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LOCALIZATION OF THE CENTRAL SITES OF ACTION OF ANGIOTENSIN II ON RELEASE OF ANTIDIURETIC HORMONE

by

Christine Agnes McGuire Gregg

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Physiology)
in The University of Michigan
1973

Doctoral Committee

Professor Richard L. Malvin, Chairman
Professor John A. Jacquez
Professor A. Rees Midgley, Jr.
Assistant Professor David R. Mouw
Professor Arthur J. Vander

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
INTRODUCTION	1
A. Overview	1
B. Historical Perspective	3
C. Proposed Studies	16
MATERIALS AND METHODS	18
Part I: Equipment and Procedures	18
A. Animals	18
B. Preparation of Tissues for Incubation	18
C. Solutions	20
D. Chemicals	21
E. Common Experimental Procedures	23
1. Equillibration	23
2. Incubation	23
3. Weighing	26
4. Counting Procedures	26
5. Calculations	28
Part II: Experimental Protocols	29
A. In vivo Studies with I^{125} Angiotensin I	29
B. In vitro Incubations with C^{14} Angiotensin II	33
C. In vitro Incubations with I^{125} Angiotensin I and Angiotensin II	34
D. The Effect of Rinsing Tissue After Incubation with I^{125} Angiotensin II	35
E. The Effect of Angiotensin II on the Release of ADH from Isolated Neural Lobes and Intact Hypothalamo-hypophysial Systems in vitro	38

Page

RESULTS AND DISCUSSION..... 42

Part I: In vivo Studies with I^{125} Angiotensin I..... 42

Part II: In vitro Binding Studies..... 54

A. Uptake of C^{14} Angiotensin II..... 54

B. Effect of Incubation with an Excess of Unlabelled Hormone on the Uptake of I^{125} Angiotensin I and II.. 59

C. Effect of Rinsing Tissue After Incubation with I^{125} Angiotensin II..... 65

Part III: The Effect of Angiotensin II on in vitro ADH Release..... 74

A. Intact Hypothalamo-hypophysial Systems..... 74

B. Isolated Neural Lobes..... 76

LIST OF REFERENCES..... 87

LIST OF TABLES

Table		Page
1.	Effect of incubating anterior and posterior pituitary for 10 minutes in 1 ng/ml of I^{125} angiotensin I in the presence and absence of 75 ng/ml of unlabelled angiotensin I	60
2.	Effect of incubating anterior and posterior pituitary for 10 minutes with 0.5 ng/ml of I^{125} angiotensin II in the presence and absence of 1 ug/ml of unlabelled angiotensin II	64
3.	Residual T/M ratio of various tissues after 30 minutes of incubation with 0.25 ng/ml of I^{125} angiotensin II followed by 25 minutes of rinsing in buffer	66

LIST OF FIGURES

Figure		Page
1.	Schematic diagram of apparatus used for in vitro tissue incubations.....	24
2.	Tissue to Plasma Ratios for various tissues four minutes after injection of 3 or 6 ng of I^{125} monoiodoangiotensin I.....	43
3.	Effect of incubating different tissues with 100 ng/ml of C^{14} angiotensin II for varying lengths of time.....	55
4.	Washout of label into buffer from anterior (AP) and posterior (PP) pituitary after 30 minutes of incubation with 0.25 ng/ml of I^{125} angiotensin II.....	68
5.	The effect of angiotensin II on release of ADH from intact hypothalamo-hypophysial systems in vitro.....	75
6.	The effect of angiotensin II on release of ADH from isolated neural lobes in vitro.....	77

INTRODUCTION

A. Overview

One fact cannot fail to impress anyone who seriously attempts the study of physiology--that is, the subtle and enormous complexity of biological control systems. A prime example is the regulation of the volume and composition of the extracellular fluid. Despite enormous variation in quantity and quality of intake, these parameters of the internal environment are normally maintained stable to within $\pm$ 1% throughout life.

To accomplish this end, a great number and variety of inputs are perceived and integrated. Examples of afferent stimuli proven or proposed include osmolality of plasma,[141,142] concentration of sodium in cerebrospinal fluid,[5,103] blood volume,[126,127] emotional stimuli,[80,96] pain,[28,83] and exercise.[114] No less diverse are the effector limbs which are both neural and hormonal. Central nervous system output comes from at least three separate areas. There is central autonomic output to the heart, blood vessels, adrenals, and kidney; cortical outflow influencing thirst and behavior; and hypothalamic outflow which stimulates release of antidiuretic hormone (ADH) [78] and may also indirectly influence aldosterone secretion.[149] Another vitally important effector limb is the hormonal renin-angiotensin-aldosterone system.

The ultimate complexity comes with the realization that there are even numerous interconnections between the various afferent and efferent limbs of this system. This work will focus on the relationship between

two important hormonal effectors of salt and water balance, viz. antidiuretic hormone and angiotensin.

The control of water balance is the primary mechanism for preservation of extracellular tonicity. Osmolality is directly proportional to the quotient of dissolved, osmotically active solute and total body water. Therefore, a change in tonicity may be produced by excessive loss or gain of water. Two of the major mechanisms which control water balance are the plasma levels of ADH and the intake of water as stimulated by the sensation of thirst.

On the other hand, extracellular volume is mainly governed by the total amount of osmotically active solute which is dissolved in it. The most abundant electrolytes in this volume are sodium and chloride, but the movement of the latter is often passively determined by sodium movement. We may say, then, that sodium balance and the mechanisms which preserve it are the major determinants of the extracellular volume. These factors are numerous, but this discussion will concern itself only with the renin-angiotensin-aldosterone system.

However, it is artificial and simplistic to attempt to separate sodium balance and volume control from water balance and the maintenance of tonicity. In fact, the two systems are inextricably interrelated. In addition to osmolality there is also a volume component which affects water balance via ADH secretion.[126, 127] Furthermore, there is some evidence this and not osmolality may be the preeminent afferent input.[70]

The newest subtlety in this interaction is the realization that the

renin-angiotensin system, in addition to being involved with volume control, now is believed to play a direct role in water balance as well. More specifically, angiotensin has now been shown to be capable of influencing two of the major determinants of water balance--ADH secretion and thirst.

B. Historical Perspective

The earliest suggestion that renin might stimulate ADH release was made by Whitney in 1954.[148] He injected renin into dogs and noted a prompt and persistent antidiuresis. Hypophysectomized animals exhibited a similar antidiuresis, but the duration was much briefer. He reasoned that ADH could not account for the initial decrease in urine flow because it was also seen in the hypophysectomized animals. The explanation offered was the intact dogs might have increased ADH release stimulated by renin and this could have sustained the later diuresis.

Thirteen years later Bonjour et al.[21] noted a "vasopressin-like" antidiuretic response to angiotensin infusion in rats. They observed a decrease in free water clearance, the effect of which was enhanced if rats were anesthetized with alcohol. The antidiuretic response to ADH itself is also potentiated by alcohol anesthesia. The authors suggested that angiotensin might have a direct effect on free water clearance, or that it might somehow enhance the endogenous secretion of ADH.

The explanation of the data by the latter hypothesis is a bit unclear since ethanol is a potent inhibitor of ADH secretion.[52,86] However, it is possible that the angiotensin stimulus might override the

inhibition and permit release of ADH. Then, if the kidney were sensitized to the effects of ADH by the ethanol anesthesia, the enhancement would be explained.

Bonjour and Malvin[20] were struck by the fact that angiotensin and ADH vary together under a number of circumstances, such as hemorrhage,[14,22,26,44,79,81,111,121,146] redistribution of blood volume,[122,33] and certain clinical conditions associated with secondary aldosteronism.[2,33]

One explanation for the covariation could have been that both hormones are released by identical or similar stimuli. The common denominator to all these situations is a change in extracellular volume which is a known stimulus for release of both hormones.[126,131,137,140]

There was, however, a more subtle possibility which could also explain the data--that renin or angiotensin might directly or indirectly influence the release of ADH. They made the first direct test of such a hypothesis. Using conscious dogs, Bonjour and Malvin[20] were able to show that intravenous infusions of either renin or angiotensin II were consistently associated with significant elevation of plasma ADH levels. Doses as small as 10 ng/kg-min consistently produced this effect. Moreover, there was a dose-response relationship in the sense that the magnitude of increase in mean arterial pressure which was also observed was correlated with the size increase in plasma ADH concentration.

This rise in blood pressure was not unexpected since angiotensin is a potent pressor agent, and this dose is about twice the threshold dose

for both dogs and man.[42,43,45] The point must be raised that perhaps the increase in ADH secretion was somehow related to the change in arterial pressure. This seems unlikely, since it is known that elevations in mean arterial pressure are usually accompanied by depression of vasopressin secretion.[127] If anything then, the rise in mean arterial pressure should have tended to attenuate the amount of ADH secreted rather than increase it.

With the dose of angiotensin II given, plasma levels of ADH approximately doubled. The criticism has been made that this is a very small change, but, in fact, this is an amount of ADH sufficient to reduce urine flow by about 60%--a not insignificant amount.[155]

The intravenous injections indicated that there is an ADH-releasing effect of angiotensin not caused by changes in plasma osmolality, sodium concentration, or probably mean arterial pressure. They do not rule out the possibility that the effect is due to redistribution of blood volume or flow caused by the vasoactive action of angiotensin, or some other unknown effect of this drug.

The next logical step was an attempt to localize the site of the response more discretely. Mouw, Bonjour, Malvin, and Vander[97] noted a given dose of angiotensin was a more potent stimulus for ADH release if given in the carotid artery rather than in the jugular vein. This was true even though there was no significant difference in the blood pressure response produced by either injection route. No other changes in parameters known to affect ADH secretion were observed.

The failure to see a difference in mean blood pressure when different injection routes are used is puzzling. Usually the pressor response to intra-arterial injections is smaller than to intravenous administration of the same dose.[25] Hodge[82] has advanced one theory to explain this common observation. He reports that up to 50% of an injected dose of angiotensin disappears in one pass through the cerebral circulation. This solitary result is open to question, and furthermore, the results of these experiments clearly contradict Hodge's contention. The absence of a difference in blood pressure response to different routes of administration of angiotensin cannot readily be explained, and the meaning of these data in relation to ADH release is also not clear. It is, however, consistent with the idea that the change in arterial pressure is not involved in the increase in ADH secretion, since MBP did not change but ADH did. More importantly, these data imply that the area sensitive to angiotensin seems to be in the head, or at least above the common carotid artery.

Claybaugh, Share, and Shimizu[31] questioned whether the release of ADH seen by Bonjour and Malvin might have been due to emotional reactions of the dogs to the experimental procedure, rather than to angiotensin administration. They were also concerned that ADH and angiotensin do not invariably change in a parallel fashion.[30, 129] Therefore, they elected to attempt to duplicate the results under different circumstances.

Angiotensin was infused intravenously into anesthetized, normally

hydrated dogs which had been subjected to rather extensive surgery. No increase in plasma ADH was observed after several doses and durations of infusions were tested. The authors concluded that under these circumstances ADH is not released by angiotensin.

There are several possible reasons for these results--the first of which was proposed by the authors. That is, plasma levels of ADH, even in control animals, were markedly elevated. It was possible that this could have obscured any response to angiotensin. However, there are other questions as well. The animals which received test infusions seemed to differ from control animals in several respects even before the angiotensin infusion was begun. Plasma ADH and osmolality, as well as mean blood pressure were almost always higher. It is not clear whether these differences are significant since the authors apparently did not do a between groups analysis of variance. Finally, at a dose of 10 ng/kg-min, there was no increase in mean blood pressure--something which has been consistently observed by other workers using the same dose. This, in addition to the dramatically elevated ADH levels, suggests the conditions of the experiment are quite different from other workers who have observed ADH release in response to angiotensin administration.

A second series of experiments was also done in which they attempted to lower ADH levels to a more normal range. It was reasoned that if the high levels of ADH in the first experiments obscured the response to ADH, it should be observable if ADH levels were in a more

physiologic range. By infusion of glucose solutions, ADH was lowered to about 1/5 of the blood levels seen in the first series of experiments. Then two doses of angiotensin were given. Again, no increase was seen in ADH release.

The possible explanations for this result are rather complicated, but perhaps may be summarized by stating that there are too many variables operant at one time. For example: (1) all the stimuli, including anesthesia and surgery which elevated ADH so markedly in the first group of experiments still existed; (2) a large inhibition to ADH release has been superimposed by expanding the blood volume by approximately half its total volume; and (3) the solution infused is hyposmotic, which should stimulate ADH release. Presumably, the secretory rate of a hormone is determined by integration of numerous stimuli, some more potent than others and present in variable amounts. Conceivably, any ADH response to angiotensin could, therefore, have been obscured by the interplay of these other inputs which affect vasopressin release.

Subsequently, these authors reported in abstract form that angiotensin infusions could potentiate the ADH release response seen with infusion of hypertonic saline.[130] This apparent relationship between angiotensin and sodium is of great interest and will be discussed more thoroughly in another section.

In addition to stimulating ADH release when injected intravascularly, renin and angiotensin have also been demonstrated to stimulate

drinking. This effect has been well documented and a considerable body of literature has evolved.[60-63] This finding will not be discussed in any detail. The point to be made is that here is another effect of angiotensin on water balance which is mediated at least in part by hypothalamic osmoreceptors. Of course, an alternative possibility is that release of vasopressin stimulates thirst receptors, but there is good evidence that such is not the case.[112]

All the previous studies have involved angiotensin given outside the blood brain barrier. That ADH is released following this administration is evidence that a central response eventually occurs. It is not yet clear, however, whether the response is a direct central effect of the hormone on nervous tissues, or whether it causes some other physiological change which then effects ADH release. Therefore, the next logical approach was to apply angiotensin to the central nervous system directly and observe if ADH were released. There is both indirect and direct evidence that ADH release is increased by this treatment. This suggests the receptor is centrally located, but it still does not rule out the possibility that the effect is due to some vascular action of angiotensin.

Severs and his colleagues[125] independently arrived at the same conclusion as Bonjour and Malvin,[20] i.e., that angiotensin can release ADH by a central mechanism. Although ADH was not measured, strong indirect evidence was provided that the hormone was, in fact, released. Moreover, this work was done on unanesthetized, unrestrained rats

which were acclimated to the experimental environment and which had not recently had surgery. All these factors may affect ADH release and are normally difficult to control.

In a first series of experiments[125] a dose of 500 ng in 5 microliters of artificial CSF was administered into the lateral ventricle via a previously placed cannula. This treatment elevated mean blood pressure by an average of 33 mmHg. When the experiment was repeated after the rats were either hypophysectomized or lesions placed in the supraoptic nuclei, the pressor response was reduced by 50%. The conclusion was that at least part of the hypertensive response to angiotensin was mediated by release of ADH. Indirectly, this suggests that a large outpouring of vasopressin must have occurred because it has pressor effects only at high doses.[115,134]

Further experiments from this laboratory have examined more fully the effects of intraventricular angiotensin on other aspects of fluid balance. It has been clearly shown that a single intraventricular dose of angiotensin into the lateral ventricles inhibits for up to 90 minutes the water diuresis normally induced by giving an intragastric water load. Sodium and potassium excretion, as well as drinking behavior were all significantly elevated.[124] These results suggested to the authors that intraventricular angiotensin may cause the appropriate receptors to "perceive" a state of hyperosmolality. The desired physiologic responses would then be what was experimentally observed--conservation of water by ADH secretion, excretion of osmotically active solute, and increased

water intake.

Prolonged intraventricular infusions of angiotensin were also done under the same experimental conditions.[110] In addition to the results described above, it was noted that volume retention and drinking persist even though the plasma has become hyposmotic. The usual response to volume expansion and hypotonicity is inhibition of ADH secretion and thirst. Perhaps the stimulus of angiotensin is prepotent or the large doses mask the responses that would be expected under more normal conditions.

Andersson and colleagues[4-6] have also noted a pronounced antidiuretic effect when angiotensin in very low concentration was infused into the third ventricle of conscious, hydrated goats. They likewise presumed the effect was due to ADH release. That this is so, was demonstrated one year later. When the experiment was repeated and the supraoptic nuclei electrolytically lesioned, the antidiuretic effect was abolished.[53]

Mouw, Bonjour, Malvin, and Vander[97] have been the only workers to put angiotensin into the cerebrospinal fluid and directly measure changes in plasma ADH produced. Cerebroventricular perfusions[107] into the lateral ventricle were done using concentrations from 0.3-443 ng/ml of artificial CSF (total amount infused 0.01-5ng/kg-min). All doses resulted in a rise in plasma ADH without conconcomitant alterations in the sodium concentration of plasma, osmolality of plasma, blood pressure, pulse pressure, or heart rate. There was also no

significant change in plasma renin activity. Moreover, the infused angiotensin disappeared rapidly from the perfusate at a rate which suggested that virtually unrestricted diffusion into brain tissue was occurring. This is not surprising, since for all practical purposes there is no CSF-brain barrier.[13,41] That is to say, virtually any molecule when placed into the cerebrospinal fluid diffuses quite readily into brain ECF. Such a situation would theoretically permit angiotensin to exert general or specific effects on some or all nerve cells in the area, on specific receptor cells or neurons, or on blood vessels--any or all of which might affect ADH release.

It is now clear that angiotensin given peripherally and centrally can have an effect upon water balance by stimulating ADH secretion and thirst. The evidence does strongly suggest that the effect is a central one, since very small doses produce marked results. It is the nature and locus of the angiotensin—ADH interaction that remains to be elucidated.

Precise information about the location of possible receptor location cannot be gained by intravascular or even intraventricular injections. A more direct approach is to apply angiotensin directly to suspected receptor areas. In the case of ADH, the most logical

receptors would be either in the supraoptic nuclei or in the neurohypophysis itself.

Nicoll & Barker[101] identified supraoptic neurosecretory cells which are believed to manufacture and secrete ADH via the neurohypophysis. Using a multiple barrel micropipette, they recorded changes in firing rates of neurons after local injection of angiotensin II by microiontophoresis.

