induce drinking may be due to a masking by other effects of the peptide. For example, it is known that after intravenous infusion AII produces pressor effects which may inhibit drinking (Fitzsimons & Kucharczyk, in press). In the rat, AII infused at rates of 10,000 and 1000 X 10^{-12} mole/kg/min caused rises in blood pressure (BP) of 5, 25 and 55 mmHg, respectively (Fitzsimons, 1979). Similar increases in blood pressure have been reported in the dog at dipsogenic doses of AII. In support of these findings are those of Fitzsimons & Kucharczyk (1978). They found that in the dog large increases in BP are observed following i.v. infusions of AII but not after central AII injections.

Therefore, it is possible that in those species most sensitive to intravenous AII, the pressor effects of the peptide in the systemic circulation may inhibit water intake.

In the present study, AII infused at 250 X 10^{-12} mole/ml at 2.5 ml/min did not produce drinking. Although blood pressure was not measured, several changes suggestive of autonomic arousal were noted. These included increased respiration rate, agitated behaviours, and unsteady gait. For these reasons further tests were not carried out at this dose level. These results, while not conclusive, demonstrate one possible reason for the ineffectiveness of AII when given intravenously.

4.2. Central Receptor Sites for AII

The controversy over the location of the central receptors for AII has existed since AII was found to arouse thirst in rats when injected into the POA (Epstein et al., 1970). In opposition to the idea of a 'forebrain thirst receptor' were proposals that two periventricular structures, the SFO (Felix & Ahert, 1975; Simpson & Routtenberg, 1973) and the OVLT (Buggy et al., 1975; Nicolaidis & Fitzsimons, 1975) were involved. Johnson & Epstein (1975) concluded that drinking after injection of AII into the POA of the rat results from reflux of the peptide along the outside of the cannula shaft into the ventricles from where it can reach and stimulate the SFO. The ventricular hypothesis is an attractive one, mainly because it is known that the SFO lies outside the blood-brain barrier (Akert, 1969) and could, therefore, be readily acted upon by blood-brain substances.

The problems with an exclusive SFO thirst receptor is that Kucharczyk & Mogenson (1976) produced drinking in rats with POA cannula angled to avoid penetrating the ventricles. The dose was 2.5×10^{-11} moles of AII in a volume of 0.2 µl. This suggests that the AII did not reflux up the cannula shaft to the ventricles. Swanson, Kucharczyk & Mogenson (1978), moreover, caused reliable short-latency drinking in rats after 6.6×10^{-12} mole AII in volumes as low as 0.01 µl, and they confirmed by autoradiography that the spread of the injected solution was restricted to the POA.

In light of this controversy, cannula implants were targeted for the POA and the LCV in the pig. Intracranial spread of a 1 µl injected solution is not restricted to the vicinity of the injection (Fitzsimons & Kucharczyk, 1978), even if the injector tip itself lies within tissue. Thus, in the small brain of a rat, reflux of 1 µl of injected solution along the sides of the implanted guide cannula and diffusion in all directions inevitably causes spread to the ventricles. In the pig, on the other hand, the larger brain means that spread of a 1 µl solution injected into neural tissue is proportionately less.

Drinking elicited by POA administration of AII is unlikely to be the result of spread of the peptide to the ventricles and SFO for the following reasons. In the dog, AII dose-response curves for POA and SFO injections were similar, whereas the LV was not as steep (Fitzsimons & Kucharczyk, 1978). Also in the dog, the latencies for drinking were greater following LV injections than those observed

with POA injection (Fitzsimons & Kucharczyk, 1978). Injection into the LV cannot be less effective than into the POA if it is hypothesized that POA effects are a result of spread to the ventricles. As this has been shown not to be the case, then there must be multiple receptors for drinking induced by AII in the brain. This interpretation is supported by the results of the present study since injections of RAS components into forebrain and ventricular loci were both effective in inducing water intake (Table 3).

4.3. The Nature of the Central Angiotensin Thirst Receptor

It is now accepted that in most species the central receptor which initiates drinking in response to intracranial angiotensin is similar to the membrane bound myotropic AII receptor in the periphery (Fitzsimons, 1979). There is now considerable evidence that AII is the biologically active component in the RAS which is produced by a central RAS. The two lines of evidence providing support for this hypothesis were a) studies of dipsogenic potency of RAS components, alone and in the presence of inhibitors, and b) localization of the different RAS components in the brain.

It was beyond the scope of this study to evaluate and measure the relative concentrations of the RAS components within specific brain areas. The pig brain has not yet been evaluated for localization of RAS components, with the exception of brain renin (Hirose et al., 1980). Although the pig brain has not been examined, all of the components have been reported to be present in several other animal species (rat, dog, monkey, human) (see Introduction). In most species AII is recognized by the dipsognic receptors but it is not produced directly or stored. The presence within the brain of the substrates and enzymes needed to biosynthesize AII supports the existence of an iso-RAS to initiate centrally-induced thirst.

The present work indicates that the pig brain is capable of converting precursors of AII to the biologically active octapeptide. The spectrum of drinking latencies for the different RAS components suggests that the conversion is accomplished as quickly and efficiently as in the dog (Fitzsimons & Kucharczyk, 1978).

The intracranial injection of the components of the RAS helps to serve two functions: First, it gives clues about the localization of particular substrates and enzymes. Second, it helps to characterize the nature of the receptors responsible for the initiation of drinking.

Previous studies have established that AII is the molecule recognized by the receptor which initiates drinking. In rats in which vigorous drinking to RS had earlier been initiated, pretreatment with either a competitive AII antagonist, saralasin, or a converting enzyme blocker, SQ20881, significantly decreased drinking responses (Epstein et al., 1974). Moreover, the fairly short latencies and similar response to equimolar RS, AI and AII (10^{-12} to 10^{-10} mole) injections in the POA or SFO suggest that in the rat there is very efficient conversion of precursors to AII (Fitzsimons, 1971). The type of receptor responsible for initiating drinking in the rat is thought to be similar to the AII myotropic receptor in the periphery. This conclusion is based on the ineffective dipsogenic capacity of AIII (Fitzsimons et al., 1977), a known promoter of aldosterone release in the periphery, but a weak stimulator of pressor activity (Chiu & Peach, 1974).

The pigeon responds in a similar dose-dependent manner to intracranial administration of RS, AI and AII (10^{-12} to 10^{-10} mole) (Evered & Fitzsimons, 1977). As in the rat, the precursors to AII

were equidipsogneic with AII, and AIII was a relatively poor dipsogen, particularly at low doses (10^{-12}, 10^{-11} mole) (Evered & Fitzsimons, 1977). The pigeon, like the rat, appears to quickly convert the inactive peptides into the biologically active AII.

In the dog, unlike the rat and pigeon, drinking in response to equimolar injections of precursors clearly peaks at AII, suggesting that in this species there is less efficient conversion of precursors to AII (Fitzsimons & Kucharczyk, 1977). Responses were observed at 10^{-12} mole AII, but not for the other components. As well, the latency to drink was significantly less following SFO or POA injections of AII than for the other components (Fitzsimons & Kucharczyk, 1977). AIII in the dog is a very weak intracranial dipsogen. At 10^{-11} mole AIII only one of four dogs responded, while at 10^{-10} mole only 6 of 9 dogs drank within one hour. Only at the highest dose, 10^{-9} mole, did all dogs respond, but the intake was significantly less than the same dose of the other components (Fitzsimons & Kucharczyk, 1978). The results in the dog suggest that like the other species examined, the dipsogenically active component of the RAS is AII. The relative lack of dipsogenic effect following AIII administration adds support to the hypothesis that the angiotensin thirst receptor is similar to the AII myotropic receptor in the peripheral circulation. The relative ineffectivness of RS and AI in the dog suggests th-t the localization of necessary enzymes is not the same as in the pigeon and the rat, and this results in less efficient conversion of precursors to AII (Fitzsimons et al., 1977).

In the present study, it was found that the pig, like all other species, is sensitive to each of the RAS components. As in the dog, AII

is clearly the most dipsogenic, particularly at low doses (10^{-12}, 10^{-11} mole). AI is not as dipsogenic as AII, and RS is even less potent. Although the pig resembles the dog in terms of the relative dipsogenic potential of the different RAS components, it appears to be more sensitive. In the dog, reliable drinking could not be initiated at doses lower than 10^{-10} mole, RS or AI (Fitzsimons & Kucharczyk, 1977). In the pig, on the other hand, drinking responses were initiated in more than 50% of animals injected with 10^{-12} mole of AI and in all 9 animals at 10^{-11} mole. RS was as dipsogenic in the pig as in the dog. In the Fitzsimons and Kucharczyk (1978) study of the dog, 3 of 5 animals drank following 10^{-11} mole of RS, 8 of 10 after 10^{-10} mole, and all 4 tested following 10^{-9} mole. The pig displayed a similar sensitivity. Two of 3 pigs drank after 10^{-11} mole RS, 10 of 13 drank following 10^{-10} mole and 9 of 10 after 10^{-9} mole of RS. In both species the responses were significantly less than that of equimolar AII injections.

Intracranial injections of renin produced moderate drinking response in the pig. Water intake induced by 1 mU of renin injected into the forebrain or LCV was approximately equal to the response after 10^{-12} or 10^{-11} mole of AI. In the dog, AI was ineffective at 10^{-12} mole and only slightly effective at 10^{-11} mole (Fitzsimons & Kucharczyk, 1978). The pig thus seems to be more sensitive to renin given intracranially than is the dog. In addition it appears that the pig does have adequate RS present to utilize the injected renin.

One of the most significant findings of this study concerns the dipsogenic ability of AIII. Of all species previously tested, only the gerbil has displayed any sensitivity to intracranial AIII

injections (Wright et al., 1984; Fitzsimons et al., 1977). In comparison to the dog, the most similar animal thus far examined, the pig is much more sensitive to AIII intracranial injections. In the dog, drinking could not be initiated at 10^{-11} mole AIII and only 1 of 4 responded at 10^{-11} mole. In contrast, 3 of 4 pigs responded at 10^{-12} mole and 4 of 4 at 10^{-11} mole of AIII injected intracranially. In the dog, even at high doses (10^{-10} or 10^{-9} mole) where most dogs drank (6 of 9 and 6 of 6, respectively), the water intakes in 60 min were significantly less than after AII (Fitzsimons & Kucharczyk, 1978). In the pig, at the higher doses (10^{-10} or 10^{-9} mole) almost all of the pigs drank (8 of 9 and 4 of 4, respectively) and the water intakes were not significantly different from those observed following equimolar AII injections.

These findings are unique to the pig, and thus demonstrate a definite species specific trait. There are some species in which AIII at high doses (10^{-10} mole) does produce good drinking responses, however, the responses are significantly less than equimolar injections of RS, AI and AII (Evered and Fitzsimons, 1977). These results suggest that the pig has an angiotensin receptor which is less specific than the AII-myotropic-like receptor described by others (Fitzsimons et al., 1977) which can also accommodate AIII. The other possibility is that there are two types of receptor, an AIII receptor as well as an AII receptor, and both are able to initiate drinking.

The latency responses to drink to the injected RAS components, although they may seem long, are comparable to those that have been reported in other species (Fitzsimons, 1979). Time is required for the

injectate to reach the receptors in adequate concentration to initiate a response. Additional time is required for the conversion of the precursors to the most biologically active component AII.

The latency to drink following injections into the POA and LCV were not significantly different. If diffusion from one area to the other was required to elicit drinking, longer latencies would be observed at one site. This did not occur. This supports the idea that there are multiple neural receptors mediating AII-induced water intake (Mogenson and Kucharczyk, 1978).

The large variability in some of the results is a result of intra-species variation. In general, the response in one animal was consistent at a given dose and site. Although individual animals exhibited dose and component dependant responses, some animals were more responsive than others.

4.4. AIII as an Intracranial Dipsogen

In general, this study supports the possibility that there are separate AII and AIII receptors involved in mediating thirst in the pig. Pretreatment with the AII competitive antagonist, saralasin, is known to abolish drinking to central injections of RS, AI and AII when administered in an antagonist to agonist ratio of 10:1 in the rat (Fitzsimons et al., 1978). In this study, two pigs were injected l.c.v. with 10^{-9} mole of saralasin, five minutes before 10^{-10} mole of AIII into the same cannula. The pretreatment with the antagonist had no effect on the drinking response to AIII.

Earlier work in the rat had demonstrated that pretreatment with saralasin did not affect AIII-stimulated aldosterone release, whereas it blocked AII-induced aldosterone release (Freeman et al., 1976). AIII is known to be equipotent with AII in stimulating aldosterone synthesis and release in the periphery (Peach & Chin, 1974). Considered with the above findings the present work suggests that the two peripheral angiotensin receptors which mediate aldosterone release (Braley et al., 1983) may have a central equivalent which mediates drinking behavior.

The pig appears to be different from other animals tested thus far in 3 respects: first, it is sensitive to low doses of AI; second, it does not efficiently convert components, particularly RS, to AII; third, AIII is a strong dipsogen in the pig. Although the experiments have not yet been carried out, one might predict that brain renin levels in the pig are low, since RS-induced responses were not strong. As well, CE would probably be low compared to the rat and pigeon, but relatively high compared to the dog brain.

The responses to injections of the RAS components were specific and identifiable. As described earlier (see Results) the behavioural changes were predictable and unmistakeable. Although testing procedures were standardized, behavioural conditioning did not appear to take place. The ineffective sham injections indicate that schedule-induced polydipsia did not occur. The gilts' behaviour prior to the injections were variable, ranging from active and routing to sleeping. Regardless of activity state, at the beginning of the injection, a short time later, the pig would head directly to the drinking nozzles and remain there until sated.

One further piece of evidence that demonstrates a lack of conditioning can be found in the follow-up study of post-pubertal gilts (see Appendix 1). Presumably if schedule-induced polydipsia had occurred there would be at least some response following every test. However, it was observed that there are a number of days during the Estrous cycle, when there is no response to the injected dipsogen. Therefore, the goal-directed behaviour following the injection; as well as the lack of response following the sham injections and during some phases of the reproductive cycle, suggest that the response observed to injections of RAS components were not a result of conditioning.

Additional work is required to more accurately characterize the types of angiotensin receptors present in pig brain. The use of competitive-AII and -AIII antagonists could help elucidate this problem. If one could selectively abolish AIII-induced drinking responses with a specific antagonist, it would add considerable support for the suggestion that AIII can elicit water intake without acting on central AII receptors.

4.4. The RAS and Salt Appetite

The means by which sodium appetite is stimulated has not yet been identified. It is known that the concentration of sodium in the ECF does not have a direct effect on sodium appetite (Fitzsimons, 1979). However, cellular dehydration or infusions of hypertonic saline or sucrose inhibit sodium intake in sodium-deprived rats (Fitzsimons & Wirth, 1976). Hypovolemia is the only known stimulus to consistently stimulate sodium appetite (c.f. Fitzsimons, 1979). Since hypovolemia is also a known stimulus for renin release, it has been hypothesized that the RAS is involved in salt appetite.

The peripheral RAS, however, has not been shown to have any direct effect on sodium appetite (Fitzsimons, 1966a; Fitzsimons & Wirth, 1978). The RAS is a potent modulator for Na^+ retention by stimulation of synthesis and release of aldosterone (Peach & Chin, 1974). AII and AIII are potent in their abilities to induce aldosterone release in vitro (Peach & Chin, 1974) as well as in vivo (Blair-West et al., 1980). If the RAS plays an important role in salt appetite it must do so centrally.

Since angiotensin is a potent intracranial dipsogen, it is attractive to postulate a role for it in sodium appetite. Delayed responses for sodium have been reported following intracranial injections of AII, whereas they are not observed following peripheral administration (Fisher & Buggy, 1978). In all studies to date, primary sodium appetite has not been observed following intracranial injection of an RAS component. In most cases it has been observed that increased salt intakes

were merely keeping pace with water intakes to maintain an approximately, isotonic mixture of water and saline (Fitzsimons, 1979). However, it has been shown that AII (5-40 X 10^{-12} mole) injected into the third ventricle restored sodium appetite in the sodium-depleted rat in which the appetite had been abolished by nephrectomy (Chiaraviglio, 1976).

In the present study, pigs which were fed a Na^+-free diet and had become experienced at acquiring their sodium needs via a 1.8% NaCl solution, were tested for sodium appetite. Peripheral infusions of AII failed to stimulate any drinking of the 1.8% NaCl solution in the hour following the infusion. This finding is consistent with observations in the rat (Fisher & Buggy, 1978).

On the other hand, drinking of 1.8% NaCl was significant in the pigs when RAS components were administered centrally. The dose-response curves for 1.8% NaCl intakes induced by RAS components were similar to water intakes elicited by the same components. AII was the most effective peptide for stimulating salt appetite followed by AIII, AI and RS. An interesting finding is that AIII, at the two highest doses tested (10^{-10} and 10^{-9} mole), elicited significantly more NaCl intake than AI ($p \leq 0.05$) or RS ($p \leq 0.01$).