Cortical neurons, which have no known function in water balance, were either unaffected or showed a decreased rate of action potentials. For the neurosecretory cells of the supraoptic nucleus the situation was quite different. Virtually all of these cells which responded to angiotensin (about 40%) did so by significantly increasing their firing rate. Release of ADH is generally believed to accompany depolarization of supraoptic neurons, and presumably increased secretion rate would accompany elevated firing rates.[51]

All this data is exciting and of great interest, but it would perhaps be well at this point to ponder a considerable problem. Traditionally, all of the stimuli for the release of ADH are believed to be mediated by way of the hypothalamic nuclei.[136] At first blush this seems no problem at all since angiotensin can apparently excite

neurosecretory neurons. The difficulty arises when one considers there is a barrier to the passage of very many molecules from the blood to the brain.[13] The crux of the issue is whether angiotensin passes from the blood into the environment of the supraoptic nuclei where it could have an effect. Considerable evidence at this point suggests that angiotensin does not pass the barrier either into the CSF or the extracellular fluid of brain, but the matter is still open to question. This point will receive more detailed consideration subsequently.

However, the fact remains that angiotensin does act on ADH release when given into the vascular tree. The question then becomes, where does it act, and how, and--perhaps most important--does it have any physiological meaning in the day to day regulation of ADH release? If the answer to the latter question is affirmative, then it is meaningful to try to answer the other questions.

Two additional lines of evidence suggest that angiotensin may be involved in the day to day regulation of water balance via ADH secretion, just as it is known to be critically involved in salt balance via stimulation aldosterone.

First of all, physiologically functional hormone regulatory

systems are typically possessed of some type of negative feedback loop. The angiotensin-ADH relationship seems to be no exception. If angiotensin stimulates ADH release, then it might be reasonable to hypothesize that ADH in turn might inhibit renin or angiotensin. Vander[138] first demonstrated this in anesthetized dogs and the results were confirmed and extended by Bunag, Page, and McCubbin.[27] This inhibition of renin secretion occurred whether plasma levels were normal or elevated by experimental manipulation. However, under these experimental conditions inhibition only occurred when rather high doses of ADH were given.

Tagawa, Vander, Bonjour, and Malvin[133] redesigned the experimental procedure and tested the hypothesis in unanesthetized, sodium-deprived dogs. In this case, as little as one microunit of ADH could be shown to inhibit renin release. This is well within the physiological range of ADH levels. Thus, there exists a rather typical negative feedback loop in this system such that: ↑ renin → ↑ angiotensin → ↑ ADH → ↓ renin → ↓ angiotensin. To complete the other side of the picture, Gutman and Benzakeen[75] have observed that very low levels of ADH are associated with elevated plasma renin levels.

Another recent and exciting discovery is the presence of a brain renin-angiotensin system which appears to be completely functional and quite independent of the peripheral system.[58,66-69] Thus, it is not surprising that some central nervous tissue is responsive to angiotensin. Of particular interest is the high degree of negative correlation between brain renin activity and cerebral water and electrolytes such as K^+, Ca^{++},

Na^+, and Mg^+ -- all of which are critical in regulating neural activity.[67]

This suggests that angiotensin, both centrally and peripherally, may play a basic and critical role in maintaining extracellular volume and tonicity of peripheral and central nervous system tissue. The inter-relationships between the central and peripheral renin-angiotensin systems will no doubt be a fertile field for future research. This is to say, it seems perfectly clear that the relationships between angiotensin and ADH may have very real physiological importance and thus merit further study.

This being so, a most basic question was asked in this work. What is the probable locus of the action of peripherally administered angiotensin on ADH secretion?

C. Proposed Studies

This work was designed to test the hypothesis that peripherally administered angiotensin releases ADH by a direct central effect at the level of the hypothalamus and/or the neurohypophysis. The former site is postulated because it is known to be the final common pathway for ADH release and the site of most stimuli for release.[136] Possibly there is some permeability of the blood-brain barrier at this level. This has been proposed,[5,53] but has never been clearly shown.

On the other hand, the target may be the neurohypophysis itself. It is from the storage granules in this area that ADH is ultimately released. More importantly, this area has no blood-brain barrier,[119] so angiotensin could freely contact the neuronal endings. The known

depolarizing action of angiotensin on some types of cells[50] may be sufficient to cause hormone release.

Three general types of experiments were done:

(1) In vivo injection of isotopic angiotensin to locate sites of increased uptake.

(2) In vitro incubation studies of isolated neural lobes with labelled angiotensin to determine the extent of uptake or binding. Attempts were then made to show this binding was specific.

(3) In vitro incubation of isolated neurohypophyses and intact hypothalamo-hypophysial systems with cold angiotensin and the measurement of changes in ADH secretion stimulated by such treatment.

MATERIALS AND METHODS

Part I: Equipment and Procedures

A. Animals

Sprague Dawley rats were obtained from Spartan Laboratories, Lansing, Michigan. Male rats weighing 250-350 g were used except where otherwise indicated. When used, anesthesia was accomplished by intraperitoneal injection of 40 mg/kg sodium pentobarbital. Supplementary doses of 5-10 mg were given as required.

B. Preparation of Tissues for Incubation

Two general types of studies were done using in vitro tissue preparations: (1) incubation of isolated anterior and posterior pituitary lobes and cerebral cortex with labelled angiotensins to study hormone uptake, and (2) incubation of isolated neural lobes and intact hypothalamo-hypophysial systems in unlabelled angiotensin to study its effects on release of antidiuretic hormone (ADH).

1. Isolated pituitary lobes and sections of cerebral cortex.- Unanesthetized rats were decapitated and the entire hypophysis quickly removed. The infundibular stalk was broken at the point where it penetrates the dura mater. The gland was placed in a petri dish containing warmed, aerated buffer. Using a 23 gauge needle, the pars neuralis was carefully dissected free from the pars distalis. The intermediate lobe remains attached to the posterior pituitary and cannot readily be removed.

When several glands (usually three) were to be used in a single experiment, the other glands were kept in warmed, aerated buffer until preparation of all tissue was completed. The total time required to isolate three glands in this manner is approximately five minutes.

When cerebral cortex was to be included in the incubations, fine scissors were used to cut a thin section from the surface of the neocortex. These samples were about 0.5 mm thick and weighed 3-5 mg. This is comparable in thickness and weight to the anterior lobe of the pituitary.

2. Intact hypothalamo-hypophysial systems. - The cerebellum and cerebrum were exposed by removing occipital and parietal bones. Coronal cuts were made just caudal to the cerebral hemispheres and through the spinal cord, and the cerebellum, pons, and medulla were removed. A small incision was made in the dura lining the floor of the skull about 3 mm caudal to the pituitary. The cut edge of the dura was grasped with jeweler's forceps and retracted upward until the pituitary, which lies beneath the dura, is visualized. A #11 blade was used to divide the cranial nerves and to cut the dura overlying the pituitary.

The entire brain and attached pituitary were reflected upward, and the remaining attachments of nerves, blood vessels, and olfactory lobes were severed with curved scissors. The entire preparation was placed in warmed, aerated buffer and the remaining fine dissection completed there.

Scissors were used to cut away all of the brain surrounding the

hypothalamus and pituitary. Sufficient hypothalamic tissue was left to insure inclusion of the supraoptic and paraventricular nuclei. Although this section is approximately 3 mm square, the nuclei of interest lie less than 0.1 mm below the surface of the section,[88] and therefore, most of their cells can be expected to receive sufficient oxygen by diffusion to remain viable. In later experiments, the third ventricle was opened at the top to further insure good circulation of buffer to the paraventricular nuclei.

Finally, the anterior lobe was dissected off as previously described. The preparation was then floated in buffer to ascertain that the fragile infundibular stalk was intact. This procedure was repeated at the end of each experiment. The time required to remove the brain and pituitary and place them in buffer is 5-7 minutes. An additional 4-5 minutes is required to complete the fine dissection, but during this time the tissue is being aerated.

C. Solutions

The standard incubation medium was Kreb's-Ringer bicarbonate buffer, pH 7.4, plus glucose 200 mg%. The composition of this buffer is: NaCl 120 mM, $NaHCO_3$ 26 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.8 mM, $MgSO_4$ 1.3 mM (osmolality = 299 mOs/kg $H_2O \pm 2.7$ (SD). Solutions were made fresh for each experiment from concentrated stock solutions which were stored in the cold. The cold buffer was saturated with 95% O_2 - 5% CO_2 gas and warmed to 37°C. All incubations were done at this temperature and the solution was continuously

gassed to prevent fluctuations in pH.

This buffer was used as the diluent for all solutions of angiotensin in which tissues were incubated. A high potassium solution used to test for ADH release was prepared by adding sufficient amounts of this reagent to make its final concentration 60 mM. No compensation was made for the increase in osmolality which would result from this method. Increased osmolality does not stimulate release of antidiuretic hormone from isolated neural lobes in vitro.[50]

Materials coming in contact with angiotensin were either plastic or had been coated with a 1% solution of Siliclad (Clay-Adams). This is done to retard the considerable tendency of angiotensin to adsorb to almost any surface. Micropipettes which had not been so treated were rinsed three times with angiotensin solutions before taking samples for counting. To retard breakdown of angiotensin, all solutions were kept cold until 15 minutes before use. They were then warmed and again equillibrated with gas before being added to the tissues for incubation.

D. Chemicals

1. Angiotensin I (Asp^1-$Ileu^5$), Calbiochem #176801, crystalline, A grade.

2. Angiotensin II (Hypertensin - Asp^1-val^5 angiotensin II amide), Ciba Pharmaceuticals. Stored in the cold as a concentrated stock solution. New solutions prepared monthly as recommended by the manufacturer. Intermittent purity checks were run using instant thin layer chromatography. No evidence of breakdown was ever seen.

3. I^{125} Angiotensin I (4-tyrosyl monoiodoangiotensin I), Schwartz/Mann #0750-53), 0.4 μCi/ng angiotensin I.

4. C^{14} Angiotensin II (5-L-Isoleucine-C^{14} (U) angiotensin II), New England Nuclear #NEC 570, 0.00025 μCi/ng angiotensin II.

5. I^{125} Angiotensin II (4-tyrosyl monoiodoangiotensin II)

   a. Cambridge Nuclear #CN 5357; Batch 239: 1.11 μCi/ng and Batch 247: 0.977 μCi/ng angiotensin II.

   b. Gift of T. E. Goodfriend, approximately 0.5 μCi/ng angiotensin II.

All labelled angiotensins were routinely checked for purity by paper chromatography on Whatman #1 paper using n-butanol:acetic acid: water (25:4:10) (J. T. Baker analyzed reagents). With the exception of angiotensin II from Cambridge Nuclear, all material showed a uniform peak of radioactivity with an R_f consistent for the material in question. The angiotensin II from Cambridge Nuclear showed a distinct tendency to break down. Secondary and tertiary peaks of radioactivity were present within two to three weeks after manufacture and these continued to increase steadily in size. This material was discarded when the area of the impurity peaks approached 10-12% of the total area. This preparation contains no scavenger resin to bind the impurities which are released (usually free I^{125}), as do some of the other labelled angiotensins, and this may account for the considerable difficulties encountered.

All labelled preparations were thawed upon arrival and diluted to a convenient volume according to the manufacturer's instructions.

Individual doses were then pipetted into plastic microcentrifuge tubes and stored at -20°C. This procedure obviated repeated freezing and thawing which disrupts the angiotensin molecule.[120]

E. General Experimental Procedures

Procedures common to several different types of experiments are described here to avoid repetition in experimental protocols.

1. Equilibration. - After removal, tissues were equilibrated at 37°C in aerated buffer for either 20 minutes (I^{125} uptake studies) or 30 minutes (ADH bioassay studies). The longer time was chosen for the latter studies because there is a spontaneous increased release of ADH from isolated neural lobes which occurs about 10 - 20 minutes after incubation has begun.[37] During this equilibration time the incubation medium was changed every 10 minutes.

2. Incubation. - For the equilibration period and for incubations with unlabelled material, a modified muscle bath was used. This is shown in Figure 1. A warm water jacket surrounds the incubation chamber proper and keeps the contents at 37 $\pm$ 1°C. A mixture of 95% O_2 - 5% CO_2 is continuously bubbled through the medium. The chamber is drained by a side outlet.

The pituitary glands are so light that they must be contained in some fashion during the incubation. If this is not done, the gas bubbles sweep the glands to the surface and they adhere to the side of the bath above the fluid level. Initially, siliconized glass wool was used to

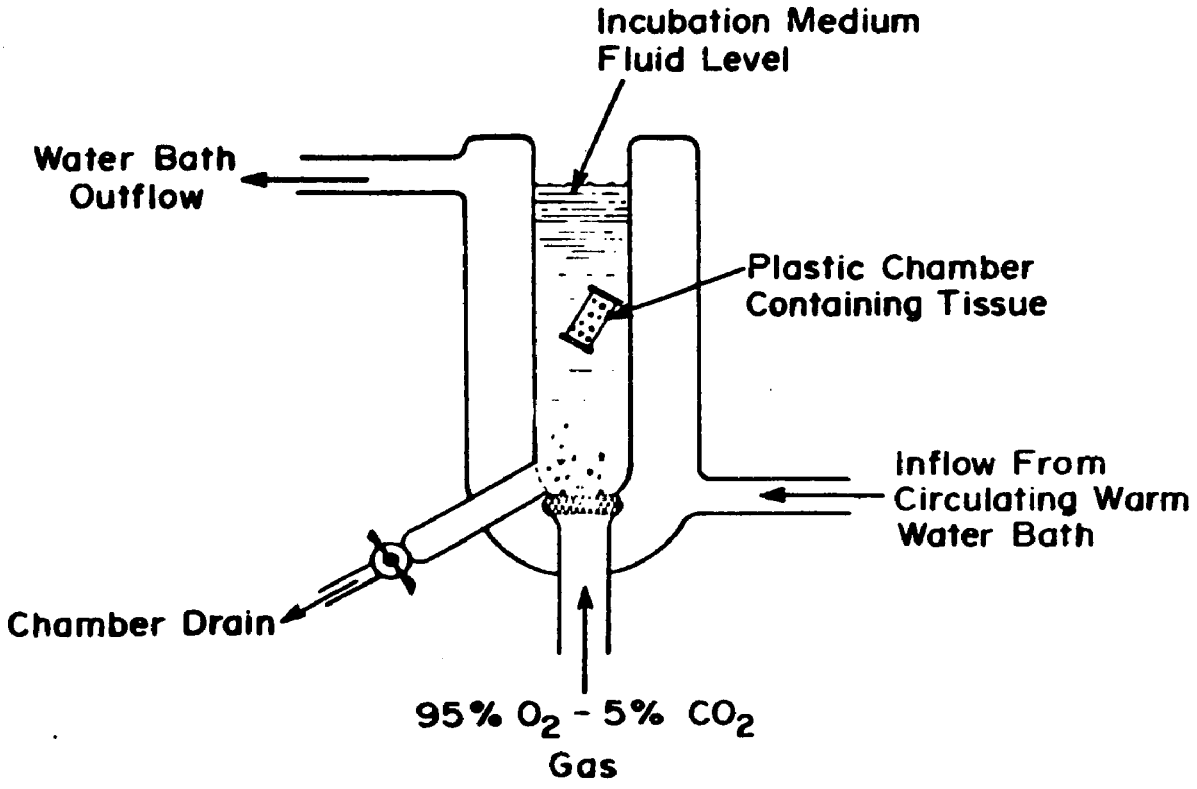


FIGURE 1. Schematic diagram of apparatus used for in vitro tissue incubations.

restrain the tissue, but this proved unsatisfactory if the glands had to be retrieved and weighed. A small plastic chamber drilled with many small holes was fashioned from a microcentrifuge tube. Circulation of incubation medium through the chamber was checked by the use of dyes. Mixing appeared to be thorough and rapid. The only difficulty encountered was that the tissue tended to adhere to the sides of the small chamber, and this would restrict diffusion to that side.

Unless the tissues were to be rinsed after incubation, both anterior and posterior lobes were placed in the same plastic chamber and incubated together. The volume of the incubation medium was many times the tissue volume. Thus, uptake by tissue did not effectively change the concentration of label in the medium.

In some experiments the tissues were incubated in a series of different solutions, one after the other. When this was the case, the solutions were pre-measured into test tubes and kept cold until 15 minutes prior to use. After being warmed and gassed, these were poured into the chamber, left the desired incubation period, and then recollected in the same tube for analysis. One or two drops of the solutions were usually lost by being left in the drain tube, but this amount was only a very small per cent of the total volume. To minimize solution contamination, the chamber and tissues were rinsed with two volumes of buffer in between the addition of each new solution.

When the glands were incubated with isotopes, the equilibration was done as usual in the muscle bath. Then the small chamber containing

the tissues was removed to a test tube containing the labelled hormone, and the incubation was completed there. The solution was kept warm by partially immersing the test tube in a warm water bath. Gassing was accomplished via a narrow glass capillary tube. Parafilm was used to cover the test tubes and the mouth of the muscle bath to retard evaporation during the incubation procedure.

3. Weighing.- Tissues weighing more than 25 mg were weighed to the nearest 0.1 mg on a Mettler H-5 balance. Pituitaries and small tissue samples were weighed to 0.01 mg on a Roller-Smith 25 mg precision balance.

Initially, tissues were weighed on tared strips of aluminum foil. The neural lobe was always weighed first because it was smallest and has a large surface area. Therefore, it had the most potential for weighing errors due to evaporative water loss. The solution to this concern was to place the tissues in small gelatin capsules to prevent loss of water vapor. These were then weighed as quickly as possible. Before counting, all samples were placed in identical containers to preserve uniform counting conditions.

4. Counting Procedures.-

a. Beta counting - C^{14} samples

After weighing, tissues were placed in liquid scintillation counting vials and digested for several hours at 55°C with Protosol 0.5 ml (New England Nuclear #NEF-935) and 0.1 ml of water. The vials were cooled and 16 ml of scintillation cocktail and 2 ml of absolute ethanol were added to each. The scintillation fluid contained:

(1) PPO (2-5 diphenyloxizole): 7 g. Research Products International, scintillation grade.

(2) POPOP (1,4-bis-2-(5 phenyloxazolyl)-benzene): 0.1 g. Packard Instrument Company, scintillation grade.