In the majority of tests, the intracranial injection of one of the peptide components first caused drinking of water followed within 1-2 min by one or several draughts of NaCl solution. The animal would then typically alternate between water and 1.8% NaCl. It appeared that in these cases the pig was trying to maintain isotonicity of the solution being ingested (Fitzsimons, 1979). In about 25% of the tests, however, the animal first drank some salt solution, then water, followed by a period of alternately drinking the 1.8% NaCl and the

water. The behavior was quite specific and occured particularly after AIII injections.

Primary salt appetite has not previously been reported as a result of intracranial angiotensin injections. The stimulation of salt appetite before water following AIII injections complements the sodium retaining role of AIII in the periphery. AIII has been shown to be a potent stimulator of aldosterone synthesis and release (Blair-West et al., 1980) and its ability to also stimulate salt appetite centrally suggests an overall role for AIII in sodium homeostasis.

The antagonist experiments described earlier support the notion of separate AII and AIII receptors in the brain. As well, the pretreatment with central saralasin in the two pigs produced drinking of the NaCl solution first. One explanation for the findings may be that in the pig, AIII acts on two different types of receptors, one which stimulates drinking of water, and one that stimulates salt appetite. The receptor for drinking water might be the same one that accomodates AII. Thus a competitive AII-antagonist would block an AIII-initiated drinking response. The second AIII receptor would subserve salt appetite. Stimulation of this second type of AIII receptor would provoke Na^+ appetite. Pretreatment with the AII-antagonist, followed closely by an AIII injection produced findings in agreement with this hypothesis in one pig. The pig drank 1.8% NaCl first, followed by water. In the one hour post-injection the pig drank 1410 ml of water and 1130 ml of saline.

An examination into AIII's central salt appetite stimulatory action has not yet been attempted. Previous work was focussed on

AII-induced salt intakes. It has been observed that central injections of AII into adrenalectomized rats induced significant increases in NaCl as well as water intakes over 1 hr (Fitzsimons & Worth, 1978) and that the increase in sodium was too rapid to be a result of natiuresis (Fitzsimons, 1979). Only in longer term experiments are increased sodium appetites conclusively demonstrated. L.c.v. injections of AII (1×10^{-12} mole/h for two weeks) produced striking increases in intakes of 2.7% saline in rats offered the choice of water and saline to drink (Avrith & Fitzsimons, 1979). In light of the results obtained in the present study, it seems possible that the effects of AII infusions on salt appetite in the rat were, in fact, the result of AII conversion into AIII. The study of AIII's effects in other animal species would assist in establishing a role for the RAS in the regulation of salt appetite.

5.0 SUMMARY

The research results presented in this thesis have shown that the prepubertal female pig, like other mammalian species, is much more sensitive to the dipsogenic action of RAS components given centrally than given peripherally. This suggests either that the thirst receptor for angiotensin is located within the brain, or that systemic angiotensin-induced thirst is partially inhibited by the pressor action of the peptide in the peripheral circulation.

In an attempt to characterize central angiotensin thirst receptors, the components of the RAS were injected intracranially into the forebrain or the lateral cerebral ventricle. The responses were similar to those reported in the dog (Fitzsimons & Kucharczyk, 1978), except that the pig appeared to be more sensitive. As in the dog, efficient conversion of precursors to AII was not observed, as reflected in the decreased responses to equimolar AI and RS (Fitzsimons, 1979). The sensitivity to AI, RS and renin appears greater in the pig than the dog. Both components elicited drinking at lower doses than in the dog, although not as large as AII. These differences may be a reflection of location of precursors and enzymes in the brains of the two species.

A potential role for AIII may have been unearthed in the pig. Unlike other animals AIII is very dipsogenic, paralleling equimolar AI injection in terms of 60 min intakes and is not significantly different from AII at high doses (10^{-9} or 10^{-10} mole). As well, AIII has been shown to stimulate primary salt appetite in the pig. The majority of injections of AIII in the pig produced saline drinking first. It is possible that this stimulation of salt appetite may be tied in with its known aldosterone stimulating effects (Peach & Chiu, 1974).

In order to assess the type of receptor that AIII was acting upon, pretreatment of two pigs with an AII-competitive antagonist was used in a ratio of antagonist to agonist of 10:1. No differences were observed in water intake between the pretreatment and untreated pigs to AIII injections. The pretreatment did cause primary salt appetite in both pigs and copious drinking of 1.8% NaCl. This evidence supports a hypothesis that in the pig there are separate receptors for AII and AIII, which may perform different functions. The receptors could be similar to the steroidogenic and myotrophic one proposed in the periphery (Fitzsimons et al., 1977).

The results support a centrally located role for the RAS system in the control of fluid intake in the pig, as well as multiple sites for thirst receptors. The findings point to a role for the RAS, in particular AIII, in salt appetite in the pig. The latter requires further study into the nature of the AIII receptor and as to whether AIII's role is a physiological one.

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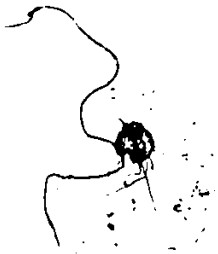
Appendix A

"Neuroendocrine Mechanisms Mediating Fluid Intake During the Estrous Cycle of the Pig"



Introduction

Studies carried out during the last decade have shown that there is a complex relationship between hormones which maintain fluid-electrolyte balance through renal and behavioural mechanisms, and the endocrine controls of the mammalian reproductive cycle. Wade (1976) has reviewed the evidence showing that all body weight regulating behaviours (water intake, food intake and voluntary exercise) predictably vary with the stage of the estrous cycle. During proestrus and estrus, daily food and water intakes decrease while activity increases and female rats lose weight (Tartellin & Gorski, 1973). The behaviours are reversed during metestrus and diestrus, when the two principal ovarian steroids, 17β-estradiol and progesterone, are relatively low in blood. Food and water intakes increase during these stages of the cycle, while voluntary exercise is greatly reduced (Tartellin & Gorski, 1973).

Previously, the cyclical nature of drinking behavior during the reproductive cycle was thought to be secondary to changes in food inake, since most of drinking (approx. 70% over 24 h) occurs in association with feeding and appears to be triggered by it (Fitzsimons & LeMagnen, 1969). However, it has been shown that there are independent central mechanisms for the initiation of each behaviour. For example, electrolytic lesions of the midlateral hypothalamus in rats significantly attenuate ad libitum drinking and water intake induced by angiotensin injections, but have no effect on ad libitum feeding behaviour (Kucharczyk & Mogenson, 1975).

The precise nature of the interaction between ovarian hormones and fluid-electrolyte homeostasis has not yet been elucidated. Estrogens increase plasma renin substrate in rats (Menard & Catt, 1973) and humans (Beckerhoff et al., 1972) and this is followed by a rise in circulating angiotensin II (Cain et al., 1971). The cyclic variations in drinking during the estrous cycle may therefore parallel changes in fluid and electrolyte excretion or alterations in peripheral angiotensin biosynthesis (Kucharczyk, 1984). Another possibility is that the ovarian steroids interact with central thirst receptors, since the gonadal steroids have been shown to alter neuronal discharge frequency in areas of the brain that are involved in drinking behaviour (Kawakami & Kubo, 1971).

Findlay et al. (1979), using female rats, have obtained support for the latter alternative. They demonstrated that in cyclical female rats with chronically implanted cannulae directed at the preoptic (POA) of the brain, equimolar doses of AII given over the estrus cycle elicited cyclical responses. One hour drinking responses were reduced at proestrus and estrus, compared to diestrus or metestrus. The cyclical nature of AII-induced drinking was abolished by ovariectomy and was absent prepubertally. The same estrous cycle-related differences in drinking were observed following peripheral injections of isoprenaline, a β-adrenergic agonist which stimulates the renin-angiotensin system (Lehr, Mallow & Kurkowski, 1967). In contrast, water intake induced in the same animals following preoptic administration of the dipsogen carbachol, or after subcutaneous injections of hypertonic NaCl,

did not vary with the stage of the estrous. These data indicate that only the extracellular mechanisms for body fluid regulation are sensitive to the estrous cycle and changes in ovarian hormones.

The area in the brain at which an interaction between ovarian steroids and the physiological mechanisms for extracellular thirst mediated by renin angiotensin occurs may be the preoptic area in the forebrain. Its possible role as a 'thirst center' has been described earlier in the Introduction. This region of the forebrain is critical for triggering the preovulatory surge of LH (Barraclough, 1973; Halasz, 1969). As well, it has been shown that estrogen, a known modulator of LH and LHRH (Gross, 1980), is concentrated by preoptic neurons following systemic administration of the labelled steroid (Pfaff & Keiner, 1973). In addition, POA neurons have been shown to have a differential sensitivity to microiontophoresed estradiol hemisuccinate at various stages of the estrous cycle (Kelly et al., 1976). It therefore appears that the POA may play a role in the maintenance of extracellular fluid balance in synchrony with the ovarian cycle (Kucharczyk, 1984).

Other vasoactive hormones may also be involved in mediating fluid-electrolyte balance during the estrous cycle. Prolactin, for example, may have primary dipsogenic activity in humans (Horrobin et al., 1971) and rats (Ensor et al., 1972). Kaufman and MacKay (1983) found that the increased drinking results from an exaggerated sensitivity of hyperprolactinemic rats to extracellular fluid deficits. At estrous and proestrous, when prolactin levels are increased, decreased drinking responses to isoprenaline are observed in rats (Findlay et al., 1979; Amenomori et al., 1970). Isoprenaline-induced thirst is also attenuated

in rats following estrogen treatment (Thrasher & Fregly, 1976), which is known to significantly increase serum prolactin levels in this species (Chen & Meites, 1970). Although prolactin and its receptors have been identified in the brain (Clemens & Sawyer, 1974), their exact relationship to neural thirst mechanisms is not known.

The effect of the mammalian reproductive cycle on circulating levels of vasopressin has also been studied. Plasma vasopressin levels fluctuate with the female menstrual and rat estrous cycle (Forsling et al., 1981; Skowsky et al., 1979). In the rat, changes in vasopressin parallel circulating estradiol levels (Skowsky et al., 1979). Human males on estradiol administration showed enhanced vasopressin levels, Legros et al. (1973). As well, post-menopausal women, and rats given low doses of estradiol, had elevated vasopressin levels, while progesterone appeared to have no effect (Skowsky et al., 1979; Forsling et al., 1981). The elevated vasopressin titre is probably due to increased release rather than decreased clearance, since the rises are rapid (Skowsky et al., 1979), and estradiol has been shown to be taken up by magnocellular neurons (Pfaff, 1978).

One possibility is that gonadal steroids may alter the osmotic threshold required for vasopressin release. In the first trimester of pregnancy, there appears to be a re-setting of the osmotic threshold for vasopressin secretion, as well as thirst, in the rat and human (Durr et al., 1981; Davidson et al., 1984). At the same time, there is a decrease in blood osmolality, and thus to prevent the pregnant female from continual diuresis a new steady state is developed

Davidson et al., 1984). The precise mechanisms by which this occurs has yet to be elucidated. In particular, the influence of the renin-angiotensin ststem on vasopressin release and osmotic thirst thresholds remains to be studied.

The objective of this study was to compare the responsiveness of prepubertal and postpubertal female pigs to plasma hyperosmolality, induced by intravenous infusion of hypertonic NaCl, and to simulated plasma hypovolemia, induced by intravenous infusion of AII. It is well established that either stimulus effectively provokes thirst, vasopressin release, and antidiuresis, in both male and female vertebrates (Fitzsimons, 1979). The precise nature of the ovarian hormone effects on induced vasopressin stimulation and thirst have not been determined. Thus, it is of interest to establish the relationship between the ovarian steroids and plasma osmolality, plasma vasopressin and thirst.

Most previous related work has been done in the rat (cf. Kucharczyk, 1984). Other animals, for example, the ewe and goat, have been tried as models with some success (Michell 1981; Olsson et al., 1982, respectively). In this study the gilt was selected because its large size enables frequent testing and withdrawal of blood samples. As well, the female pig has a reproductive cycle similar in hormonal profile to the human female although on average the pig's cycle is 6 days shorter (Van der Weil et al., 1981).

Finally, a regime for inducing ovulation in late prepubertal pigs has been developed (Baker et al., 1969) which conveniently synchronizes the estrous cycles of the individual animals to facilitate testing and data collection.

Materials and Methods

Eight prepubertal pigs were used in the study. Four of the animals were allowed to come into puberty naturally, while the other 4 had their first ovulation induced. Following the regime described by Baker et al. (1965), 750 IU of Pregnant Mare Serum Gonadotrophin (PMSG) (Equinet; Ayerst Labs, Montreal) given i.m. was followed 72 h later with 500 IU of human chorionic gonadotrophin (hCG). This treatment synchronizes the production of a population of uniformly developing follicles which ovulate approximately 42 h after hCG (Baker et al., 1969). Signs of estrus (red and swollen vulva) were usually evident 48-72 h after hCG, and the number of ovulations is similar to that observed in the spontaneously ovulating gilt (Baker and Coggins, 1968).

In order to assess the length of the animals reproductive cycle, 17β-estradiol and progesterone measurements were made from sera collected daily at 09:00 h. Blood was collected from chronically implanted saphenous catheters, and immediately transferred to chilled siliconized whole blood tubes for steroid analysis. The serum was then separated and frozen at -4° C until the assay was performed. It was found that with 3 ether extractions of a 1 ml serum sample, approximately 85% recovery could be obtained (E2 = 84 ± 6, p4 = 85 ± 5, n = 10). The steroids and ether were blown down with nitrogen gas and then resuspended in 1 ml of 99.7% ethanol. Duplicate 100 µl aliquots of the ethanol extracts were evaporated and assayed by direct

radioimmunoassay (RIA) using the method developed by Orczyk et al. (1974).

The P_4 antiserum used shows negligible cross reactivity (< 0.1%) with androgens and estrogens. The dilution of the P_4 antibody required to achieve a sensitivity of 0.4 ng/ml was 1:1000, prepared against deoxycortico monohemisuccinate:bovine serum albumin (BSA). The E_2 antibody used was diluted 1:1000, prepared against E2-17β hemisuccinate:BSA. There was < 10% cross reactivity with other phenolic steroids and less than 0.1% cross reactivity with neutral steroids. The sensitivity of the E_2 assay was 2.5 pg/ml. The assays were validated by estimation of recovery by the recovery of added nonradioactive steroids (E_2 = 25, 250 pg, P_4 = 1, 32 ng).

The animals were tested twice a week as follows: At 09:00 h a blood sample was taken for analysis of E_2, P_4, plasma osmolality and plasma vasopressin. At 12:00 h the food was removed from the pen. At 13:00 h a blood sample was taken for measurement of plasma osmolality and vasopressin. Following the blood sample, an infusion of either 3 M NaCl or AII (125 or 250 ng/min) was begun and continued for a half an hour or until the animal drank, whichever came first. At this time, and again 2 h later, another blood sample was taken for plasma osmolality and vasopressin evaluations.

Plasma osmolality was measured in 8 ml duplicate plasma samples on a Wescor 5100 CXR Vapour Pressure Osmometer (Johns Scientific). If samples had greater differences than 5 m·Osm/kg H_2O, then two more measurements were taken. The machine was re-calibrated after every

10 samples back to the 290 m·Osm/kg H_2O standard provided by the manufacturer.

Plasma lysine vasopressin (LVP) was determined using an RIA kit (Immunonuclear, Minnesota). This kit is a delayed tracer RIA that uses octadecasilyl-silica for plasma extraction. After the extraction procedure, the RIA was performed employing addition of sample and guinea pig anti-vasopressin, followed by an 12-24 h incubation at 2-8° C. ^{125}I vasopressin was then added, followed by a second incubation for 18-24 h at 2-8° C. Pre-precipitated carrier, second antibody and polyethylene glycol were added in a single step. The assay was then centrifuged and decanted after a minimum of 15 min second antibody intubation at room temperature. The assay had a sensitivity of 2.5 pg/ml and recovery better than 85% of unlabelled vasopressin. The antibody detects 100% of added AVP and LVP.