(3) Toluene: 1 liter. Mallinckrodt, analytical grade.

(4) Absolute ethanol: 200 ml.

All samples were counted in a refrigerated Nuclear Chicago 7200 series liquid scintillation counter.

Samples were counted to at least 10,000 counts (1 SD = 1% of total counts). Before any calculations were done, low activity samples were tested statistically using the Student t-test to determine if they were higher than background. Each time samples were counted, a quench curve was constructed by using a set of variably quenched C^{14} standards of known disintegrations/minute (Nuclear Chicago). Raw counts were converted to disintegrations per minute (DPM) by means of the channels ratio technique for determining quenching, as described in the Nuclear Chicago instruction manual. Background was subtracted before any calculations were done on the data.

b. Gamma counting - I^{125} samples

Tissues were weighed and placed in plastic microcentrifuge tubes. These small tubes were, in turn, placed inside larger tubes for the actual counting procedure. Samples of incubation medium (or plasma or cerebrospinal fluid) were similarly treated. This protocol

was adopted to prevent evaporation from the samples during the long periods of time during which they were counting. Counting was done in a Nuclear Chicago well-type, solid scintillation counter, model 4216.

Pilot studies were done to demonstrate that the physical state of the samples, i.e., whether they were liquid or solid, had no effect on counting efficiency. The same tissue samples were counted before and after liquification with Protosol. No differences in counting rates were observed. Since all samples are counted with similar efficiency, no quench correction need be made. Efficiency of counting was routinely monitored using an I^{129} standard (mock I^{125}; Nuclear Chicago #184671), and was found to range consistently between 60-63%.

All samples were counted to 40,000 counts except very low level samples. These were counted to 10,000 counts. As with the C^{14} samples, statistical testing was done to determine if low activity samples differed from background. Background was accounted for in all samples.

5. Calculations

a. T/P Ratio

This parameter was used in a series of in vivo experiments with C^{14} angiotensin II which will shortly be described. It is defined by:

$$T/P = \frac{\text{DPM/mg fresh tissue}}{\text{DPM/ml plasma}}$$

The point must be made that this calculation cannot, in fact, be

accurately made since the plasma level is not stable (nor for that matter, is the tissue level). This parameter must be recognized for what it represents -- an instantaneous relationship which holds at the time of sampling.

b. T/M Ratio

This measurement was made for in vitro incubation studies and is calculated as:

$$T/M = \frac{\text{CPM } (I^{125}) \text{ or DPM } (C^{14})/ \text{ ml tissue water}}{\text{CPM (or DPM)/ ml incubation medium}}$$

The water content of brain and pituitary was taken as 0.76 ml H_2O / g of fresh tissue.[150] Implicit in the value of T is label which is specifically bound to the plasma membrane and to intracellular material, dissolved in tissue water, and non-specifically bound (probably to cell proteins and lipids).

Part II: Experimental Protocols

A. In vivo Studies with I^{125} Angiotensin I

These studies were done to determine what tissues, if any, would accumulate significant amounts of angiotensin within a short period of time.

Rats of both sexes were anesthetized and the trachea (PE-205) and the carotid artery (PE-10) were cannulated with polyethylene tubing. The animal was placed in a stereotaxic device (David Kopf Instruments) and the muscles of the dorsal aspect of the neck and skull were reflected

aside to expose the cisterna magna.

At time zero, a bolus of monoiodoangiotensin I containing either 3 or 6 ng of angiotensin (1.25 or 2.5 μCi) was injected over a period of 15-20 seconds into the previously placed carotid catheter. A Hamilton microliter syringe was used and the total volume injected was either 125 or 250 microliters. The tubing was cleared with a small amount of normal saline and clamped.

Four minutes after completion of the injection, the cisterna magna was entered with a 21 gauge needle and a small sample of CSF was aspirated. As rapidly thereafter as possible, the animal was removed from the stereotoxic and the chest, heart, and great vessels were opened. The blood was collected into a petri dish containing 100 μl of EDTA (50 ng/ml). The entire animal was then dropped into liquid nitrogen and allowed to freeze solid. Average total elapsed time from beginning the injection to beginning of freezing was 5.5 min.

While the animal was freezing, blood samples were collected from the petri dish into heparinized capillary tubes. The volume of EDTA diluted the plasma by a small amount, and the estimated error introduced is about 3.5-5%. The capillary tubes were centrifuged and measured samples of plasma were withdrawn using Lang-Levy micropipettes. These were placed in plastic microcentrifuge tubes and frozen until counting. Measured CSF samples were taken from the aspirating syringe and similarly treated.

Within a day of the experiment, organs and tissues of interest

(still frozen) were removed, weighed, and counted. The ratio of tissue to plasma radioactivity (T/P) was calculated, and the various samples were compared on this basis.

The original series of experiments planned were designed to use C^{14} angiotensin II. However, this material is of very low specific activity (0.25 μCi/μg of angiotensin II), and an isotopically traceable dose was usually fatal when administered into the jugular vein. This effect was not observed if the same dose were given via the carotid artery. In fact, doses up to five times higher (up to 5 μg of angiotensin) were tolerated without overt difficulty. This is consistent with the observation that the pressor response to the same dose of angiotensin is usually greater when the intravenous route is used.[25] The reason for this is not known.

Although large doses of angiotensin could be tolerated if given by the carotid route, a dose of the size that would be required for counting purposes would be 10,000 times the threshold pressor dose in rats.[94] A few such experiments were attempted, but the results were grossly inconsistent, most likely because of the profound effects that such a dose would cause. Thus, the choice of an iodinated preparation was made. The problems inherent in the use of this iodinated material will be discussed subsequently.

The only iodinated preparation available at that time was angiotensin I. Although this decapeptide is usually regarded as being generally inactive itself, there are many tissues, such as lung,[100] plasma,[36]

intestine,[47] kidney,[3,46,71,100] hindlimb,[3] and even brain,[154] which rapidly convert angiotensin I to II by cleaving the COOH- terminal dipeptide. The assumption was made that this reaction would occur in vivo, although perhaps at a slower rate, and iodinated angiotensin II would be the result. The great virtue of this preparation is that one can use doses in the range that produced ADH secretion in the experiments by Bonjour and Malvin. Assuming the blood volume of the rat to be

$$\frac{57.5\text{-}67.0 \text{ ml}}{\text{kg body wt.}},^{34,55}$$

the dose administered would correspond to a blood level of 208-386 pg/ml blood or 12-24 ng/kg-min. This amount is perhaps 30 times the normal circulating level.[20]

Conversion of native angiotensin I to II is virtually complete in one pass through the circulation.[100] Once converted, the active octapeptide has a circulating half life of 1-2 minutes.[84] It was decided to sample the tissues and blood at four minutes after the completion of the injection. This time was chosen on the assumption that conversion of the iodinated peptide might reasonably occur at a slightly slower rate than that of the native hormone. Once conversion to angiotensin II is accomplished, if this preparation is active it should circulate and rapidly bind to its receptors. One can observe the onset of a pressor response within 15-20 seconds after intravascular injection of angiotensin II.[84]

Freezing was done to stop molecular movement and enzyme degradation as quickly as possible. Angiotensin is rapidly inactivated by

Variable		Normal Group (30)	CNSD Group (30)
Grade Placement			
Pre-School	N	3	7
	%	10.0%	23.3%
Kindergarten	N	5	7
	%	16.7%	23.3%
First	N	13	13
	%	43.3%	43.3%
Second	N	8	2
	%	26.7%	6.7%
Special Ed.	N	1	1
	%	3.3%	3.3%
Referral Source			
Local Medical Doctor	N	26	10
	%	86.7%	33.3%
Pediatric Cardiology	N	1	1
	%	3.3%	3.3%
Pediatric Neurology	N	0	6
	%	-	20.0%
Pediatric Rehabilitation	N	0	5
	%	-	16.7%
Speech and Hearing Clinic	N	2	0
	%	6.7%	-
Parental Request	N	1	3
	%	3.3%	10.0%
Pediatric Clinic	N	0	4
	%	-	13.3%
Orthopedic Clinic	N	0	1
	%	-	3.3%

Table 3 shows the Normal Group to be on the average about 5 months older than the CNSD group. The average achievement levels of the Normal Group are at the beginning first grade levels. The average achievement levels in spelling, arithmetic, and reading in the CNSD

group are at the latter half of the kindergarten level.

The mean Binet Mental Age for the Normal Group is 75.13 months, which is 6.2 months higher than the Binet Mental Age for the CNSD Group (68.93 months). The Mean Binet IQ scores are very similar (Normal Group - 92.63 and CNSD Group - 91.57). There are considerable differences in AALIPS IQ scores and Binet Mental Age minus AALIPS Mental Age scores which will be discussed in the Analysis of the Results chapter.

One can see from Table 4 that the two groups are roughly comparable in terms of socio-economic status. The grade placement ranges from pre-school through second grade, with one child in each group being in a Special Education program for the educable mentally retarded. There were eight different referral sources. Twenty-six of the 30 normal children were referred by their local physicians, while only 10 of the CNSD Group were referred by their local physicians. The other 20 in the CNSD Group were referred by various departments within the medical center or by parental request.

Measures

The achievement levels in spelling, arithmetic and reading were assessed by the Wide Range Achievement Test, which is a short standardized individually administered achievement measure which yields raw scores and grade equivalence. Socio-economic status was roughly assessed by the child's father's occupation (the mother's occupation was used when there was no father in the home) in terms of the occupation being unskilled, semi-skilled or professional in nature. Grade placement was the child's grade placement in school at the time

the psychologicals were administered.

Design and Analyses

An analysis of variance was run between the two main groups (Normal and CNSD) using all quantitative variables such as chronological age, achievement scores, Binet Mental Age, Binet I.Q., AALIPS Mental Age, AALIPS I.Q., and Binet Mental Age minus AALIPS Mental Age. Particular attention was given to the difference between the two groups with regard to Binet Mental Age minus AALIPS Mental Age, which is a discrepancy score pointing up the phenomena with which the first major hypothesis deals.

An analysis of variance was also run on the two groups, the groups being sub-divided into male and female to determine if there were differences between the sexes on all the above mentioned quantitative variables. An analysis of variance was run using sex alone (not sex within each group) as the stratifying variable and looking at Binet Mental Age minus AALIPS Mental Age. Another analysis of variance was run between the three socio-economic levels to see if the groups differed in their Binet minus AALIPS scores.

Within the Normal and CNSD Groups the children were further divided into low, average and high I.Q. groups based on their Binet I.Q. scores. All children below a Binet I.Q. of 86 were considered low I.Q., children with a Binet I.Q. of 86 to 110 classified as average, and children above a Binet I.Q. of 110 were considered above average. An analysis of variance using the above mentioned quantitative variables of chronological age, Binet Mental Age, spelling achievement, arithmetic achievement, reading achievement, AALIPS I.Q., and Binet Mental

Age minus AALIPS Mental Age was run on the low, average and high I.Q. groups.

Correlation matrices were computed for the nine above mentioned variables for the two groups, and scatter grams on significant and relevant correlations were run.

In order to determine which tests items discriminate between the Normal and CNSD Groups, a chi square was run on each item on the Binet and the AALIPS, and the ones showing a significant relationship between groups in responses correct are reported.

Testable Hypotheses

Research Hypothesis I:
Children with Central Nervous System Dysfunctioning will show significantly lower AALIPS Mental Age scores as compared to their Stanford Binet Mental Age scores than will children diagnosed as Normal with regard to central nervous system functioning.

Null Hypothesis I:
There will be no difference between children diagnosed as having Central Nervous System Dysfunctioning and Normal children in their Binet minus AALIPS Mental Age scores.

Corollary Research Hypothesis A:
Regardless of the sex of the children, children with CNSD will show lower AALIPS Mental Age scores as compared to their Binet Mental Age scores than will children diagnosed as Normal.

Corollary Null Hypothesis A:
There will be no difference in the Binet Mental Age scores minus AALIPS Mental Age scores for boys and girls.

Corollary Research Hypothesis B:
Regardless of the socio-economic status of the children, children with CNSD will show lower AALIPS Mental Age scores as compared to their Binet Mental Age scores than will children diagnosed as Normal.

Corollary Null Hypothesis B:
There will be no difference in Binet Mental Age scores minus AALIPS Mental Age scores for children of low, average, and high socio-economic status.

Corollary Research Hypothesis C:
Regardless of whether the children have low, average, or high I.Q. scores as measured on the Binet, the phenomenon of children with CNSD scoring lower AALIPS Mental Age scores as compared to their Binet Mental Age scores than do Normal children will be observed.

Corollary Null Hypothesis C:
There will be no difference between low, average, and high I.Q. children in their Binet Mental Age scores minus AALIPS Mental Age scores.

Research Hypothesis II:

Children diagnosed as Normal with regard to central nervous system functioning and children with Central Nervous System Dysfunctioning will show differential response patterns on the AALIPS.

Null Hypothesis II:

There will be no differences between the Normal children and children diagnosed as having Central Nervous System Dysfunctioning in their response patterns on the AALIPS.

Summary

The two main statistical techniques used in this study are an analysis of variance between the two groups (Normal and CNSD),on all quantitative variables, and the chi square procedure to investiage the items on the AALIPS and the Binet which discriminate between the two groups.

The two main groups are subdivided into sex, socio-economic status, and low, average, and high I.Q. groups in attempts to further investigate the nature of the variance by the use of an analysis of variance.

Scatter plots are presented to further clarify how the two tests

correlate with each other, achievement levels and chronological age.

A thorough analysis of the results of the study is presented in the following chapter.

CHAPTER V: RESULTS

In this chapter the results of the study are presented. The first major hypothesis and the three corollary hypotheses, which concerns the discrepancy between the Stanford Binet Intelligence Scale[1] MA scores as compared to the Arthur Adaptation of the Leiter International Performance Scale[2] MA scores for the Normal children and children in the Central Nervous System Dysfunctioning[3] group, is investigated by use of the analysis of variance statistical technique.

The first central hypothesis is the following:

> Children with Central Nervous System Dysfunctioning will show significantly lower Arthur Adaptation of the Leiter International Performance Scale Mental Age scores as compared to their Stanford Binet Mental Age scores than will children diagnosed as normal with regard to Central Nervous System functioning.

In order to test this main hypothesis and to look at how the two groups differ in respect to all quantitative variables, an analysis of variance on all quantiative variables for the two groups was calculated. The results of these analyses are shown in Table 5. It can be seen that the two groups do not differ significantly in their average chronological ages, although the Normal Group is on the average 4.93 months older than the CNSD Group.

[1] Also referred to as Binet

[2] Also referred to as AALIPS

[3] Also referred to as CNSD

Table 5

Means and Standard Deviations On Chronological Age, Spelling Achievement, Arithmetic Achievement, Reading Achievement, Binet Mental Age, Binet I.Q., AALIPS Mental Age, And AALIPS I.Q., For Normal And Central Nervous System Dysfunctioning Children

Variable	Statistic	Normal Group	CNSD Group	F-Ratio
Chronological Age (in months)	Mean Score	80.70	75.77	3.69
	S.D.	8.84	10.93	
Spelling Achievement (Raw Score and Grade Equivalence)	Mean Score	19.07 (gr.1.1)	14.10 (Kg. 6)	10.79**
	S.D.	4.90	6.67	
Arithmetic Achievement (Raw Score and Grade Equivalence)	Mean Score	16.40 (gr.1.0)	13.43 (Kg. 6)	6.50**
	S.D.	5.00	3.95	
Reading Achievement (Raw Score and Grade Equivalence)	Mean Score	24.00 (gr.1.0)	20.37 (Kg. 8)	.07
	S.D.	6.96	8.27	
Binet Mental Age (in months)	Mean Score	75.13	68.93	6.05*
	S.D.	10.56	8.90	
Binet I.Q.	Mean Score	92.63	91.57	.80
	S.D.	14.22	15.02	

* Significant at the .05 level

** Significant at the .01 level

Variable	Statistic	Normal Group	CNSD Group	F-Ratio
AALIPS Mental Age (in months)	Mean Score	76.00	59.07	37.82**
	S.D.	10.32	10.99	
AALIPS I.Q.	Mean Score	94.73	78.63	22.53**
	S.D.	12.26	13.96	

* Significant at the .05 level

** Significant at the .01 level

The two groups do show significantly different mean spelling achievement and arithmetic achievement scores as measured on the Wide Range Achievement Test, with the Normal Group scoring significantly higher achievement scores than the CNSD Group. There is no significant difference in reading achievement levels between the two groups.

An examination of the mean Binet Mental Age scores shows the Normal Group has significantly higher average mental age scores as compared to the CNSD Group, significant at the .05 level. This suggests that the two groups are not comparable with regard to Binet M.A. However, the author does not see this as a serious problem to the study, as a closer look at the mean Binet Mental Ages shows the Normal Group to score an average M.A. of only 6.2 months higher than the CNSD Group. Further, as mentioned above, the Normal Group has also a slightly higher chronological age (4.93 months) than the CNSD Group. Therefore, the Normal Group children are slightly older and have somewhat higher Binet Mental Ages. The two groups, however, are very similar with regard to Binet I.Q. scores, the Normal Group having an average Binet I.Q. of 92.63 and the CNSD Group scoring an average Binet I.Q. of 91.57.