Results

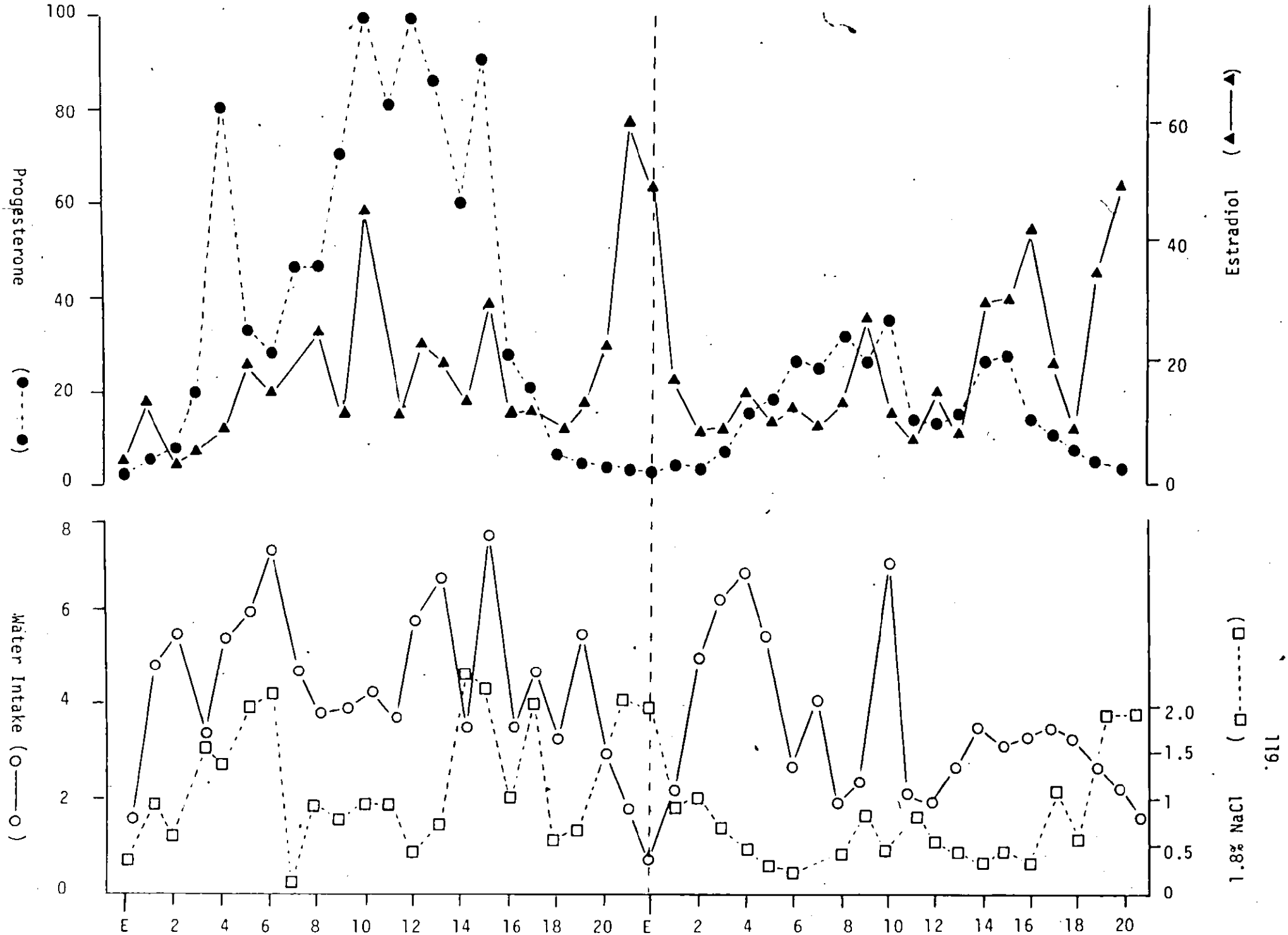
In order to determine the length and hormonal profile of the postpubertal female pig, 4 pigs were followed through two complete cycles (see Fig. 1). The first cycle was initiated by the synchronizing regime described in the Introduction, the second followed spontaneously. The two major ovarian hormones, estradiol and progesterone, were measured at 09:00 h every day during the two cycles.

Estradiol levels were variable throughout the mid-portion of both cycles, but rose abruptly and fell just as quickly after day 20. The two cycles were approximately equal in terms of magnitude of estradiol levels throughout the cycles. At the end of either cycle a peak of approximately 60 pg/ml was measured.

Progesterone levels were typically low (less than 2 ng/ml) around the time of estrus, but after day 3 began to rise and plateau between day 6 and 9. The progesterone levels in the first cycle are almost double those observed in the second cycle, although the same pattern of hormonal fluctuations was found. Just prior to estrus, progesterone levels decreased from 90 mg/ml in the first cycle and 20 mg/ml in the second, to less than 2 ng/ml.

The profiles produced are similar to those reported by others (Van der Weil et al., 1981). The length of the estrous cycle in these pigs was 21 or 22 days, which is the normal length for a young adult female pig (Canadian Council on Animal Care, 1983). From the above data, it appears likely that pigs used in this study were reproductively hormonal.

Figure 1. Blood levels of progesterone (ng/ml), estradiol (pg/ml) (upper panel), 24 h water intakes (l) and 24 h intakes of 1.8% NaCl (l) (lower panel) of four pigs. Blood samples were taken at 09:00 h each day over two complete estrous cycles. Values shown are means of the measurements.


Progesterone (●----●)
Estradiol (▲——▲)
Water Intake (○——○)
1.8% NaCl (□------□)
100
80
60
40
20
0
60
40
20
0
8
6
4
2
0
2.0
1.5
1
0.5
0
E 2 4 6 8 10 12 14 16 18 20 E 2 4 6 8 10 12 14 16 18 20

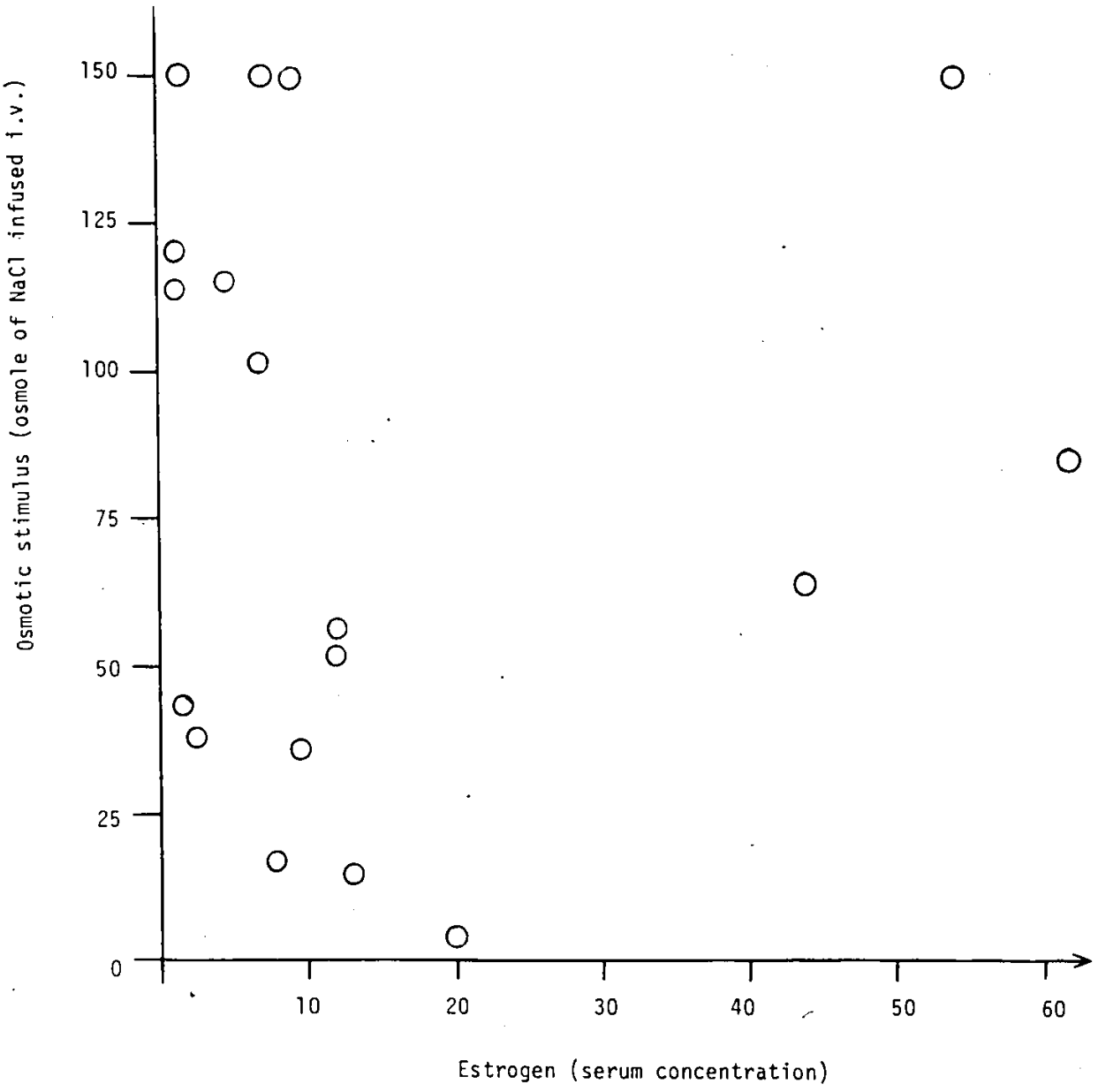
Daily intakes of water in female mammals have been shown to be dependent on the stage of the estrous cycle (Findlay et al., 1979; Michell, 1981). An attempt was therefore made to establish the relationship between the stage of the estrous cycle, as reflected by estrogen and progesterone levels, and the ad libitum intakes of water and 1.8% NaCl.

A cyclical pattern in 24 h water intakes was observed in the post pubertal pigs (see Fig. 1c). Water intakes on the day preceding estrus, the day of estrus, and the day following estrus, were significantly lower than at any other three day period during the cycle ($p < .05$, ANOVA), or than for the cycle as a whole ($p < .01$, ANOVA). However a significant correlation between either ovarian hormone and water intake was not found.

Salt appetite, measured by daily intakes of 1.8% NaCl in Na^+-free chow-fed gilts, appears to be independent of the estrous cycle (see Fig. 1a). Peaks were observed at different times in both cycles as were the nadirs. However, these did not correlate with any physiological parameter measured.

Post pubertal gilts tested twice weekly with i.v. 3 M NaCl did not appear to be affected by the state of their reproductive cycle. A significant linear correlation between estradiol (Figure 2) and/or progesterone (not represented) and the amount of osmotic stimulus required to initiate drinking was not observed. Over the range from 1 to 70 pg/ml of estradiol and 0.1 to 60 ng/ml of progesterone, the gilts responded to the osmotic stimulus in 20 of 25 trials. The insensitive periods were during the upper, mid and lower ends of the

Figure 2. The osmotic stimulus required to initiate drinking is plotted against serum estradiol concentration. 3 M NaCl was infused at a rate of 2.5 ml/min until the pigs drank, or for 30 min, whichever occured first.


Osmotic stimulus (osmole of NaCl infused i.v.)
150
125
100
75
50
25
0
10
20
30
40
50
60
Estrogen (serum concentration)

values of ovarian hormones. The responsiveness to dehydration-like stimuli thus appears to be independent of the cycle.

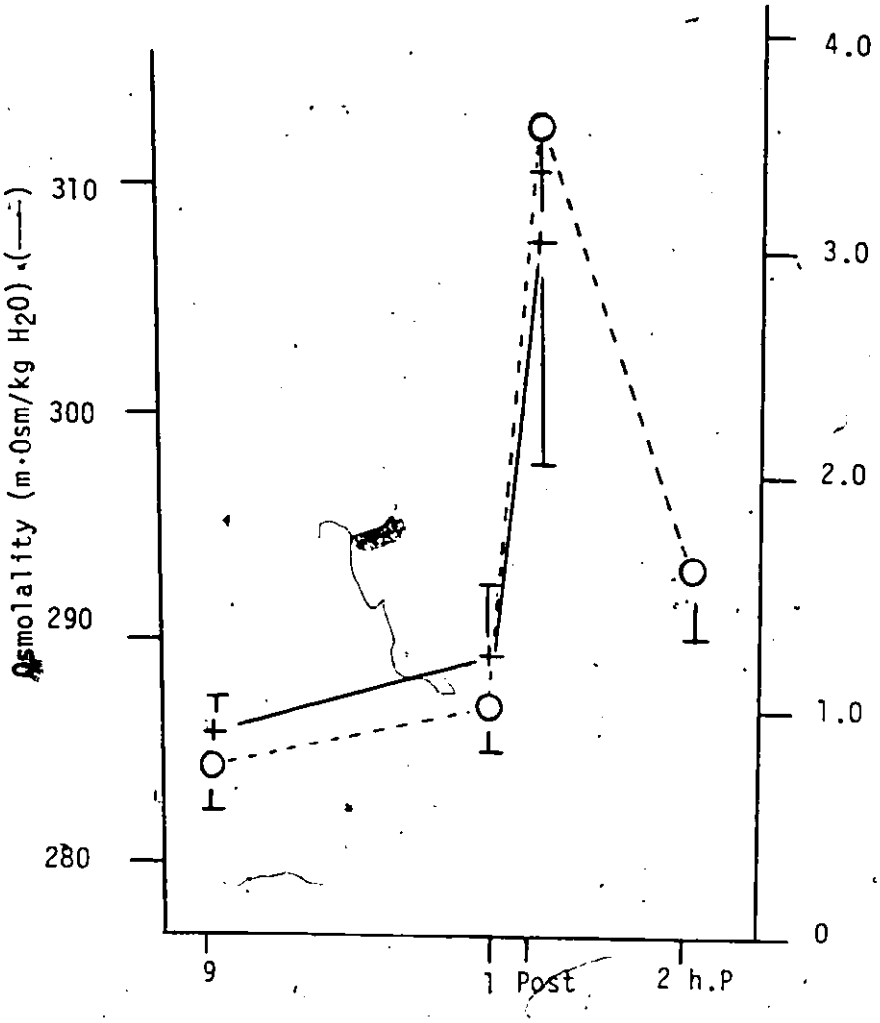
The strength of stimulus required to initiate drinking in post-pubertal pigs is approximately double that required to do the same in prepubertal pigs (see Table 1). The response to the stimuli, in terms of water drunk for post pubertal gilts, is also double that of prepubertal female pigs. This is undoubtedly a size related phenomenon, since the prepubertal pigs weighed an average of 45 kg, and the post-pubertal pigs weighed between 70 to 100 kg. According to the Canadian Council on Animal Care blood volumes range from 60-80 ml/kg, and thus it is possible that the average blood volume of the post pubertal female pigs was twice that of the prepubertal pigs.

The change in osmolality in both pre- and post-pubertal pigs required to initiate drinking was the same (approximately 7% from resting). The pretest osmolalities were not significantly different from each other, nor were the post-test values. Plasma osmolalities were relatively stable throughout the day, around 290 m·Osm/kg H_2O. In the post pubertal tests, blood osmolalities two hours after the test were not different from either the 09:00 or 13:00 h levels.

Vasopressin levels for the most part paralleled the blood osmolalities (see Figure 3). In prepubertal animals, the plasma vasopressin levels rose with increasing osmolality, and fell two hours later (Fig. 3a). If the plasma osmolality had been measured two hours later, it would probably show that it was back to about 290 m·osm/kg H_2O or similar to that observed in the post pubertal pigs. In the prepubertal pigs it

Figure 3. Changes in plasma osmolality (solid line) and vasopressin concentration (dashed line) are shown for (a) prepubertal and (b) postpubertal pigs infused with i.v. with 3 M NaCl for 30 min or until drinking occured. Values shown are means ± S.E.M. Numbers of observations are 8 for prepuberta- pigs and 20 for postpubertal pigs.