Of central importance to the present study are the mean AALIPS M.A. scores of the two groups. The two groups show a highly significant difference in the average AALIPS Mental Age scores, the Normal Group scoring a mean M.A. of 76.00 months (6 years 4 months) and the CNSD Group scoring a mean AALIPS M.A. of only 59.07 months (4 years 11 months). The difference is significant at the .0000

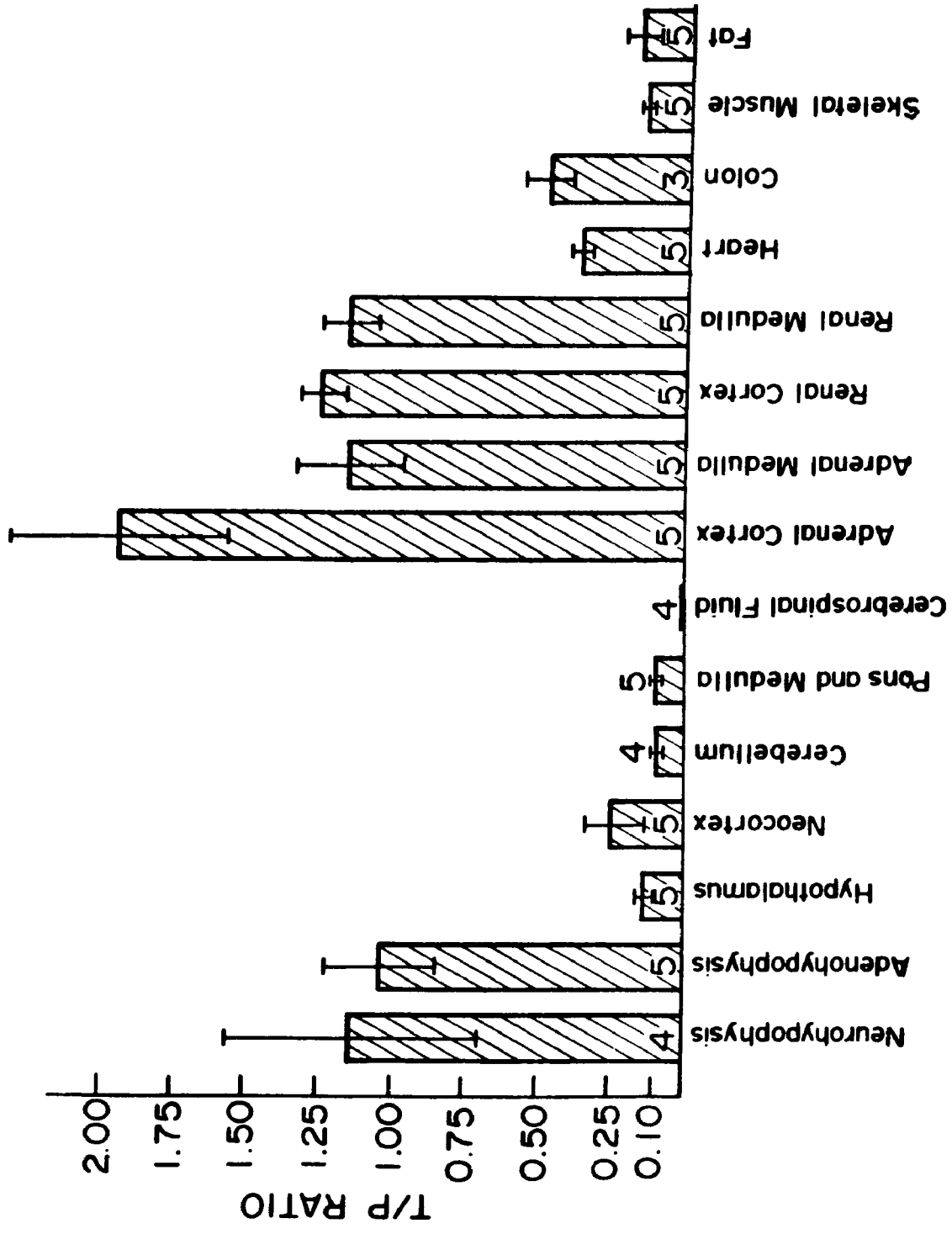


FIGURE 2: Tissue to plasma ratios for various tissues four minutes after injection of 3 or 6 ng of I^{125} monoiodoangiotensin I (1.25 or 2.50 μCi). Values are means ± SEM. The numbers at the bars indicate the number of experiments.

T/P ratios are more than double those of intestine and heart, which are also targets of angiotensin,[84] also strengthens this view.

Ratios for liver, lung, and spleen are not illustrated, but these are also intermediate in value, ranging from 0.55 to 0.80. These tissues are not known target tissues of angiotensin. However, all share the characteristic of being extremely vascular. For this reason it is difficult to know if the activity represents binding, or reflects the relatively larger volume of blood which would be contained within these organs. A further consideration is that some of the activity in liver may represent metabolism. That of lung might be concerned with activity of converting enzyme which is known to be present in large amounts there.[100]

The question may reasonably be asked whether there is more activity in the gland than could accumulate if diffusion equilibrium were to obtain between plasma and total pituitary water. These data do not support that finding. Although the T/P ratio for the gland is approximately 30% higher than would be expected had diffusion equilibrium occurred between plasma and extracellular and intracellular fluid, that increase is not statistically greater. In this regard, the point must be made that the sample is small and the standard error rather large. Thus, it would take quite large differences to attain significance. Possibly more experiments would have clarified this matter, but this is in the realm of conjecture. There are other points more firmly based in fact or theory which need to be made.

First, it is unlikely that a large, polar, lipid-insoluble molecule, such as

angiotensin, would rapidly diffuse through the cell membrane. Secondly, apparent accumulation of a substance against a concentration gradient is evidence for active transport, but not a sine qua non for specific binding to occur. Tissues such as colon and heart, which are known to be affected by angiotensin in vivo,[84] have T/P ratios which are lower than 0.75-0.80. This is the approximate value which would be expected if the hormone concentration were just equillibrated between total tissue water and plasma. These values for colon and heart are considerably less than pituitary values. On the other side of the coin, skeletal muscle and fat, where angiotensin has no known effect, have quite low T/P ratios. These are of a magnitude (0.2) which might be expected if angiotensin were equillibrated with plasma concentrations only (or primarily) in the extracellular fluid of the tissue.

Thus, there would appear to be some sort of direct correlation between the T/P ratio of a tissue and its responsiveness to angiotensin. Although it is not yet proven, the relatively high degree of accumulation of label by pituitary tissue suggests this hormone may have some action there. However, one must be extremely cautious in drawing this conclusion. This is so because of the impossibility of calculating an accurate T/P ratio.

Although concentration of a substance by a tissue is not necessary per se to show that specific binding exists, the fact remains that it would be a nice piece of evidence to have. It is known that target tissues often accumulate hormones to levels several times those of

plasma. If pituitary does, in fact, usually accumulate angiotensin *in vivo*, one possible reason why this was not seen more clearly in these studies is because the angiotensin used was labelled with iodine which is not a normal constituent of the molecule.

Even before this point is discussed, a still more basic issue must be considered. A very valid question is whether the addition of a bulky iodine molecule to this small polypeptide might alter the conformation sufficiently to render it questionable for biological studies. Before proceeding, it must be established that this iodinated preparation has at least some of the biological activity of the native hormone.

Lin et al.,[91] using highly purified monoiodoangiotensin II tested its effects on three different standard bioassay systems. Results showed that the iodinated preparation had from 25-80% of the effect as the same dose of native hormone, depending on the assay system used. They concluded that this preparation may be used as a qualitative indicator of the behavior of native angiotensin. On the other hand, diiodoangiotensin had little if any biological activity and was not adjudged suitable for tracer studies.

The material used in this work was checked for purity before each experiment by paper chromatography in n-butanol: acetic acid: and water (25:4:10). This procedure invariably revealed a narrow uniform peak of radioactivity with an R_F consistent with angiotensin. There were no minor peaks which could have represented diiodoangiotensin or free iodine.

Since the biological activity of the iodinated preparation is less than the native hormone, it would not be unreasonable to expect that T/P ratios might be lower as well--especially for very sensitive target tissues. When tritiated angiotensin has been used in similar studies by other workers, T/P ratios as high as 3 to 4 have been reported for adrenals and kidney.[104] Values found in my studies are about 1/3-1/2 as high, depending on the organ in question.

With respect to the pituitary, Epstein[104] has shown that two minutes after an intravenous injection of tritiated angiotensin II the entire pituitary has a T/P ratio of approximately 3.88. He made no attempt to examine the lobes separately. In this study the data for neurophypophysis and adenohypophysis separately are again about 1/3 the values seen when the more physiological H^3 angiotensin is used.

These data suggest that the neurohypophysis may well be capable of accumulating angiotensin against a concentration gradient. The most plausible reason it could not be demonstrated more clearly in this study is that the material used was of lower biological activity. These studies indicate that introduction of an iodine molecule into angiotensin reduces the uptake in known target tissues significantly. It may well do the same in the neurohypophysis, but this remains to be demonstrated.

For completeness, a final point merits some discussion. It has not been demonstrated that the radioactivity in the tissues represents intact hormone rather than metabolites. However, the very brief time lapse between injection and freezing of the samples make this unlikely.

There is also evidence that iodinated angiotensin may have a longer half life than native angiotensin. Labelled molecules with the same electrophoretic mobility as standards of monoiodoangiotensin have been recovered from blood several hours after injection.[152] Furthermore, Kahirallah[85] has found that labelled metabolites of tritiated angiotensin tend to accumulate in brain tissue rather quickly. Within 30 minutes of injection, radioactivity in brain was seven times higher than plasma, and this activity had very different electrophoretic mobility from the originally administered angiotensin. The observation that T/P ratios found for brain in these experiments are 30 to 50 times lower than those of Kahirallah suggests that there are very few metabolites present.

If not metabolites, the radioactivity could be free iodide which has been enzymatically cleaved from the molecule. That there may be some deiodination is suggested by the observation that T/P ratios for 2 thyroid glands which were tested were 2.97 and 4.12. However, this is much below the known T/P ratios of 30/1[65] that have been measured using labelled iodide. It suggests that little of the circulating radioactivity present is likely to be free iodide. Furthermore, the neurohypophysis binds iodide only to a minor degree,[65] and kidney and adrenals do not accumulate it to any significant degree either. It seems likely that at least the majority of the radioactivity represents angiotensin.

An unexpected finding was that isotope levels in the anterior lobe were not significantly different from the neurohypophysis. The reason for this finding is not clear, but several possibilities may be offered:

1. Angiotensin could stimulate ACTH release from this gland. When the hormone is administered intraventricularly plasma corticosterone levels increase significantly within 30 minutes and corticosterone content of adrenal tissue is elevated after one hour. These changes are not observed in hypophysectomized animals.[39]

2. In rats, the adenohypophysis, and ACTH in particular, seem to play a more important role in aldosterone secretion than it does in many other mammals.[149] One of the primary actions of angiotensin is well known to be regulation of aldosterone secretion.

3. The adenohypophysis is also the site of negative feedback inhibition of TSH secretion by the iodine-containing thyroid hormones. After binding, triiodothyonine and tetraiodothyonine are often inactivated by deiodination.[65] Perhaps deiodinoses in the pars distalis are particularly active and some of the radioactivity represents iodine rather than angiotensin.

4. This may be non-specific binding, but the high T/P ratio mitigates against this.

Thus, there are several possible explanations for the observed data, but there is no evidence which permits any conclusion as to which, if any, is correct. The first two possibilities seem most plausible.

Aside from the posterior pituitary itself, another logical central target for angiotensin would be the ADH-manufacturing neurons of the hypothalamic supraoptic nuclei or their osmoreceptor cells (which may or may not be anatomically different entities). If the hormone were to

work at this locus, a higher uptake in hypothalamus relative to the rest of brain would be predicted. This was not found. Tissue to plasma ratios are not significantly higher than those of neocortex, cerebellum, or brainstem (pons and medulla).

These data are in close agreement with those of Epstein.[104] He gave H^3 angiotensin intravenously and two minutes later sampled regions of hypothalamus known to be most sensitive to the dipsogenic action of angiotensin. Uptake in this area was compared to the rest of hypothalamus and also to thalamus, neocortex, hypocampus, and the floor of the fourth ventricle. He found no differences between any of these regions.

These findings are in accord with the well known fact that a great majority of substances are effectively excluded from contact with brain by a blood-brain barrier.[13] It has been postulated that the supraoptic nuclei may be outside this barrier, but there is no good evidence which permits a conclusion to be made.

The failure to demonstrate preferential uptake in hypothalamus does not support the view that the central action of angiotensin is at the hypothalamic level. On the other hand, the possibility still cannot be completely dismissed. The clear fact remains that when angiotensin is put into cerebral spinal fluid it does stimulate ADH release, thirst and elevation in blood pressure. That is, it can have an effect on neural tissue. At the crux of the issue is whether peripherally administered angiotensin has access to these sensitive tissues <u>in vivo</u>.

In addition to entering the neurophypophysis, angiotensin could

also enter the CNS at some site which was not studied or which could not be detected by the techniques employed. With respect to the latter position, the anatomic area of the supraoptic and paraventricular nuclei is very small. For example, if a coronal section of brain is cut at the level where the area of the supraoptic and paraventricular nuclei is maximal, the combined area for these regions is only about 0.0475% of the total area. The sample of hypothalamus used in this work was quite large and really included part of the thalamus and other surrounding tissues. If the receptor areas were very small in relation to the total mass of tissue sampled, a high T/P ratio there might be obscured when averaged in with a low ratio from a much larger weight of tissue.

A less theoretical argument that angiotensin may selectively penetrate the barrier comes from the realization that there is more activity in brain samples than can be accounted for by what would have been in blood contained in tissue at sampling time. Calculations were made using blood volume estimates from two sources,[34, 55] and there is 5-10 times more activity present than would be expected from blood content alone. This excess could represent metabolites which are known to accumulate in brain over time.[85] It could also mean that some angiotensin may leave blood and enter extracellular fluid where it might interact with receptors on cell membranes.

Volicer and Lowe have contributed data which is in accord with the latter view.[144] They measured brain plasma space, inulin space, and volume of distribution of angiotensin. The angiotensin space was

greater than plasma volume, but less than extracellular volume as measured by inulin. Their interpretation was that since angiotensin space was less than extracellular space, the hormone penetrated brain only in some areas. Because their brain samples were homogenates of entire brain (including CSF), it is not possible to gain any clue as to where such regions might be. They also did not demonstrate that the activity was angiotensin and not metabolites.

Finally, there is the possibility that angiotensin could enter cerebrospinal fluid at some location and exert an effect on structures close to the surface of the third ventricle, such as the supraoptic nuclei. This is a particularly appealing possibility since intraventricularly administered angiotensin has many demonstrated effects.

Unfortunately, there are no data to support this position. In this work, levels in cerebrospinal fluid were not different from background. Mouw et al.[97] were also unable to detect any renin or angiotensin in cerebrospinal fluid. Ganten et al.[66] have confirmed this finding even in dogs which had very high plasma renin activity induced by various experimental manipulations.

Perhaps the only comment left at this point is that all CSF samples were taken from the cisterna magna fluid rather than directly from ventricular fluid. CSF is modified as it passes through the ventricular system. It is possible that the hormone could enter somewhere in the ventricular system and be removed and/or metabolized before it ever circulates to the cisterna magna fluid, but this is unlikely. Mouw

et al.[97] observed that substantial amounts of angiotensin disappeared from CSF perfusate as it passed through the ventricular system. However, some was still detectable, even when quite small doses were given. Furthermore, Ganten et al.,[66] gave very large doses of angiotensin intravascularly (200 ng/kg-min) for an hour at a time. No angiotensin was detected in CSF, using an assay sensitive to a concentration of 1 picogram/ml. Therefore, it seems unlikely that angiotensin enters CSF and has its effect via that route.

What, then, may be concluded at this juncture about the central target of angiotensin with regard to ADH release? The data so far indicate that one locus of action may well be the neurohypophysis itself. The balance of this work will direct itself to further testing this hypothesis by two general types of experiments: (1) binding studies designed to see if the uptake of hormone by this gland represents specific binding, and (2) attempts to correlate the binding activity with the release of ADH from isolated neural lobes and intact hypothalamo-hypophysial systems.

Part II: In vitro Binding Studies

In the in vivo studies, there was observed preferential uptake of label presumed to represent angiotensin in several organs, including both lobes of pituitary. However, the major concern of such studies is that accumulation of label may represent non-specific binding, e.g., adsorption of the hormone to cell protein or lipid. Alternatively, the binding might be specific, but reflect some process such as hormone transport or metabolic degradation. Destruction of angiotensin is rapidly and efficiently carried out by a great number of tissues.[25,84]

The fact that labelled angiotensin tended to accumulate in tissues in vivo in proportion to its known physiological activity suggests that the uptake by pituitary may also indicate some hormone-receptor interaction. However, more evidence is required before making such an assertion.

Thus, it was deemed necessary to attempt a more detailed characterization of the nature of the apparent binding of angiotensin by pituitary. The unexpected uptake of label by the anterior lobe was similarly examined. The basic question to be addressed was whether or not the binding was specific.

A. Uptake of C^{14} Angiotensin II

Figure 3 summarizes the result of incubating various tissues with 100 ng/ml (.025 μCu/ml) of angiotensin II for various lengths of time. For both lobes of pituitary the ratio T/M increases to a maximum at 30 minutes, and then for posterior pituitary begins to decline ($p=.03$). The apparent decrease for adenohypophysis at 40 minutes is not significant.