a) Pre pubertal



b) Post pubertal

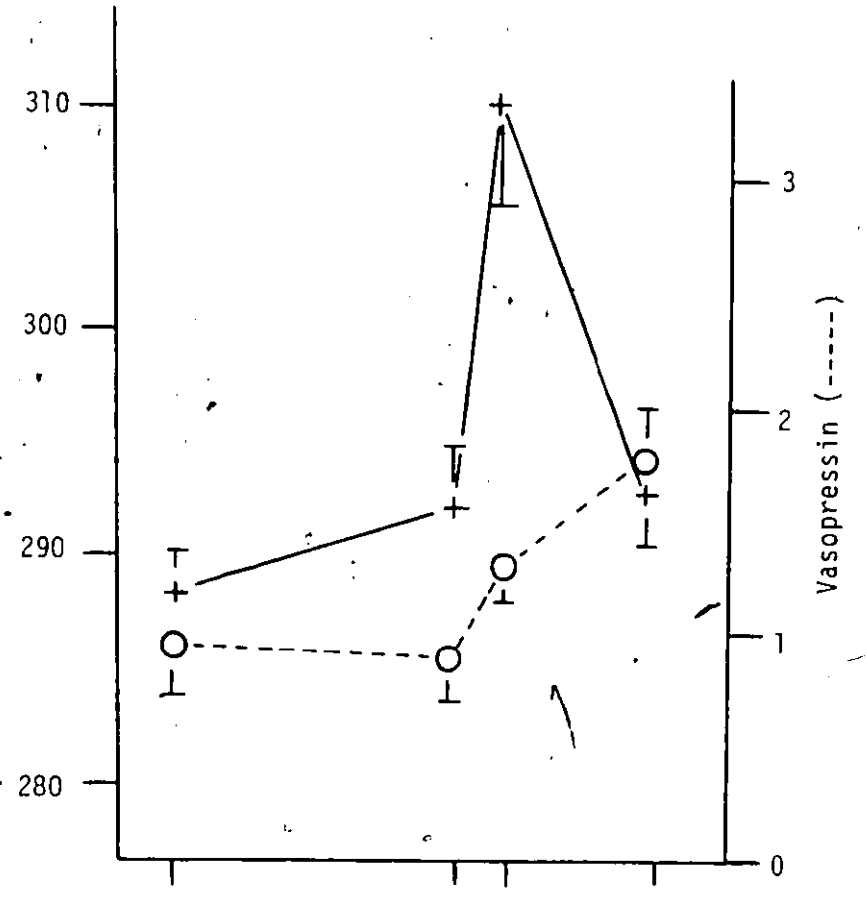
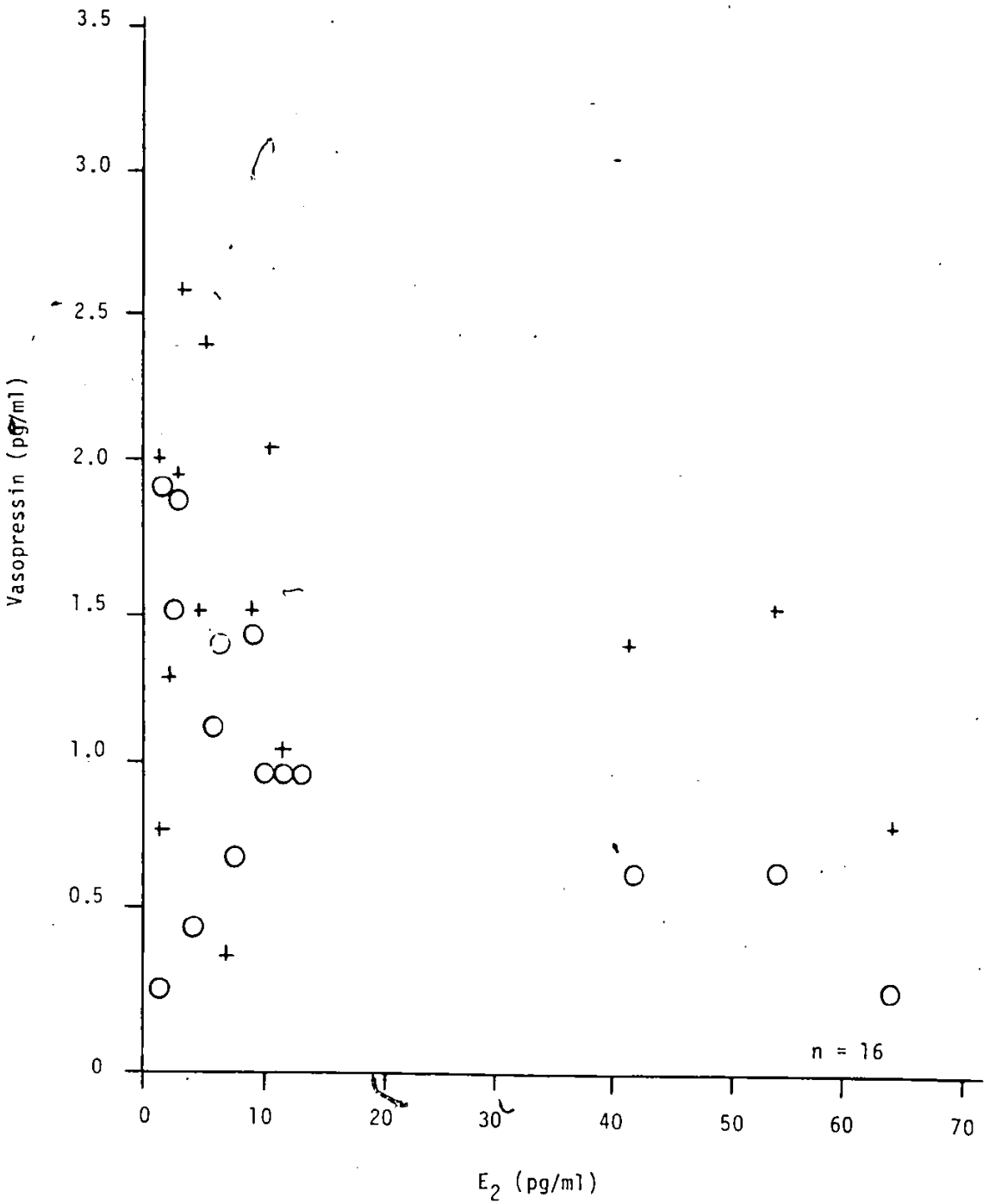


Table 1. Changes in plasma osmolality and vasopressin concentration in prepubertal and postpubertal female pigs infused i.v. with 3 M NaCl. The number of Osm of NaCl infused before drinking occured, and the volume of water ingested by the pigs during the 30 min period after the infusion was started, as also noted. Values are means ± S.E.M. Numbers of observations in parentheses.

Osm (m·Osm/Kg H_2O)				Vasopressin (pg/ml)				Osmotic load (Osm)	Water Intake (ml/30 min)
09:00	Pre	Post	2 h	09:00	Pre	Post	2 h		
285 ±1 (8)	289 ±2 (8)	308 ±5 (8)	-	0.80 ±.17 (8)	1.02 ±.09 (8)	3.69 ±.94 (8)	1.67 ±.28 (8)	97.6 ±11.0 (8)	380 ±50 (8)
POSTPUBERTAL									
289 ±2.0 (16)	292 ±1.0 (22)	310 ±3.0 (21)	293 ±1.0 (16)	1.08 ±.13 (19)	0.95 ±.11 (20)	1.35 ±.14 (20)	1.77 ±.29 (19)	80.0 ±1.0 (25)	640 ±90 (25)

Figure 4. Plasma vasopressin levels taken at the time of the test (o) and just proceeding the test test (+) as compared with the estradiol level of the same morning. The number of observations is at the bottom.


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Vasopressin (pg/ml)
n = 16
0
10
20
30
40
50
60
70
E_2 (pg/ml)

therefore appears as though changes in blood osmolality lead to changes in plasma vasopressin.

In the post pubertal pig the plasma vasopressin levels do not 'mirror' the plasma osmolality values (see Fig. 3b). At the time of drinking the average plasma osmolality is 310 ± 3.0 mOsm/kg but the vasopressin value is only 1.35 ± 0.14 pg/ml (Table 1). Two hours later, when the osmolality had almost returned to normal (293 ± 1.0 mg/ml), the vasopressin level has risen to 1.77 pg/ml. It is possible that the vasopressin peak occured between the two sampling times. If this were the case, then the values obtained might better represent the true picture.

Vasopressin levels were examined in different endocrine states (Fig. 4). Plasma vasopressin has been shown to be related to the level of circulating estradiol (see Introduction). In this study, however, no significant correlation was found between E_2 and vasopressin. At low plasma levels of estradiol (less than 20 pg/ml), vasopressin levels ranged from less than 1 pg/ml to more than 5 pg/ml. At high estradiol values, the vasopressin levels for the most part were low.

Discussion

In order to be able to make quantative assessments about the role of the estrous cycle on fluid and electrolyte homeostatic parameters a careful documentation of the reproductive cycle of the pigs had to be made first. During the course of the study, external manifestations of estrus, red and swollen vulva and lordosis response, were used as indicators. More accurate indicators (vaginal biopsy) were not used as stress-free conditions were desired. However, in order to more accurately pinpoint the time of estrus, ovarian hormone measurements were made. On the whole, measurements of blood estradiol (E_2) and progesterone (P_4) were supportive of the earlier observations.

The ovarian hormone measurements made during two consecutive cycles were similar in magnitude to those published by others (Van der Weil et al., 1970; Hendricks et al., 1971). Progesterone levels typically remained low from the time of estrus until about day 3 after estrus, rose rapidly until day 7 or 8, and then continued to rise more slowly until day 16 of a 22 day cycle (Rayford et al., 1971). On day 16 or 17, progesterone declined rapidly from 30-35 ng/ml to 1 ng/ml or less, within about a 48 hour period. Plasma E_2 levels showed a peak from 55-80 pg/ml on the day preceding estrus, and then declined to levels of 5 to 20 pg/ml during the remainder of the cycle.

Differences between the measurements of the animals in this study and earlier published work are that the hormonal profiles are not smooth, and the concentration of progesterone in the second cycle was lower than in the first cycle (See Fig. 2). Numerous aberations, particularly

in the estrogen profile, can be observed (See Fig. 1). This is a result of using only four animals to generate the profiles. If one animal was needlessly stressed one morning, estradiol levels, which are very stress sensitive, could become quite elevated. Some examples of possible stresses include the cleaning of the pens, routine intramuscular injection, corrective surgery, and illness. Care was taken to exclude data obtained from known stressful situations, but as many stresses are unseen, some of what appeared to be abnormally high mid-cycle values were used in the calculation of hormonal means for each of a particular day of the cycle.