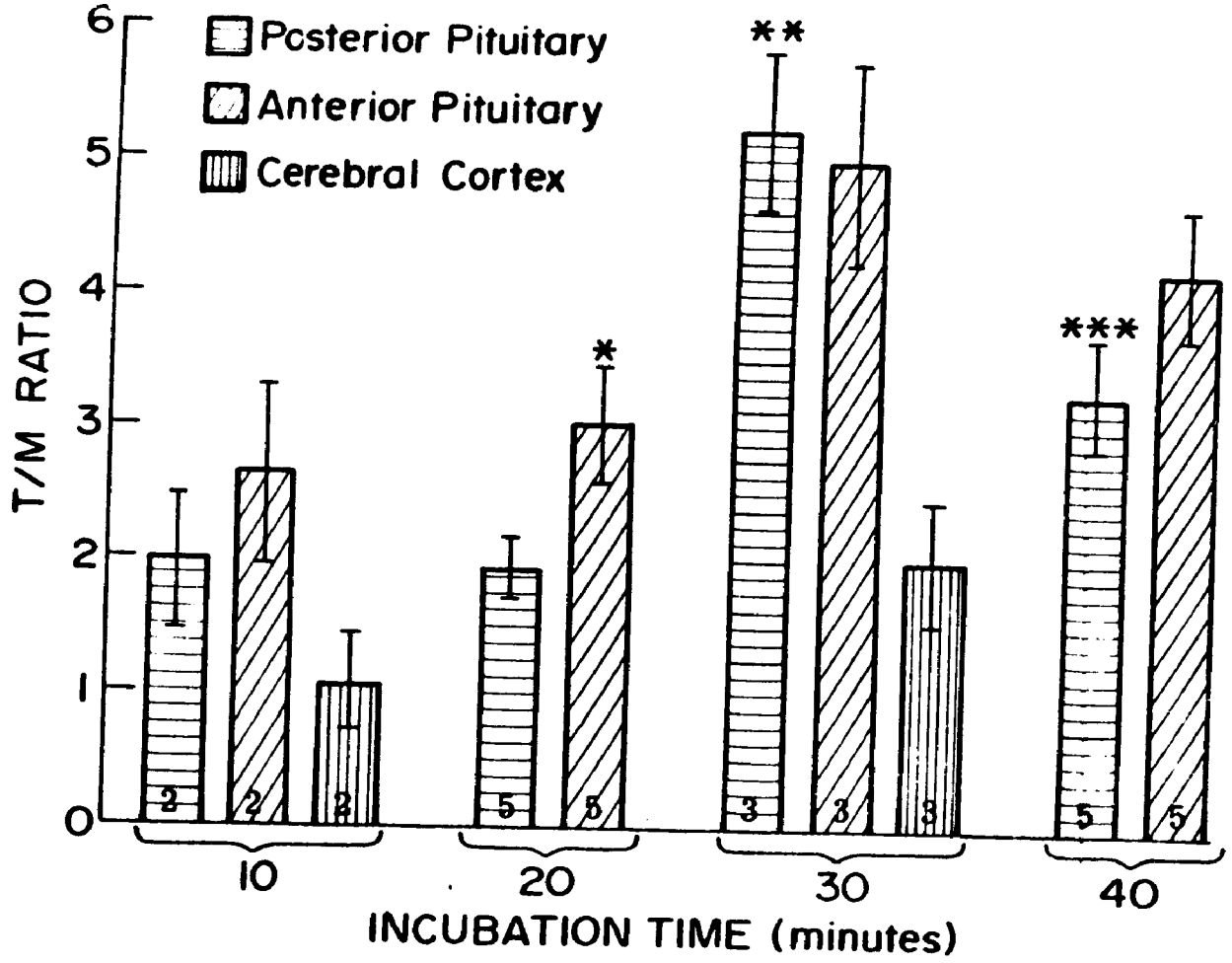


FIGURE 3. Effect of incubating different tissues with 100 ng/ml of C^{14} angiotensin II for varying lengths of time. Values are means ± SEM. Numbers at the base of bars indicate the number of experiments.

* p=.05 anterior pituitary (AP) > posterior pituitary (PP)

** p=.01 AP and PP > cerebral cortex

*** p=.03 PP (30 min) > PP (40 min)

There is no difference between the amount of activity in either lobe of the pituitary at any time except for the 20 minute period ($p=.05$). Why there should be a difference only at this time is not known. Accumulation at 30 minutes in cerebral cortex is significantly less than either lobe of pituitary ($p=.01$).

Cerebral cortex, henceforth referred to as brain, was incubated for comparison to neurohypophysis for two reasons. First, both contain neural elements and thus, should have some similarities in biochemical composition. It was reasoned if the binding were primarily non-specific and due to adsorption to some chemical component of tissue, then brain uptake might tend to resemble neurohypophysis more closely than adenohypophysis. The latter is chemically and histologically very different from the posterior lobe. Secondly, cerebral cortex was not then known to be affected by angiotensin. If there were differences observed between brain and pituitary, this would be consistent with the concept that the latter possessed angiotensin receptors. Brain proved to be an unfortunate choice since it has since been shown that the CNS has its own renin-angiotensin system.[58,66-69] Unless brain and peripheral angiotensin are very different, there is reason to suppose that brain will also bind this hormone.

The most obvious point to be made from the data is that both lobes of the pituitary accumulate label to a significant degree. After 30 minutes of incubation, there is more than six times the amount of activity in tissue than could be accounted for by diffusion (assuming tissue to be

76% water by weight). This is evidence for binding of some type. The question is whether or not it is binding related to some physiological phenomenon.

The peak in uptake at 20 to 30 minutes has been seen by other workers studying specific binding of angiotensin to known target tissues.[92] Using adrenal cortex and uterus, Lin and Goodfriend have separated out the fraction of the total binding which is thought to represent hormone-receptor interaction. They observed that this specific binding reaches a maximum in 20 to 30 minutes and then slowly declines (Goodfriend, personal communication). The latter effect is explained by presuming that the receptors slowly become non-functional.

Because of the overt similarities between uptake curves for known targets and those of pituitary, it is tempting to speculate that the latter tissue also shows evidence of specific binding. However, the matter deserves further analysis.

One of the general characteristics of hormonal receptors is that they bind the hormone very quickly. This should be especially true for angiotensin, since one can observe an effect beginning within 30-45 seconds after intravenous injection of a pressor dose.[84] It is not necessarily true that the time course of the ADH-releasing effect of angiotensin is also that swift, but on the other hand, there is no good reason to predict that it should be significantly slower either. Recall that Epstein[104] observed concentration of label in pituitary up to four times plasma levels within two minutes of injection. The initial slopes

of the uptake curves from Lin and Goodfriend's[92] work were also very steep, a fact which indicates rapid binding. In their experiments uptake reached about 80% of maximum in less than ten minutes. The corresponding figure after ten minutes of incubation in these experiments is 40%.

There are at least two possible reasons for the difference in initial uptake rates. First, the tissue may have been in an unstable condition during the first few minutes of incubation. These tissues were only equilibrated for approximately ten minutes before being placed in isotope. Perhaps even more important is the fact that in these experiments the angiotensin must diffuse over relatively "large" distances (0.1-0.5 mm) to contact the postulated receptors. This situation does not exist in vivo or in the situation where angiotensin is incubated with cellular particles. Much of the work of Goodfriend and colleagues which has been cited was done on preparations of this type.[73,74,92]

Another matter which should be considered is that pituitary uptake curves represent total binding, both specific and non-specific. The data of Lin and Goodfriend[74,92] had already subtracted off the non-specific component of binding in their tissues. There is good reason to suspect that non-specific binding may make up a considerable fraction of the total binding in my experiments.

Angiotensin is infamous for its ability to bind to everything. The use of plastic and siliconized glassware only reduces the problem somewhat. For example, in pilot studies for autoradiography, angiotensin bound not only to plastic, but even to plastic which had been siliconized

or otherwise treated. Furthermore, angiotensin will bind to some extent to any tissue with which it is incubated.[73,74,92] These facts make it imperative to evaluate the magnitude of the non-specific component of binding before coming to any conclusions.

B. Effect of Incubation With an Excess of Unlabelled Hormone on the Uptake of I^{125} Angiotensin I and II

Table 1 summarizes the result of 10 minute incubations of anterior and posterior pituitary with 1 ng/ml of 1^{125} angiotensin I with (hot + cold) and without (hot) 75 ng/ml of unlabelled angiotensin I. Both anterior and posterior pituitary showed a significant reduction in binding in the presence of cold hormone. The decrease in binding for neurohypophysis was nearly twice that of the anterior lobe. The absolute value of T/M for posterior pituitary for both the hot and hot plus cold incubations is also significantly greater (p .001).

If the assumptions implicit in this technique are valid, it may be concluded that both lobes of the pituitary are capable of specifically binding angiotensin I. In known target tissues when binding is measured by this technique, the size of the decrease generally correlates well with the known physiological potency of angiotensin II in vivo. In general, tissues most sensitive to angiotensin II show the greatest per cent decrease in binding in the presence of cold hormone.[73,74,92]

However, very few studies of this type have been done with angiotensin I, no doubt because until recently it was believed that the decapeptide was essentially inactive. Goodfriend and Lin[74] have examined

TABLE 1. Effect of incubating anterior and posterior pituitary for 10 minutes in 1 ng/ml of I^{125} angiotensin I in the presence (hot+cold) and absence (hot) of 75 ng/ml of unlabelled angiotensin I.

Tissue	T/M Hot	T/M Hot + Cold	% Decrease in Binding
Posterior Pituitary n = 6(3)	2.59 ± 0.20	1.74 ± 0.06	32.8*
Anterior Pituitary n = 6(3)	1.55 ± 0.06	1.30 ± 0.04	16.1**

Values are means ± SEM

n = number of experiments; ( ) = number of glands/experiment

* p = .003

** p = .003

the relative abilities of adrenal cortex, renal cortex, and uterus to bind angiotensin I and angiotensin II specifically. Of these three target organs, only adrenal cortex (62% decrease) showed greater binding of angiotensin I over angiotensin II. This would appear to be an important finding. However, angiotensin I does not apparently have the capability of releasing aldosterone.[73,74] Perhaps it is bound as angiotensin I and converted to angiotensin II which then has an effect on aldosterone release.

Actually, the question of which form of the molecule, angiotensin I or II, binds to the receptor has never really been studied. Because of their chemical similarity, one would predict that both forms might bind to the same receptor sites. However, when the terminal dipeptide is cleaved from angiotensin I to form angiotensin II, a conformational change may occur. Now that a converting enzyme inhibitor is available, this problem may receive some attention.

There is one more aspect which deserves some comment. That is, it is not sufficient to show that angiotensin is specifically bound in a tissue. One must address the question: to what is the hormone bound? In addition to the postulated angiotensin receptors in pituitary, converting enzyme as well as the ubiquitous tissue "angiotensinases" all will specifically bind the hormone. Of particular concern in this regard is the observation by Yang[154] that the pituitary has rather large amounts of converting enzyme. Moreover, it is thought that the bulk of this enzyme is concentrated in the neurohypophysis (Yang, personal

communication). These facts alone could account for the findings in these experiments.

The logical test of this hypothesis is to block the converting enzyme and repeat the study. However, at the time no such agent was available. Failing that, it was decided to repeat the studies using angiotensin II. If the results were very different, this would be evidence that at least part of the specific binding seen was due to the converting enzyme. It was even more important to do these studies for another reason, i.e., angiotensin II is the most physiologically important form of the molecule.

Twelve experiments were done using the same protocol and substituting I^{125} angiotensin II. Samples of cerebral cortex were also included in these studies to characterize the nature of the binding noted in the in vitro C^{14} angiotensin II studies. Unfortunately, it was later discovered that this batch of material was highly impure. Chromatographic analysis demonstrated that only 30 to 40% of the radioactivity was present as angiotensin II. Conversation with the manufacturer (Cambridge Nuclear) revealed that the other radioactive peaks were most likely free iodine and a combination of labelled tetrapeptide and labelled pentapeptide. These fragments are formed when the molecular bonds are ruptured by radiolysis on either side of the labelled tyrosine in position 4. No conclusions could be drawn from this work because there is no way to know what the radioactivity in the tissue represents.

Sufficient newly manufactured angiotensin II was obtained from

another laboratory (gift of T. E. Goodfriend) to permit one set of these angiotensin II experiments to be repeated. The values reported are the average for three glands incubated together in one experiment. The protocol was unchanged except that a huge excess of cold angiotensin II (20,000:1) was used in order to obtain a maximal effect. Results are shown in Table 2.

The data here are almost the reverse of the results from the angiotensin I experiments. Here the per cent decrease for the anterior lobe is more than double that of the neurohypophysis. These data, therefore, imply that some part of the specific binding of angiotensin I by posterior pituitary may have been due to binding by the converting enzyme. The point should be made that it is not strictly appropriate to compare quantitatively the per cent decreases from these two sets of observations. The data are not from a homogeneous pool of experiments in that the doses of hormone used are not identical, nor are the specific activities of the angiotensin I and II preparations the same. These factors could not account for the reversal seen, however, and it is appropriate to make qualitative comparisons.

All that can really be concluded is that under the conditions of this one experiment the adenohypophysis seems to display a greater degree of specific binding of angiotensin II than does the neural lobe. Conversely, the neurohypophysis specifically binds relatively more angiotensin I than the anterior lobe. The presence of specific binding suggests that there are angiotensin receptors in pituitary.

TABLE 2. Effect of incubating anterior and posterior pituitary for 10 minutes with 0.5 ng/ml of I^{125} angiotensin II in the presence (hot+cold) and absence (hot) of 1 µg/ml of unlabelled angiotensin II.

Tissue	T/M Hot	T/M Hot + Cold	% Decrease in Binding
Posterior Pituitary n = 1(3)	1.36	1.08	20.6
Anterior Pituitary n = 1(3)	1.63	0.82	49.5

Values are means + SEM.

n = number of experiments; ( ) = number of glands/experiment.

C. The Effect of Rinsing Tissue After Incubation With I^{125} Angiotensin II

Shown in Table 3 is a summary of the activity left in various tissues after 30 minutes of incubation in 0.25 ng/ml of I^{125} angiotensin II, followed by 25 minutes of rinsing. The T/M ratio is calculated from the residual counts in tissue and the counts originally present in the incubation medium. Anterior and posterior pituitary are not different from each other, but both have significantly greater residual counts than does skeletal muscle ($p=.05$). Unexpectedly, cerebral cortex had more residual counts per gram than all of the other tissues ($p=.05$).

If this technique gives an index of the avidity and specificity of binding, then it must be concluded that cerebral cortex binds angiotensin II most strongly. However, these data are in apparent conflict with the earlier C^{14} studies. Recall that the T/M ratio for brain was considerably lower than pituitary after 30 minutes of incubation with a much higher concentration of angiotensin and without rinsing. It is certainly possible that brain could bind angiotensin specifically, since it has its own renin-angiotensin system,[58,66-69] but if this were so, one might expect more uptake than was seen in those early studies. It is possible that brain lipid may have some great affinity for the iodinated portion of the molecule, but there is no evidence as yet to support that view.

The answer may be that residual binding is not a good measure of specific binding--or perhaps it is only valid for certain tissues. A closer review of the relevant papers[73,74,92] suggests that the assertion about avidity of binding and resistance to washing out may have come

TABLE 3. Residual T/M Ratio of various tissues after 30 minutes of incubation with 0.25 ng/ml of I^{125} angiotensin II followed by 25 minutes of rinsing in buffer.

Tissue	T/M Ratio
Posterior Pituitary n = 16(3)	0.835 ± .075 *
Anterior Pituitary n = 16(3)	0.681 ± .063 *
Cerebral Cortex n = 11	1.427 ± .182 **
Skeletal Muscle n = 4	0.296 ± .053

Values are means ± SEM.

n = number of experiments; ( ) = number of glands/ experiment

* $p = .05$ PP and AP > skeletal muscle

** $p < .05$ cerebral cortex > AP and PP

from experiments on only one type of tissue.

As a simple test of whether non-specific binding is always easily removed by rinsing, empty plastic chambers were run through the experimental procedure and counted before and after rinsing. Only about 50% of the total counts were thus removed, and most of the counts came off in the first minute of rinsing. Furthermore, a large portion of those initial counts that came off could reasonably be attributed to small droplets of incubation medium left clinging to the inside of the chamber. Thus, non-specific binding may be quite resistant to being washed off.

It is certainly true that non-specific binding to plastic may have different characteristics than non-specific binding to tissue. However, until that hypothesis is tested, there is good reason to question whether counts left in tissue at the end of rinsing represent material which is avidly and specifically bound. On that note, it must also be concluded that the data from the rinsing experiments really contribute little to the understanding of the nature of the binding of angiotensin by various tissues.

In the hope of gaining some insight about what these residual counts in pituitary might reflect, it was decided to do individual washout curves for both lobes of pituitary and compare them. It has been a consistent finding of this work that there is often (but not always) little difference in the T/M ratios for the two glands. It was reasoned that this technique might reveal some hidden differences.

The washout curves are illustrated in Figure 4. The method for

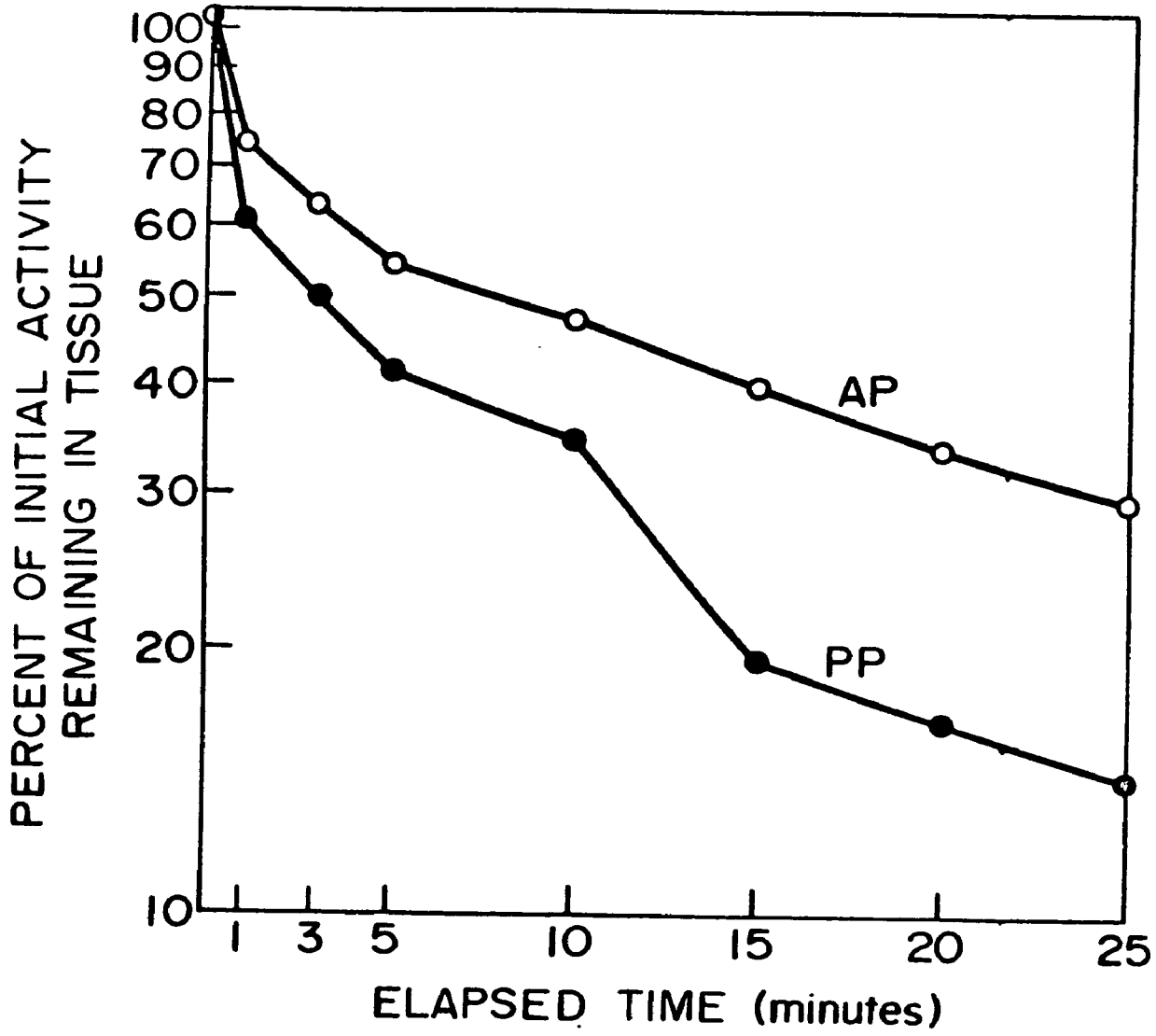


FIGURE 4. Washout of label into buffer from anterior (AP) and posterior (PP) pituitary after 30 minutes of incubation with 0.25 ng/ml of I^{125} angiotensin II.