The abnormally high level of progesterone in the first estrous cycle was due to the artificial induction of ovulation. Progesterone in the plasma during the luteal phase of the estrous cycle is positively correlated with the number of corpora lutea. The administration of exogenous gonatrophic hormones (PMSG, hCG) will increase the ovulation rate. Thereafter the progesterone levels are increased (Ellicott, 1980). This is exactly what was observed in our pigs. The first cycle progesterone levels were at about 100 ng/ml, whereas in the second cycle it peaked at less than 40 ng/ml. The ovarian measurements suggest that the gilts were in fact cyclic and the profiles were as one would expect considering the regime that they were subject to. It was found using external manifestations that the pigs had cycle lengths of either 21 or 22 days.

Daily Fluid Intakes and the Estrous Cycle

Since Sloanaker (1925), researchers have been aware that the estrous cycle plays a role in voluntary activities in rats. Tartellin (1968) found cyclical variations in food and water intake in ewes. However, all of this earlier work was unable to pinpoint the cause of the observed depression in intakes at estrus. Questions arose as to whether the depressed intakes at estrus were related directly to the neurohormonal events of ovulation or whether it was mere coincidence (Tartellin & Gorski, 1971). A detailed examination was done by Tartellin and Gorski in 1971 on rats in which they measured daily food and water intakes, body weight, and fecal and urinary output during the estrous cycle. They found that food and water intakes were significantly depressed at estrous as compared to metestrus and diestrus. Unfortunately these observations were not correlated with any pituitary or ovarian hormone measurements. However it was reported earlier that at proestrus, estrogen and progresterone reach their peak plasma level (Uchida et al., 1969). It was speculated that it is estradiol which is the key factor in the depression of voluntary intakes, but the rat's four day cycle is too short to make any correlation. What was required was an animal model with a longer, more stable ovarian hormone cycle.

The post pubertal female pig was the logical animal model for studying this possible relationship, since it has a repeating 22 day cycle, with an ovarian hormone profile similar to that of humans (Van der Weil- et al., 1978).

It was found that 24 hour water intakes in the periovulatory period were significantly less than at any other time during the cycle, as well as the cycle as a whole. These findings are similar to those which have been reported for the rat, and ewe (Tartellin & Gorski, 1971; Michell, 1981). As in other species, during diestrus, increased water intakes are observed (Findlay et al., 1979). There did not appear to be significant correlation between daily intakes of water and the ovarian hormones.

Although a definitive statement could not be made regarding blood ovarian hormone concentrations in relation to daily intakes of water during the estrous cycle, the purpose of this portion of the study has been partially fulfilled. It can be said that the pigs used in later testing had normal reproductive cycles, in terms of length and ovarian hormonal profiles. As well, even with the limited sample size, one can say that the pig, like other animals, has depressed daily intakes of water at the time of estrus. As other investigators have stated before, it appears as if the means by which the ovarian hormones in particular, and the estrous cycle in general, affect the voluntary activities of the cyclic female is a complex one, and the precise mechanism by which it acts has yet to be elucidated (Kucharczyk, 1984).

A better animal model to use might be an ovariectomized pig that has been given subcutaneous hormone implants of either estrogen and/or progesterone. This would allow for the creation of a non-fluctuating hormonal environment, as opposed to the unstable one in cyclic animals. By altering the implants, one could then develop different hormonal

mileus which might better enable the clarification of the relationship between the ovarian steroids and as yet uncorrelated parameters, ie. daily water and food intakes and induced drinking by extracellular thirst stimuli.

In contrast to thirst initiated by extracellular stimuli, ie. hypovolemia and decreased blood pressure, thirst resulting from intracellular stimuli is unrelated to the stage of the reproductive cycle. It has been shown that subcutaneous injections of hypertonic saline do not produce responses of a cycle-dependent nature, nor do one hour intakes following a twenty-four hour water deprivation period fluctuate (Kucharczyk, 1981). It appears as though only the extracellular mechanisms for body fluid regulation are sensitive to the stage of the reproductive cycle.

In an attempt to validate those findings in a larger animal, intravenous infusions of hypertonic saline were made in eight postpubertal pigs at random times during their cycle. It was found that there were no significant correlations between blood estradiol concentrations and either the osmotic stimuli required to initiate drinking (see Figure 2) or the magnitude of the behavioural response. The same was found to be true for blood progesterone levels. Thus it seems that the osmotic stimulus and magnitude of response are independent of direct ovarian steroid modulation.

An examination was made to determine if there were differences between pre pubertal and post pubertal female pigs to infusions of hypertonic saline. As it has been mentioned earlier, that the reproductive cycle does not appear to play a role in intracellularly stimulated thirst (Kucharczyk, 1981; Kaufman, 1980), it was proposed that one would not

observe any difference between pre- and post pubertal pigs.

An attempt was made to further the work by Forsling et al. (1981; 1982) and Skowsky (1979), which showed that in the human and rat, respectively, plasma E_2 levels were correlated with circulating VP concentrations. It was also shown by both groups that vasopressin levels fluctuate during the reproductive cycle in response to changing estradiol, and rise in response to exogenous estradiol administration in postmenopausal or ovariectomized females. Skowsky (1979) also observed that progesterone and high estradiol levels may have an inhibitory role.

In the present work, a correlation between E_2 and VP concentrations was not observed. This may have been due in part to the assay kit. As well, there were no estradiol values between 12 and 35 pg/ml. There appears to be a rise in VP levels with increased E_2 and E_2 is less than 12 pg/ml. At concentrations greater than 35 pg/ml, the E_2 appears to be inhibitory. This supports Skowsky's work in rats, which demonstrated that at low concentrations E_2 is stimulatory and at high concentrations it becomes inhibitory.

In summary, the post pubertal female pig appears to be similar to other mammals studied in terms of responsiveness to hypertonic saline and the effect of the reproductive cycle on daily water intakes. 24 Hr water intakes are significantly depressed at the time of estrus in all 3 species. On the other hand, the reproductive cycle and ovarian hormones do not appear to have a direct influence on drinking induced by hyperosmotic stimuli. Post pubertal and pre pubertal pigs were approximately equally sensitive (7% change in osmolality) to hypertonic stimuli when these were adjusted for differences in body

weight. The possible influence of ovarian steroids on plasma vasopressin could not be assessed accurately due to technical problems. However, the initial results are in agreement with the findings of Skowsky et al. (1979) who found at low levels, estradiol is stimulatory, but at high concentrations it is inhibitory. At high estradiol levels plasma vasopressin levels are lower than at decreased estradiol levels in this study. Unfortunately, a dose-response relationship could not be established at lower levels.

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Appendix B

1. Composition of commercially prepared pig chow:

Min. Crude Protein	15.0%
Min. Crude Fat	3.0%
Max. Crude Fiber	5.0%
Calcium (actual)	0.65%
Phosphorus (actual)	0.5%
Zinc (actual)	0.006%
Min. I.U. Vitamin A	1760/kg
Min. I.U. Vitamin E	10/kg

Ralston Purina Canada Inc.

2. Physiological and Nutritional Parameters:

Water Required Daily	Food Required Daily	Salt Required Daily	Digestible protein %	Blood vol. (ml/kg)	Light Hours	space per animal
4.5-6.5 l	1.5-3 Kg	7.5 g-15 g	14	70	10-12	2-4 m^2

Ranges derived from literature mean values for young adults (Canadian Council on Animal Care, 1983).

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2. Ovarian Cycle related changes in fluid balance in the pig. Paper presented at the Reproductive Biology Workshop, Ottawa, Ontario, May 1983. In collaboration with J. Lemoine, J. Kucharczyk and B.K. Tsang.

3. Influence of reproductive cyclicity on the renin-angiotensin system and plasma vasopressin in the post pubertal gilt. Paper presented at the Reproductive Biology Workshop, Ottawa, Ontario, May 1984. In collaboration with M. Huang, J. Lemoine, J. Kucharczyk and B.K. Tsang.

ABSTRACTS

1. Mutter J., Lemoine, J., Tsang, B.K. and Kucharczyk, J. (1983). Central angiotensin-induced water intake in the pig. Proc. Can. Fed. Biol. Soc. 26: Abst. 2.

2. Jamshaid, A., Mutter, J., Rosoph, L., Lemoine, J., Kinson, G. and Kucharczyk, J. (1983). Dianabol and exercise induced changes in fluid-electrolyte balance and reproductive cycle in female rats. Proc. Can. Fed. Biol. Soc. 26: Abst. 358.

PUBLICATIONS

Mutter, J., Lemoine, J., Tsang, B.K. and Kucharczyk, J. Central angiotensin-induced water intake and salt appetite in the pig. Brain Res. (In press).

Jamshaid, A., Kinson, G., Lemoine, J., Mutter, J. and Kucharczyk, J. Dianabol and exercise-induced changes in fluid-electrolyte balance and reproductive cycle in female rats. Submitted to Arch. Androl.