Plotted is the log % of counts present in the tissue after a given period of rinsing, relative to the total number of counts present in the tissue before rinsing began. Each point is the mean of 5 values.

obtaining these curves is described in the methods section. Counts attributable to the washout from chamber in which the glands were contained during rinsing have been subtracted.

Rather than shedding light upon the issue, however, these data raise some rather puzzling questions. First of all, the curves look very different, except for the initial phases. There is a very rapid and similar rate of washout for both tissues during the first minute of rinsing. This might be due primarily to the presence of small amounts of incubation fluid clinging to the surface of the glands. It cannot be the total explanation. On this basis more counts would be expected to come from the anterior lobes, since they have many times more surface area. Probably some of these initial counts washing out are from the extracellular fluid of the tissue.

After the initial sharp drop, the rate of washout for the anterior pituitary begins to slow and reaches a constant proportion by about six minutes. The exponential washout during the last 19 minutes of rinsing is consistent with the concept that a single compartment is being diluted by the rinsing process. It is not possible to say what this compartment is physically, but it most likely represents the intracellular fluid.

The tail of this curve was extrapolated back to zero time and subtracted off the initial part of the curve. Since the replotted points did not fall on a straight line on semilogarithmic paper, this suggests the early part of the curve represents washout from more than one compartment. However, this is a questionable technique to employ under these

circumstances, since there were only three points left to replot. The deviation of the first point from the straight line plot could well be explained by the artifact of surface contamination with incubation medium.

The pattern of washout for posterior pituitary looks very different. The most obvious peculiarity is the sudden change in washout rate which occurs between 10 and 15 minutes. This occurred in three of the five experiments, and it was quite dramatic. The amount of counts in the rinse fluid suddenly increased from 2 to 5 times the amount in the previous rinse. It was as if a little reservoir of angiotensin had suddenly been dumped out.

One explanation for such a phenomenon would be that a droplet of incubation fluid had somehow been carried along in the chamber and was suddenly released. Contamination by carryover of droplets of rinse fluid from one rinse to the next could not account for the magnitude of increase that was seen.

Two points argue against this interpretation: (1) this effect was never seen in the anterior pituitaries or during rinsing of the empty chambers, and (2) even if accidental contamination did only occur in the posterior lobe preparations, it is unlikely that the effect would always be observed at the same time interval.

If the washout medium were devoid of some ion such as might be required as a cofactor for binding, then this might explain the observation. Such an effect would occur when the ion reaches a critically low concentration in the tissue water. The bound material would be suddenly

released when all the ionic components necessary for binding are no longer present. Such cannot be the case in these experiments. The rinse solution is the selfsame buffer with which the tissue has been equillibrated and in which the incubations took place. It is also not likely that back diffusion can account for this effect. The volume of rinse is many orders of magnitude greater than the volume of the tissue water.

Possibly some organelle which has angiotensin trapped within it has suddenly ruptured and spilled its contents into the intracellular pool. This would imply that some tissue damage is occurring. There is no good reason to expect that the posterior pituitary should have a shorter life span in vitro than anterior pituitary. If rupture of cell organelles is the explanation, then one might also expect to see this effect in the adenohypophysis. In a sense, it would be even more likely that the latter would degenerate more quickly in vitro. This is so because the thickness of the gland is about at the limit for adequate diffusion of O_2 to maintain cell viability.

It must be said that there is no obvious explanation. All the interpretations made are merely speculative. The answer to the question of what this phenomenon means or represents will have to await further experimentation.

Aside from this unexplained observation, there are other differences between the two curves. It looks as if several different pools of angiotensin are washing out from posterior pituitary. Before this

assertion could be made with any degree of confidence, however, several more experiments and several more points on the curve would have to be obtained. One thing is clear, though. Label washes out of the posterior pituitary at a faster rate, and a greater fraction of the counts present at the beginning of rinsing are removed by this procedure. This is an interesting observation, but there is no way to know what it means. In fact, all that can be concluded is that the angiotensin contained in or bound to the posterior pituitary is more readily exchangeable with something in the rinse than is that angiotensin bound to the anterior lobe.

This brings me to the final puzzle. If the counts in the rinses are added back to those remaining in the tissue after rinsing, one can arrive at what the T/M ratio must have been before rinsing began or at any point at which a sample was taken. At time zero and again after one minute of rinsing values for T/M for anterior and posterior pituitary, respectively, are: 2.39 and 10.62; 1.33 and 6.66.

Because the values at time zero include some surface contamination from the incubation medium, attention will be directed toward the T/M values after one minute of rinsing. There is a considerable difference observed between tissues. This would appear to support the contention that the posterior lobe binds more angiotensin. The quandary arises when it is remembered that there was no difference between the glands in the C^{14} *in vitro* studies, and no difference in the *in vivo* uptake of angiotensin.

Perhaps the error here is in looking at the absolute value of the

T/M ratios which include specific and non-specific binding. There did appear to be a difference between the glands when specific binding was studied using the technique of measurement by displacement of label by cold hormone. This difference may be what is being reflected here in the different shape of the washout curves and the differences in the T/M ratios, but there is no factual basis for that view.

Only one conclusion is really evident at this point. That is, this mode of inquiry does not permit a definite answer to the basic question this thesis seeks to answer. These studies neither clearly refute or clearly affirm the hypothesis that angiotensin may have a direct central effect on ADH release at the level of the neurohypophysis and/or the hypothalamus. They are merely consistent with the hypothesis. Until a specific means is found to block the actual angiotensin receptor, only inferences can be made about the possible relationships between binding and hormone action. The final series of experiments is directed toward a definitive answer to the thesis question. The modus operandi is to apply the stimulus of angiotensin to the postulated target areas and observe if release of ADH is stimulated.

Part III: The Effect of Angiotensin on in vitro ADH Release

A Intact Hypothalamo-Hypophysial Systems

The effect of 200 ng/ml of angiotensin II on ADH release from intact hypothalamo-hypophysial systems is shown in Figure 5. In each of four experiments ADH secretion increased markedly ($p=.05$) when the tissue was exposed to angiotensin II, and returned toward control values when the stimulus was removed. Two similar experiments were done at a concentration of 20 ng/ml, but this dose had no observable effect.

These experiments demonstrate that angiotensin can have a direct effect on ADH release somewhere in the hypothalamo-pituitary axis. However, it is not yet possible to discern whether the site of action is in the hypothalamus, the neurohypophysis, or both. The evidence accumulated to this point has suggested that the pituitary itself may well be the target site for angiotensin.

In addition, it is not clear whether the release of ADH from this system is due to a specific effect of angiotensin, or if it may be attributed to a non-specific depolarization of neurons by this hormone. Douglas and Poisner[50] have shown that angiotensin releases epinephrine from adrenal medullary cells by depolarizing the plasma membrane. Since ADH release is also preceded by depolarization of nerve terminals containing this hormone,[51, 136] this may be the effect of angiotensin here. Such an effect could be specific or non-specific. In order to be better able to speak on these questions, the experiments were repeated using isolated neural lobes.

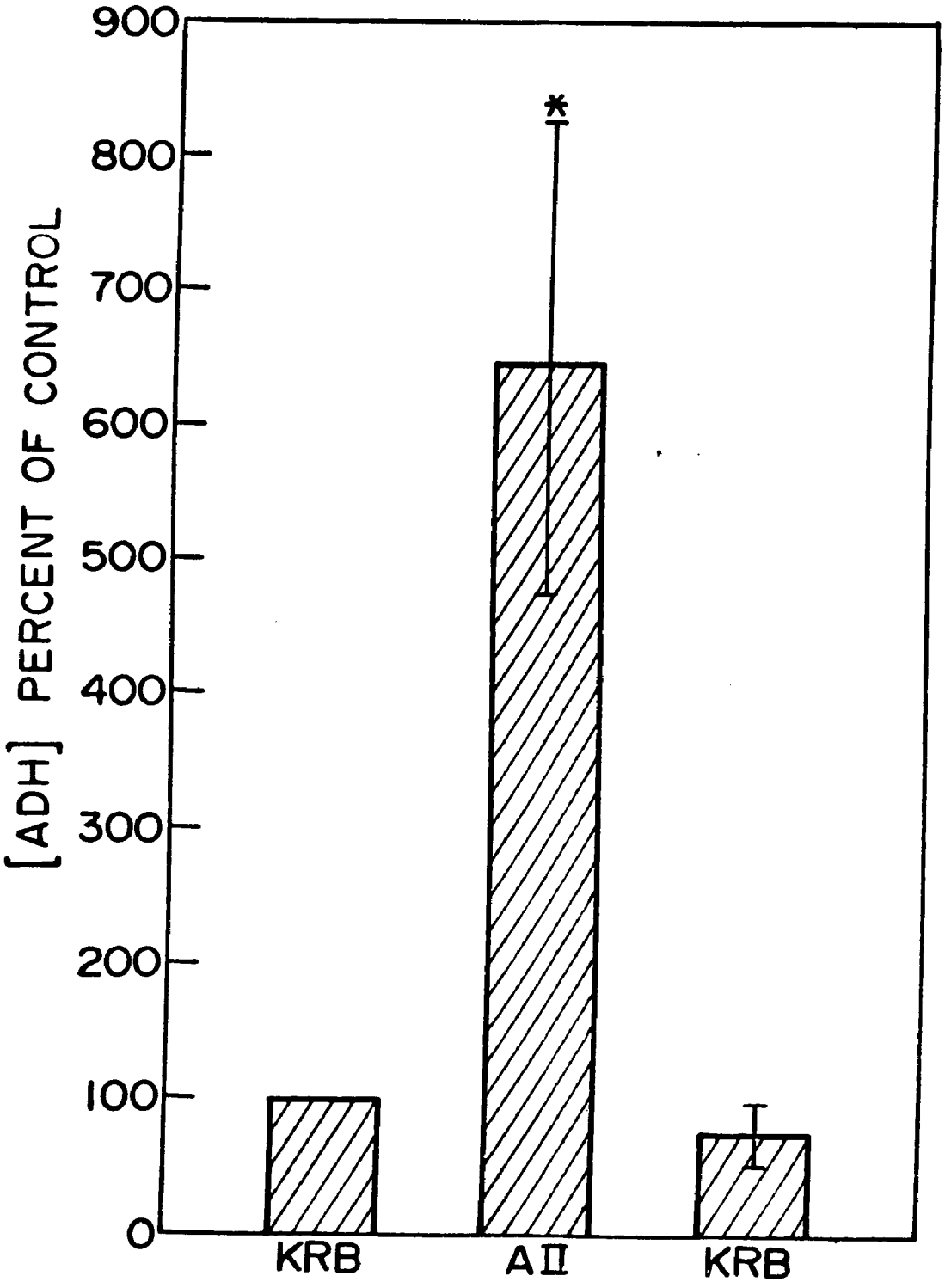


FIGURE 5: Effect of incubating intact hypothalamo-hypophysial systems in 200 ng/ml of angiotensin II for 10 minutes. Values are mean ± SEM; n = 4
* p= .05 (paired sample analysis)

B. Isolated Neural Lobes

The effect of incubating isolated neural lobes with 200 ng/ml of angiotensin II for ten minutes is depicted in Figure 6. Rather than stimulating ADH release, the data indicate that the apparent effect of angiotensin is to lower secretion. ADH concentrations decreased to about 50% of control values when angiotensin II was added to the medium. This decrease was highly significant ($p < .001$).

Two conclusions may be drawn at this juncture:

1. The site of stimulation of ADH release by angiotensin must be at the level of the hypothalamus. This is a direct effect which is not mediated by alteration of some cardiovascular parameter. Nor can it be attributed to an effect on some other area of brain which might, in turn, synapse on the supraoptic nuclei. These influences have all been removed. This stimulation in vitro may well be the same process of ADH stimulation which angiotensin evokes in vivo.

2. This release is not due to a non-specific depolarization of neural tissue by angiotensin. If this were the case, then angiotensin should have stimulated release of ADH from the isolated neural lobes as well as from the hypothalamo-hypophysial systems. Depolarization of both types of tissue preparations by high potassium solutions is known to cause a profound increase in ADH release in each case.[37] After the second control period in buffer, the isolated neural lobes were placed in a buffer containing a high concentration of KCl (60 mM). In all instances the expected increase in ADH release was observed. It was usually not

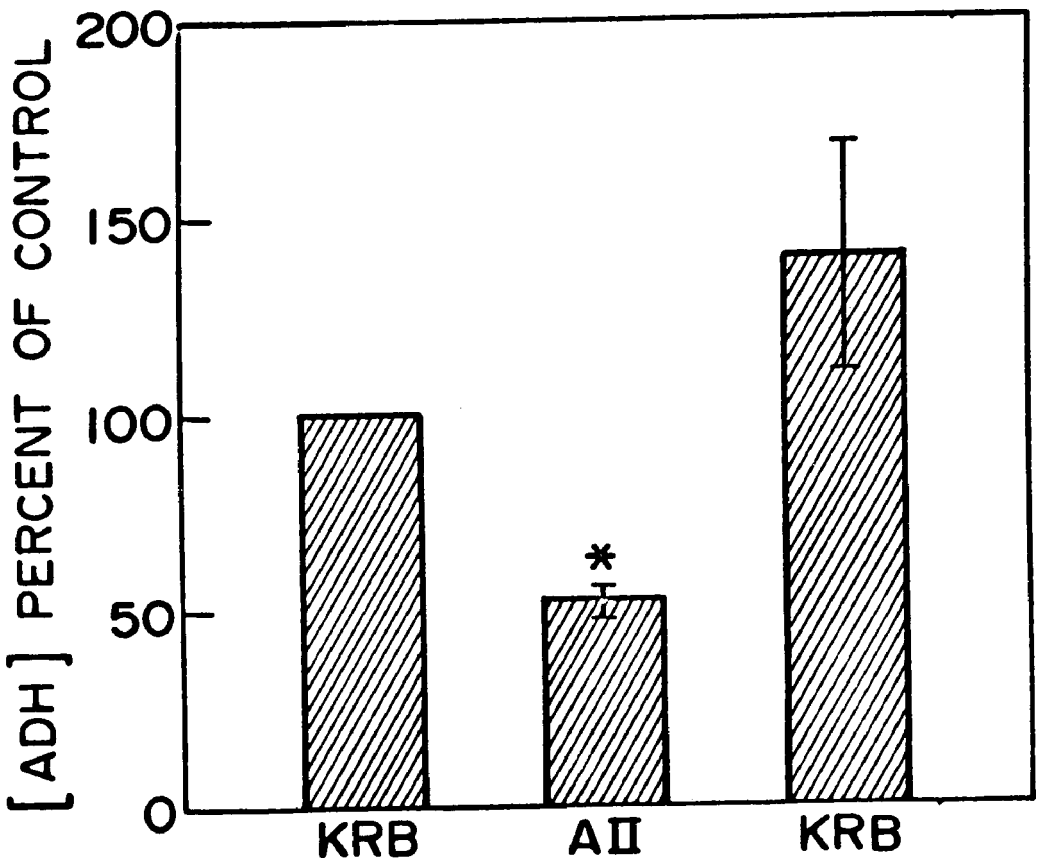


FIGURE 6. Effect of incubating isolated neural lobes in 200 ng/ml of angiotensin II for 10 minutes. Values are means ± SEM; n = 3.

* $p < .001$ (paired sample analysis)

possible to assess accurately the degree of the increase because the amount of ADH in the samples was higher than any of the ADH standards.

This effect of angiotensin at the hypothalamic level is in accord with the traditionally held view that stimuli for ADH release do not act directly on the peripheral nerve endings in the neural lobe.[136] Rather, it has long been thought that releasing stimuli act on more proximal structures such as the supraoptic and paraventricular nuclei, or on areas which synapse on these nuclei. This view is strengthened by the observation that agents such as nicotine, amyl nitrate, and ATP stimulate ADH release in vivo, but have no effect upon isolated neural lobes.[135] The fact that angiotensin also stimulates ADH release in vivo is now well documented.[12,20,97,125] Very recently it has been reported that supraoptic cells grown in tissue culture show a dose-dependent increase in spiking when exposed to angiotensin II. This effect was antagonized by 8-cys angiotensin II which inhibits the vascular effects of angiotensin in vivo.[95]

It has been proposed that angiotensin may play an integral role in the day to day regulation of ADH secretion and thereby in the regulation of the tonicity of the extracellular fluid.[19,97] Woods[153] has demonstrated that the only tissue necessary for the "osmometric" control of vasopressin release is the supraoptic nuclei and a small bit of surrounding hypothalamus connected to the hypophysis. This preparation is very similar to the intact hypothalamo-hypophysial preparations which were used in these experiments.

Another observation which strengthens the view that this *in vitro* stimulation of ADH release by angiotensin represents the same process which occurs *in vivo* is the short period of time it takes for ADH stimulation to occur in this system. In these experiments there was an average six-fold increase in release rate within ten minutes. The time course of the stimulation of ADH release by angiotensin *in vivo* is not known precisely. Andersson and colleagues[12] have noted that urine osmolality began to increase in the first ten minutes after initiation of an intraventricular infusion of angiotensin II and hypertonic saline. ADH release, as stimulated by acute hemorrhage, is also at a peak within fifteen minutes after the onset of the stimulus.[146] The *in vivo* values are consistent with the findings of these experiments.

The point should be made that a rather large dose of angiotensin (200 ng/ml) was used in these experiments. The blood level of this hormone is probably less than 20 pg/ml.[20] The question of dosage in studies such as these is somewhat academic, however, since conditions are rather artificial. The *in vivo* situation offers cells excellent exposure to the hormone, whereas in this experimental situation diffusion over rather long distances (up to 0.5 mm) must be relied upon to permit contact of the hormone with receptors.

It would not have been surprising to discover that angiotensin had little or no effect on ADH release from isolated neural lobes. This would have been consistent with the view that the primary site of action of angiotensin is at the hypothalamic level. The observation that

angiotensin seems to inhibit ADH release was totally unexpected. Furthermore, this finding is at variance with some published data.

Gagnon and colleagues[64] have reported that a concentration of approximately 1 µg/ml of angiotensin significantly stimulated ADH release from isolated neural lobes. The increase in secretion could be progressively inhibited by increasing doses of angiotensin analogues which compete for the same binding sites. There are some very serious questions to be asked about this study. First of all, it seems very likely that their result may be explained by the observation that the amount of angiotensin in the incubation solution being assayed was high enough to stimulate release of endogenous ADH from the assay animals.

The method chosen to measure ADH was a blood pressure assay. Nowhere in the protocol was there any indication that steps had been taken to prevent endogenous ADH secretion. I have calculated the amount of angiotensin present in the volume of solution which was injected into the assay animals. It approaches 200 ng/ml--a dose many times the amount Bonjour and Malvin gave intravenously to stimulate ADH release.[20] Even if the source of the increase in ADH secretion is the assay animals themselves, one would still expect to see a decline in ADH release in this situation when angiotensin inhibitors are given. These agents would simply compete for the angiotensin binding sites in the assay animals and thereby reduce the release of ADH.

There is also another serious difficulty with this work. It is puzzling that under the experimental conditions chosen that the

investigators elected to use a blood pressure assay. Angiotensin is one of the most potent pressor agents known, and its effects are commonly assayed by its effect upon blood pressure. The doses of angiotensin injected into the assay animals are far above the threshold pressor dose in rats. It is in no way clear how the authors would be able to separate out whether a rise in blood pressure was due to angiotensin or ADH. Very likely some of the pressor effects attributed to ADH are really due to the effects of angiotensin on blood pressure. This is so because the amount of ADH that the authors report as being located in the pituitary gland are about a thousand times in excess of the known amounts which are present in rat pituitaries. For these reasons, it is difficult to accept the data from these studies.

A question still remains as to why angiotensin should inhibit ADH release from isolated neural lobes. The first thought that comes to mind is that severing the neural lobes from the hypophysis might deprive them of some necessary constituent. This would result in a decline in the spontaneous secretion rate that would be unrelated to the presence of angiotensin. The fact that secretion rates return to control levels after angiotensin is removed from the incubation medium argues strongly against that possibility.

What, then, is the explanation? The answer is not apparent. A few possibilities are:

1. The neurohypophysis could be a site of negative feedback for the effects of angiotensin on ADH release. This would explain the

earlier findings that angiotensin specifically binds to posterior pituitary. However, it is difficult to envision how such a system would operate with the proximal part of the system being stimulated by angiotensin and the final common pathway for release being inhibited by the same agent.

2. Perhaps ADH inhibits its own release. If there is a site of negative feedback for ADH release, the logical place for this to occur is at the neurohypophysis. This is so because there is a blood-brain barrier to ADH which would prevent it from reaching the supraoptic nuclei.[143] If angiotensin were able to bind to the negative feedback sites that would normally be occupied by ADH, then a decrease in secretion rate would be expected. Angiotensin would thus mimic the inhibitory effect of ADH upon its own release.

Louis and Doyle[93] have provided some evidence that angiotensin and ADH may bind to the same receptor sites in vascular smooth muscle. An identical situation might occur in the pituitary. This postulated binding of angiotensin to ADH receptors would also be consistent with the earlier findings that the binding of angiotensin to pituitary is specific, i.e., that it may represent binding to a receptor of some type. Actually, some other neural tissues which are outside the blood-brain barrier, such as sciatic nerve, also specifically bind angiotensin.[73,74,92] The physiological significance of this finding is not yet known.

3. The hypothalamo-hypophysial system may exhibit a biphasic response to angiotensin. That is to say, it may stimulate ADH release

at one dose and inhibit at another. It is well known that the urinary response to renin and angiotensin is dose-dependent in several species, including rat.[105] Antidiuresis and natriuresis occur at lower doses and these effects are exactly reversed as the dose is increased. However, this difference in effects may be due to changes in other parameters such as an increase in blood pressure or a decrease in GFR, rather than simply to the difference in dose of angiotensin. The fact that the same dose has different effects in hypothalamus and pituitary could be attributed to differences in sensitivity. Vascular smooth muscle from various regions of the body reacts quite differently to the same dose of angiotensin.[84]

It is reasonable to conclude on the basis of the evidence from these last studies that angiotensin is capable of exerting a direct central effect on ADH release at the level of the hypothalamus. Very likely this in vitro stimulation of ADH release is representative of the process which occurs in vivo. The only real reason to hesitate in drawing this conclusion is the question of whether peripherally administered angiotensin can gain direct access to the supraoptic nuclei. At the moment, there is no clear evidence that angiotensin can pass the blood-brain barrier (see Part I results). However, the unalterable fact remains that peripherally administered angiotensin does stimulate ADH release and thirst. How could this be accomplished?

The supraoptic nuclei or their osmoreceptors may be outside the barrier. This has never been clearly shown. However, there is an

increasing body of evidence that the classical osmoreceptors of Verney[141, 142] may, in fact, be sodium sensitive cells near the wall of the third ventricle.[4-12, 98, 103] Since there is a blood-brain barrier to sodium, this suggests that these elements may be on the vascular side, rather than on the brain side.

The supraoptic nuclei and the perinuclear zone around them have the richest capillary supply in the brain.[38, 57] Although the vascular bed in this region has tight junctions of the blood-brain barrier type, the glial end feet in this region do not completely envelop the capillary walls.[78] Furthermore, there is an unusually intimate contact between the capillaries and neurons in this region.[32]

Thus, there is morphological evidence that there may be something unusual about the blood-brain barrier in this region. These differences might allow for passage of angiotensin into the supraoptic nuclei cells. It is also possible that angiotensin may stimulate transport of sodium or some other substance into the supraoptic nuclei or their "osmoreceptor cells" (which would be present in the in vitro tissue preparations in these experiments). This action may then result in a stimulation of ADH secretion.

For completeness it should be noted that there is at least one other way in which angiotensin could stimulate ADH release without directly passing the blood-brain barrier. That is, angiotensin given intravascularly may affect some parameter such as sodium concentration which could then affect ADH release and thirst. For example, the action

of angiotensin may be to stimulate sodium transfer across the choroid plexus cells into cerebrospinal fluid. This action would not require that angiotensin pass the blood-brain barrier or enter CSF. The following data support this hypothesis:

1. Most sodium which enters CSF does so by way of the choroid plexus.[41]

2. Autoradiography of brain after intravenous administration of tritiated angiotensin reveals a concentration of activity around the third brain ventricle.[144] This activity is most likely contained in choroid plexus cells or choroid plexus blood.

3. Choroid plexus cells have been shown to bind angiotensin specifically.[73]

4. Angiotensin can affect sodium transport in tissues such as intestinal mucosa.[35]

5. The cells of the intestinal mucosa closely resemble those of choroid plexus.[17]

6. Increased sodium in CSF stimulates both ADH release and thirst.[4-12]

7. Angiotensin in CSF markedly potentiates the effect of increasing CSF sodium concentration.[6,9,12]

In this situation the angiotensin may be stimulating entry of more sodium into sensitive cells.

8. This action on sodium transport could also explain the in vitro stimulation of ADH release by angiotensin. The hormone could be

increasing sodium transport from the medium into sodium sensitive cells contained in the tissue preparation.

This hypothesis will have to await confirmation or denial in further studies.

LIST OF REFERENCES

1. Abrash, L., N. Marks, M. Pirotta, and R. Walter. Inactivation of polypeptide hormones and analogues by purified brain enzymes. Trans. Am. Soc. Neurochem. 2:54, 1971.

2. Ahmed, A. B. J., B. C. George, C. Gonzalez-Quvert, and J. F. Dingman. Increased plasma arginine vasopressin in clinical adrenocortical insufficiency and its inhibition by glucocorticoids. J. Clin. Invest. 46:111-123, 1967.

3. Aiken, J., and J. R. Vane. Inhibition of converting enzyme of the renin-angiotensin system in kidneys and hind legs of dogs. Circ. Res. in press.

4. Andersson, B. Thirst and central control of water balance. Am. Scientist 59:408-415, 1971.

5. _____, M. F. Dallman, and K. Olsson. Observations of central control of drinking and of the release of antidiuretic hormone (ADH). Life Scie., Part I, 8:425-432, 1968.

6. _____, and L. Eriksson. Conjoint action of sodium and angiotensin on brain mechanisms controlling salt and water balance. Acta Physiol. Scand. 81:18-29, 1971.

7. _____, _____, and O. Fernandez. Reinforcement of centrally mediated hypertensive response to angiotensin II. Life Scie., Part I, 10:663-638, 1971.

8. _____, _____, _____, C.-G. Kolmodin, and R. Oltner. Centrally mediated effects of sodium and angiotensin II on arterial blood pressure and fluid balance. Acta Physiol. Scand. 85:398-407, 1972.

9. _____, _____, and R. Oltner. Further evidence for angiotensin-sodium interaction in central control of fluid balance. Life Scie., Part I, 9:1091-1096, 1970.

10. _____, M. Jobin, and K. Olsson. A study of thirst and other effects of an increased sodium concentration in the third brain ventricle. Acta Physiol. Scand. 69:29-36, 1967.

11. _____, _____, and R. G. Warner. Dissimilarities between the central control of thirst and the release of antidiuretic hormone (ADH). Acta Physiol. Scand., 71:57-64, 1967.

12. _____, and O. Westbye. Synergistic action of sodium and angiotensin on brain mechanisms controlling fluid balance. Life Scie., Part I, 9:601-608, 1970.

13. Bakay, L. The Blood-Brain Barrier. Springfield: Thomas, 1956, pp. 40-76.

14. Baratz, R. A., and R. C. Ingraham. Renal hemodynamics and antidiuretic hormone release associated with volume regulation. Am. J. Physiol. 198:565-570, 1960.

15. Berde, B. (ed.). Neurohypophysial Hormones and Similar Polypeptides. Handbook of Experimental Pharmacology. Vol. 23. Berlin: Springer-Verlag, 1968, p. 1-35.

16. Bie, P., and N. A. Thorn. In vivo studies of the release mechanism for vasopressin in rats. II: Studies of the possible release of hormone from hypothalamic tissue. Acta Physiol. Scand. 56:139-145, 1967.

17. Bittar, E. E. Membranes and Ion Transport. Vol. 3. New York: Wiley-Interscience, 1971, p. 268.

18. Bonjour, J.-P. and R. L. Malvin. Renal extraction of PAH, GFR, and $U_{Na}V$ in the rat during infusion of angiotensin. Am. J. Physiol. 216:554-558, 1969.

19. _____, and _____. Plasma concentrations of ADH in conscious and anesthetized dogs. Am. J. Physiol. 198:565-560, 1970.

20. _____, and _____. Stimulation of ADH release by the renin-angiotensin system. Am. J. Physiol. 218:1555-1559, 1970.

21. _____, G. Peters, F. Chomety, and D. Regoli. Renal effects of val_5-angiotensin II-amide, vasopressin and diuretics in the rat as influenced by water diuresis and by ethanol anesthesia. Eur. J. of Pharmacol. 2:88-105, 1967.

22. Brown, J. J., D. L. Davies, A. F. Lever, J. I. S. Robertson, and A. Verniory. The effect of acute hemorrhage in the dog and man on plasma renin concentration. J. Physiol. (London) 182: 649-663, 1966.

23. Brunner, H. R., P. Chang, R. Wallach, J. E. Sealey, and J. H. Laragh. Angiotensin II vascular receptors: their avidity in relationship to sodium balance, the autonomic nervous system, and hypertension. J. Clin. Investig. 51:58-67, 1972.

24. Buckley, J. P. Actions of angiotensin of the CNS. Fed. Proc. 31: 1332-1337, 1972.

25. Bunag, R. D. Pharmacology of angiotensin in Renal Hypertension. I. H. Page and J. W. McCubbin (eds.). Chicago: Year Book, 1968, p. 134-159.

26. _____, I. H. Page, and J. W. McCubbin. Neural stimulation of renin release. Circ. Res. 19:851-858, 1966.

27. _____, _____, _____. Inhibition of renin release by vasopressin and angiotensin. Cardiovasc. Res. 1:67-73, 1967.

28. Chalmers, T. M., and A. A. G. Lewis. Stimulation of the supra-optico-hypophysial system in man. Clin. Scie. 10:127-135, 1951.

29. Chase, G. D., and J. L. Rabinowitz. Principles of Radioisotope Methodology. Minneapolis: Burgess, 1967, p. 75-108.

30. Claybaugh, J. R., and L. Share. Role of the renin angiotensin system in the vasopressin response to hemorrhage. Endo. 90: 453-460, 1972.

31. _____, _____, and K. Shimizu. The inability of infusions of angiotensin to elevate the plasma vasopressin concentration in the anesthetized dog. Endocrinol. 90:1647-1652, 1972.

32. Clementi, F., and B. Ceccarelli. Fine structure of rat hypothalamic nuclei in The Hypothalamus. L. Martini, M. Motta, and F. Fraschini (eds.). New York: Academic Press, 1970, p. 17-44.

33. Conn, J. W., D. R. Rovner, and E. L. Cohen. Normal and altered function of the renin-angiotensin-aldosterone system in man: application in clinical and research medicine. Ann. Internal Med. 63:266-284, 1965.

34. Creskoff, A. J. and T. Fitzhugh, Jr. Hematology of the rat in The Rat in Laboratory Investigation. E. J. Farris and J. Q. Griffith (eds.). New York: Hafner, 1971, p. 413.

35. Crocker, A. D., and K. A. Munday. The effect of the renin-angiotensin system on mucosal H_2O and Na transfer in everted sacs of rat jejunum. J. Physiol. (London) 206:323-333, 1970.

36. Cushman, D. W., and H. S. Cheung. Concentrations of angiotensin-converting enzyme in tissues of the rat. Biochim. Biophys. Acta (Amst.) 250:261, 1971.

37. Daniel, A. R., and K. Lederis. Release of neurohypophysial hormones in vitro. J. Physiol. (London) 190:171-187, 1967.

38. Daniel, P. M. The blood supply of the hypothalamus and pituitary gland. Brit. Med. Bull. 22:202-208, 1966.

39. Daniels-Severs, A., E. Ogden, and J. Vernikos-Daniellis. Centrally mediated effects of angiotensin II in the unanesthetized rat. Physiology and Behavior 7:785-787, 1971.

40. Davies, N. T., Munday, K. A., and B. J. Parsons. The effect of angiotensin on the rat intestinal fluid transfer. J. Endo. 48:39-46, 1970.

41. Davson, H. Physiology of the Cerebrospinal Fluid. Boston: Little-Brown, 1967, p. 76.

42. Day, M. D., J. W. McCubbin, and I. H. Page. Limited hypertensive effect of infusion of angiotensin. Am. J. Physiol. 209: 264-272, 1965.

43. DeBono, E., G. Lee, F. R. Mottram, G. W. Pickering, J. J. Brown, H. Keen, W. S. Peart, and P. H. Sanderson. The action of angiotensin in man. Clin. Scie. 25, 123-157, 1963.

44. Dexter, L., H. A. Frank, I. W. Haynes, and M. D. Altschule. Traumatic shock. VI. The effect of hemorrhagic shock on the concentration of renin and hypertensinogen in the plasma in unanesthetized dogs. J. Clin. Investig. 22:847-852, 1943.

45. Dickinson, C. J., and R. Yu. Mechanisms involved in the progressive pressor response to small amounts of angiotensin in conscious rabbits. J. Physiol. (London) 190:91-99, 1967.

46. DiSalvo, J., M. Menta, C. B. Montefusco, and A. Peterson. Intrarenal conversion of angiotensin I to angiotensin II in the dog. Circ. Res. 29:398-406, 1971.

47. _____, and C. B. Montefusco. Conversion of angiotensin I to angiotensin II in the canine mesenteric circulation. Am. J. Physiol. 221:1576-1579, 1971.

48. Douglas, W. W. Polypeptides-angiotensin, plasma kinins, and other vasoactive agents in The Pharmacological Basis of Therapeutics. L. S. Goodman and A. Gilman (eds.). 4th ed. New York: MacMillan Co., 1970, p. 663-668.

49. _____, T. Kanno, and S. R. Sampson. Effects of acethycholine and other medullary secretogogues and antagonists on the membrane potential of adrenal chromaffin cells: an analysis employing techniques of tissue culture. J. Physiol. (London) 188:107-120, 1967.

50. _____, and A. M. Poisner. Stimulus-secretion coupling in a neuro-secretory organ and the role of calcium in the release of vasopressin from the neurohypophysis. J. Physiol. (London) 172: 1-18, 1964.

51. Dyball, R. E. J. Oxytocin and ADH secretion in relation to electrical activity in antidromically identified supraoptic and paraventricular units. J. Physiol. (London) 214:245-256, 1971.

52. Eggleton, M. C. Diuretic action of alcohol in man. J. Physiol. (London) 101:172-191, 1945.

53. Eriksson, L., O. Fernandez, and K. Olssen. Differences in the anti-diuretic response to intracarotid infusions of various hypertonic solutions in the conscious goat. Acta Physiol. Scand. 83: 554-562, 1971.

54. Everett, N. B., B. Simons, and E. P. Lasher. Distribution of blood (Fe^{59}) and plasma (I^{131}) volumes of rat determined by liquid nitrogen freezing. Circ. Res. 4:419-424, 1956.

55. Federation of American Societies for Experimental Biology. Blood and Other Body Fluids. Bethesda, 1961, p. 9-11.

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

57. Finley, K. H. Angio-architecture of the hypothalamus and its peculiarities. Publ. Ass. Res. Nerv. Ment. Dis. 20:286-309, 1939.

58. Fischer-Ferrano, C., V. E. Nahmod, D. J. Goldstein, and S. Finkielman. Angiotensin and renin in rat and dog brain. J. Exp. Med. 133(2):353-361, 1971.

59. Fisher, J. W. (ed.). Kidney Hormones. New York: Academic Press, 1971, p. 59-91.

60. Fitzsimons, J. T. The renin-angiotensin system in the control of drinking in The Hypothalamus. L. Martini, M. Motta, and F. Fraschini (eds.). New York: Academic Press, 1970, p. 195-212.

61. _____. The hormonal control of water and sodium intake in Frontiers in Neuroendocrinology, 1971. L. Martini and W. F. Ganong (eds.). New York: Oxford University Press, 1971, p. 103-127.

62. _____, and B. J. Simons. The effect of angiotensin on drinking in the rat. J. Physiol. (London) 196:39-41, 1968.

63. _____, and _____. The effect on drinking in the rat of intravenous infusion of angiotensin given alone or in combination with other stimuli of thirst. J. Physiol. (London) 203:45-57, 1969.

64. Gagnon, D. J., D. Cousineau, and P. J. Boucher. Release of vasopressin by angiotensin II and prostaglandin E_2 from the rat neurohypophysis in vitro. Life Scie., Part I, 12:487-497, 1973.

65. Ganong, W. F. Review of Medical Physiology. 5th ed. Los Altos: Lange Medical Publications, 1971, p. 230-244.

66. Ganten, D., P. Granger, U. Ganten, R. Boucher, and J. Genest. An intrinsic renin-angiotensin system in the brain in Hypertension '72. J. Genest and E. Koiw (eds.). Berlin: Springer-Verlag, 1972, p. 423-431.

67. _____, M. Kusumoto, G. Constantopoulous, U. Ganten, R. Boucher, and J. Genest. Iso-renin, electrolytes, and catecholamines in dog brain. Life Scie., Part I, 12:1-8, 1973.

68. _____, A. Marquez-Julio, P. Granger, K. Hayduk, K. P. Karsunky, R. Boucher, and J. Genest. Renin in dog brain. Am. J. Physiol. 221:1733-1737, 1971.

69. _____, J. Minnich, P. Granger, K. Hayduk, A. Barbeau, R. Boucher, and J. Genest. Presence of angiotensin-froming enzyme in brain tissue of dogs. Clin. Res. 19:531, 1971.

70. Gauer, O. H., and J. P. Henry. Circulatory basis of fluid volume control. Physiol. Rev. 43:423-481, 1963.

71. Gilmore, J. P., J. Merrill, and M. J. Peach. Further evidence for the renal conversion of angiotensin. Fed. Proc. 30:499 Abs., 1971.

72. Ginsburg, M., and H. Heller. Antidiuretic activity in blood obtained from various parts of the cardiovascular system. J. Endocr. 9:274-282, 1953.

73. Goodfriend, T. L., F. Fyhrquist, F. Gutman, E. Knych, H. Hollmans, D. Allmann, K. Kent, and T. Cooper. Clinical and conceptual uses of angiotensin receptors in Hypertension '72. J. Genest and E. Koiw (eds.). Berlin: Springer-Verlag, 1972, p. 549-562.

74. _____, and S. -Y. Lin. Receptors for angiotensin I and II. Circ. Res. (Suppl. I) 28:I-163-I-174, 1970.

75. Gutman, Y., and F. Benzakein. Effect of an increase and lack of antidiuretic hormone on plasma renin activity in the rat. Life Scie., Part I, 10:1081-1085, 1971.

76. Harris, J. W. Central control of pituitary secretion. Handbook of Physiology, Sect. I, Neurophysiology, 2. Bethesda: American Physiological Society, 1960, p. 1007-1038.

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

78. Hayward, J. N. Hypothalamic input to supraoptic neurons in Progress in Brain Res. Vol. 38. Topics in Neuroendocrinology. J. Kappers and J. Schadé (eds.). Amsterdam: Elsevier, 1972, p. 145-161.

79. Henry, J. P., P. D. Gupta, J. P. Heehan, R. Sinclair, and L. Share. The role of afferents from the low pressure system in the release of antidiuretic hormone during non-hypotensive hemorrhage. Can. J. Physiol. Pharmacol. 46:287-295, 1968.

80. Hinkle, L., C. J. Edwards, and S. Wolf. The occurrence of diuresis in humans in stressful situations and its possible relation to the diuresis of early starvation. J. Clin. Investig. 30:809-817, 1951.

81. Hodge, R. L., R. D. Lowe, and J. R. Vane. The effects of alteration of blood volume on the concentration of circulating angiotensin in anesthetized dogs. J. Physiol. (London) 185:613-626, 1966.

82. _____, K. K. F. Ng, and J. R. Vane. Disappearance of angiotensin from the circulation of the dog. Nature 215:138-141, 1967.

83. Kelsall, A. R. The inhibition of water diuresis in man by ischemic muscle pain. J. Physiol. (London) 109:150-161, 1949.

84. Khairallah, P. A. Pharmacology of angiotensin in Kidney Hormones. J. W. Fisher (ed.). New York: Academic Press, 1971, p. 129-171.

85. _____, I. H. Page, F. M. Bumpus, and R. R. Smedby. Angiotensin II: its metabolic fate. Science 138:523-525, 1962.

86. Kleeman, C. R., and E. Landin. Studies on alcohol diuresis. I. The effect of ethyl alcohol on water, electrolyte, and acid base balance. J. Clin. Investig. 34:448-455, 1955.

87. Knigge, K. M., and D. E. Scott. Structure and function of the median eminence. Am.J. Anat. 129:223-244, 1970.

88. Konig, J. F. R., and R. A. Klippel. The Rat Brain: A Stereotaxic Atlas. Baltimore: Williams and Wilkins, 1963.

89. Kumar, T. C. A., and F. G. W. Knowles. A system linking the third ventricle with the pars tuberalis of the rhesus monkey. Nature (London) 215:54-55, 1967.

90. Lauson, H. D. Metabolism of antidiuretic hormones. Am. J. Med. 42:712-744, 1967.

91. Lin, S.-Y., H. Ellis, B. Weisblum, and T. Goodfriend. Preparation and properties of iodinated angiotensins. Biochem. Pharmacol. 19:651-662, 1970.

92. _____, and T. L. Goodfriend. Angiotensin receptors. Am. J. Physiol. 218:1319-1328, 1970.

93. Louis, W. J., and A. E. Doyle. The relationship between the vascular responses to the peptides angiotensin and pitressin. Clin. Scie. 31:255-263, 1966.

94. Malvin, R. L., and A. J. Vander. Effects of angiotensin infusion in renal function in the unanesthetized rat. Am. J. Physiol. 213: 1205-1208, 1967.

95. Marks, B. H., K. K. Sakai, J. M. George, and A. Koestner. Angiotensin receptors in the organ-cultured supra-optic nucleus. Endocrine Society, 55th Annual Meeting. Abstract 49, p. A-73, 1973.

96. Miles, B. E., H. E. DeWardner, and R. R. McSwiney. Renal function during emotional diuresis. Am. J. Med. 12:659-666, 1952.

97. Mouw, D. R., J.-P Bonjour, R. L. Malvin, and A. J. Vander. Central action of angiotensin in stimulating ADH release. Am. J. Physiol. 220:239-242, 1971.

98. _____, and A. J. Vander. Evidence for brain sodium receptors controlling renal Na excretion and plasma renin activity. Am. J. Physiol. 219:822-832, 1970.

99. Munday, K. A., B. J. Parsons, and J. A. Poat. Effect of angiotensin on cation transport by rat kidney cortex slices. J. Physiol. (London) 215:269-282, 1971.

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

101. Nicoll, R. A., and J. L. Barker. Excitation of supraoptic neurosecretory cells by angiotensin II. Nature:New Biology 233:172-174, 1971.

102. Olsson, K. Studies on the central regulation of secretion of antidiuretic hormone (ADH) in the goat. Acta Physiol. Scand. 77: 465-474, 1969.

103. _____. On the importance of CSF Na^+ concentration in the central control of fluid balance. Life Scie., Part I, 11:397-402, 1972.

104. Osborne, M. J., N. Pooters, G. d'Auriac, A. Epstein, M. Worcel, and P. Meyer. Metabolism of tritiated angiotensin II in anesthetized rats. Pfleug. Archiv. 326:101-114, 1971.

105. Page, I. H., and J. W. McCubbin (eds.). Renal Hypertension. Chicago: Year Book, 1968, p. 188, 193.

106. Palic, D., and P. A. Khairallah. Inhibition of norepinephrine reuptake by angiotensin in brain. J. Neurochem. 15:1195-1202, 1968.

107. Pappenheimer, J. R. Transport of HCO_3 between brain and blood. Capillary Permeability. Transfer of Molecules and Ions Between Capillary Blood and Tissue. Alfred Benzon Symposium II. C. Crone and N. A. Lassen (eds.). New York: Academic Press, 1970, p. 454-458.

108. Peters, G. Renal tubular effect of val_5-angiotensin II amide in rats. Proc. Soc. Exp. Biol. and Med. 112:771-775, 1963.

109. Pollay, M., R. Kaplan, and K. M. Nelson. Potassium transport across the choroid ependyma. Life Scie., Part II, 12:479-487, 1973.

110. Radio, G. J., J. Summy-Long, A. Daniels-Severs, and W. B. Severs. Hydration changes produced by central infusion of angiotensin II. Am. J. Physiol. 223:1221-1226, 1972.

111. Regoli, D., and J. R. Vane. The continuous estimation of angiotensin formed in the circulation of the dog. J. Physiol. (London) 183:513-531, 1966.

112. Rolls, B. J. The effect of intravenous infusion of antidiuretic hormone on water intake in the rat. J. Physiol. (London) 219: 331-339, 1971.

113. Rosendorf, C., and W. I. Cranston. Effects of intrahypothalamic and intraventricular norepinephrine and 5-hydroxytryptamine on hypothalamic blood flow in the conscious rabbit. Circ. Res. 28: 492-502, 1971.

114. Rydin, A., and E. B. Verney. The inhibition of water diuresis by emotional stress and muscular exercise. Quar. J. Exp. Physiol. 27:343-374, 1938.

115. Saameli, K. The circulatory actions of the neurohypophysial hormones and similar polypeptides. Handbook of Experimental Pharmacology. Vol. 23. Neurohypophysial Hormones and Similar Polypeptides. B. Berde (ed.). Berlin: Springer-Verlag, 1968, p. 545-612.

116. Sachs, H. Neurosecretion. Handbook of Neurochemistry. Vol. IV. Control Mechanisms of the Central Nervous System. A. Lajtha (ed.). New York: Plenum Press, 1970, p. 373-428.

117. Sawyer, C. H., and G. R. Fuller. Electroencephalographic correlates of reflex activation of the neurohypophysial antidiuretic mechanism. Electroenceph. Clin. Neurophysiol. 12:83-93, 1960.

118. _____, and B. E. Gernadt. Effects of intracarotic and intraventricular injections of hypertonic solutions on electrical activity of the rabbit brain. Am. J. Physiol. 185:209-216, 1956.

119. Schuleman, W. Beitrage zur vitalfarbung. Arch. Mikr. Anat. 79: 223-246, 1912.

120. Schwartz/Mann. Division of Becton, Dickinson and Co., Orangeburg, N.Y. Procedure for the Radioimmunoassay of Renin Activity. 13 pp., 1971.

121. Scornik, O. A., and A. C. Paladini. Angiotensin blood levels in hemorrhagic hypotension and other related conditions. Am. J. Physiol. 206:553-556, 1964.

122. Segar, W. E., and W. W. Moore. The regulation of antidiuretic hormone release in man. I. Effects of change in position and ambient temperature on blood ADH levels. J. Clin. Investig. 47: 2143-2151, 1968.

123. _____, _____, and J. P. Buckley. On the central hypertensive effect of angiotensin II. Int. J. of Neuropharmacol. 6: 199-205, 1967.

124. Severs, W. B., A. Daniels-Severs, J. Summy-Long, and G. J. Radio. Effects of centrally administered angiotensin II on salt and water excretion. Pharmacol. 6:242-252, 1971.

125. _____, J. Summy-Long, J. S. Taylor, and J. D. Connor. A central effect of angiotensin: release of pituitary pressor material. J. Pharm. Exp. Ther. 174:27-34, 1970.

126. Share, L. Extracellular fluid volume and vasopressin secretion. Frontiers in Endocrinology 1969. L. Martini and W. F. Ganong (eds.). New York: Oxford University Press, 1969, p. 183-210.

127. _____, and M. N. Levy. Cardiovascular receptors and blood titer of antidiuretic hormone. Am. J. Physiol. 203:425-428, 1962.

128. _____, and _____. Effect of carotid chemoreceptor stimulation on plasma antidiuretic hormone titer. Am. J. Physiol. 210: 157-161, 1966.

129. _____, and R. H. Travis. Plasma vasopressin concentration in the adrenally insufficient dog. Endo. 86:196-201, 1970.

130. Shimizu, K., L. Share, and J. R. Claybaugh. Potentiation by angiotensin II of osmotically stimulated ADH release. Fed. Proc. 31:235 Abs., 1972.

131. Skinner, S. L., J. W. McCubbin, and I. H. Page. Control of renin secretion. Circ. Res. 15:522-531, 1963.

132. Stumpf, W. E. Estrogen neurons and estrogen-neuron systems in the periventricular brain. Am. J. Anat. 129:207-218, 1970.

133. Tagawa, H., A. J. Vander, J.-P. Bonjour, and R. L. Malvin. Inhibition of renin secretion by vasopressin in unanesthetized sodium-deprived dogs. Am. J. Physiol. 220:949-951, 1971.

134. Tepperman, J. Metabolic and Endocrine Physiology. 2nd ed. Chicago: Year Book, 1968, p. 44.

135. Thorn, N. A. In vitro studies on the release mechanism for vasopressin in rats. Acta Ando. 53:644-654, 1966.

136. _____. ADH synthesis, release, and action under normal and pathological circumstances. Adv. Metab. Disorders. New York: Academic Press, 1970, p. 44-68.

137. Vander, A. J. Control of renin release. Physiol. Rev. 47: 359-382, 1967.

138. _____. Inhibition of renin release by vasopressin and vasotocin. Circ. Res. 23:605-609, 1968.

139. _____, and G. W. Geelhoed. Inhibition of renin secretion by angiotensin II. Proc. Soc. Exp. Biol. and Med. 120:399-403, 1965.

140. _____, and R. Miller. Control of renin secretion in the anesthetized dog. Am. J. Physiol. 207:537-546, 1964.

141. Verney, E. B. Absorption and excretion of water. The antidiuretic hormone. Lancet II. 739-744, 781-783, 1946.

142. _____. Antidiuretic hormone and the factors which determine its release. Proc. Roy. Soc. B 135:25-106, 1947.

143. Voherr, H., N. W. Bradbury, N. Haghoughi, and C. R. Kleeman. Antidiuretic hormone in cerebrospinal fluid during exogenous and endogenous changes in its blood levels. Endo. 83:246-250, 1968.

144. Volicer, L., and C. G. Lowe. Penetration of angiotensin II into the brain. Neuropharmacol. 10:631-636, 1971.

145. Weiner, J. S. The diuretic response of men working in hot and humid conditions. J. Physiol. (London) 103:36 P, 1944.

146. Weinstein, H., R. M. Berne, and H. Sachs. Vasopressin in blood: effect of hemorrhage. Endo. 66:712-718, 1960.

147. Wesson, L. G., Jr. Physiology of the Human Kidney. New York: Grune and Stratton, 1969, p. 465-520.

148. Whitney, J., S. Smith, II, J. Marmorston, H. Goodman, and A. Sellars. Antidiuretic effect of renin in the dog. Am. J. Physiol. 176:419-422, 1954.

149. de Wied, D., M. Palkovitz, T. C. Lee, B. Vander Wal, and W. de Jong. Central nervous and pituitary control of aldosterone secretion in the rat. Progress in Brain Research. Vol. 38. Topics in Neuroendocrinology. J. Kappers and J. Schade (eds.), p. 279-290, 1972.

150. Willumsen, N. B. S., and P. Bie. Tissue to plasma rations of radioactivity in the rat hypothalamo-hypophysial system after intravenous injection of ^{3}H-Lysine ^{8}vasopressin and ^{3}H mannitol. Acta Endo. 60:389-400, 1969.

151. Winterstein, H. The actions of substances introduced into the cerebrospinal fluid and the problem of intracranial receptors. Pharmacol. Rev. 13:71-107, 1961.

152. Wolf, R. L., M. Mandelowitz, J. Roboz, N. Naftchi, and S. Gitlow. Recent studies on I^{131} labelled angiotensin II and analogues. Can. Med. Assn. J. 90:295-298, 1964.

153. Woods, J. W., P. Bard, and R. Blier. Functional capacity of the deafferented hypothalamus: water balance and response to stimuli in the decerebrate cat and rat. J. Neurophysiol. 29:751-767, 1966.

154. Yang, H.-Y. T. Angiotensin converting enzyme in rat brain. Fed. Proc. 31:512 Abs., 1972.

155. Zehr, J. E., J. A. Johnson, and W. W. Moore. Left atrial pressure and plasma osmolality in the unanesthetized ewe. Am. J. Physiol. 217:1672-1680, 1969.