Regional Changes in Rat Brain Angiotensinogen Following Bilateral Nephrectomy

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SUMMARY One approach to establish the existence and functionality of a brain angiotensin system is to demonstrate selective alterations in that system following perturbation of peripheral cardiovascular functions. The present study utilized this approach to quantify regional angiotensinogen levels in the rat brain following bilateral nephrectomy, a perturbation that severely disrupts salt and water homeostasis. Angiotensinogen, the precursor of any centrally-derived angiotensin, was analyzed since it should provide a marker for a putative angiotensin peptidergic system. Net brain angiotensinogen was determined by correcting total tissue concentrations of angiotensinogen with accurate values of contaminating plasma angiotensinogen. The latter was determined by quantifying regional plasma space utilizing tritiated inulin as a marker of cerebral vascular space. It was found that there were no detectable alterations in regional net brain angiotensinogen in the first 24 hours following nephrectomy despite over a twofold increase in plasma angiotensinogen and the absence of significant plasma renin. By 32 hours postnephrectomy, certain areas of the rat hypothalamus and midbrain exhibited significant elevations in net angiotensinogen content. These areas coincided with regions traversed by neural pathways shown to mediate angiotensin-induced drinking or blood pressure elevations. The results lend further support to the concept of an independent brain angiotensin system.

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KEY WORDS

angiotensin • blood brain barrier • brain angiotensinogen • inulin space • nephrectomy • rat

THE existence of cells in the central nervous system (CNS) that respond to and may contain angiotensin II (All) is indicated by several findings including the actions of All on structures within the blood-brain barrier, 1-3 neurons containing All-like immunoreactivity, 4 and All receptors in CNS tissue. 5-6 Recent reports demonstrating the presence of angiotensinogen (the precursor of the angiotensin peptides) in brain tissue 7-8 and cerebrospinal fluid (CSF), 9 and that plasma angiotensinogen does not cross into the CNS in rabbits 10 indicate a possible central origin of the precursor in brain. Also, the finding that adrenalectomy produces a decrease in the angiotensinogen content of specific hypothalamic and midbrain regions11 has further demonstrated that the central levels of the precursor (and presumably All itself) can be altered by changes in adrenal steroid levels.

The purpose of the studies outlined below was to investigate the effect of nephrectomy, which profoundly alters the plasma renin-angiotensinogen system, on the levels of angiotensinogen in selected brain regions. We have found that bilateral nephrectomy produces time-dependent increases in the levels of angiotensinogen in specific hypothalamic and midbrain regions while not affecting other specific regions.

Materials and Methods

Animals and Surgery

Male Sprague-Dawley rats (Hilltop Laboratories), weighing 175 to 250 g, were used for all experiments. Under light ether anesthesia the animals were either bilaterally nephrectomized or had sham surgery, both via dorsal incision. The animals were then housed individually and given rat chow and water ad libitum. Animals were sacrificed by decapitation at 5, 8, 16, 24, or 32 hours post surgery. Surgery was timed so that the animals were always sacrificed between 8:00 and 11:00 p.m. The brains were quickly removed and frozen on dry ice. Whole trunk blood was collected for plasma analysis.
Brain Dissection

Brains were freehand dissected while frozen, with anatomical reference to the König and Klippel atlas. Table 1 describes the regions taken and their content. A description of our cutting procedures is provided in the Appendix.

Brain Angiotensinogen Concentration

Brain angiotensinogen concentration was determined by a modification of the method of Lewicki et al. Each brain region was homogenized by sonication (Branson Sonifier, Danbury, Connecticut) in 225 μl sodium phosphate, 50 mM, pH 7.0. The homogenates were centrifuged at 9000 × g (Brinkman Instruments, Westbury, New York); 200 μl of supernatant were adjusted to 2.3 M in ammonium sulfate, by the addition of 270 μl of a 4.0 M ammonium sulfate solution, and incubated at 4°C overnight to permit complete precipitation of proteins. The ammonium sulfate solutions were next centrifuged (9000 × g), the supernatants discarded, and the pellets resuspended in 150 μl of incubation buffer consisting of 50 mM sodium phosphate, 7 mM disodium EDTA, and 100 μM phenylmercuric acetate, pH 7.0. A 50 μl aliquot was removed for protein determination, and 5 μl of a stock rat kidney renin solution (see below for renin purification procedure) in 25 μl buffer was added to the remaining 100 μl. Two 50 μl aliquots were then incubated 20 hours at 37°C, the reaction terminated by freezing, and the amount of angiotensin I generated was determined by specific radioimmunoassay. Data are expressed as nanograms (ng) of angiotensin I (AI) equivalents per milligram (mg) of protein in the ammonium sulfate pellet.

Regional Brain Plasma Space

Nephrectomized or sham-operated animals were cannulated in the left jugular vein to 8 hours prior to sacrifice. At 5 minutes prior to sacrifice, 100 μCi of 3H-inulin (New England Nuclear, Boston Massachusetts) in 100 μl saline were injected intravenously. The animals were sacrificed by decapitation, and brains and trunk plasma were collected and frozen as described above. The brains were dissected and weighed as above, and the tissue samples, as well as 50 μl plasma samples, were dissolved by overnight shaking with 500 μl of NCS tissue solubilizer (Amersham/Searle Corporation, Chicago, Illinois). Then 10 ml of OCS scintillation fluid (Amersh) was added and tritiated inulin quantitated in a Beckman LS-330 liquid scintillation counter. Data are expressed as % μl plasma per mg tissue (cpm/mg area per cpm/μl plasma × 100).

Simultaneous Regional Brain Plasma Space and Brain Angiotensinogen Content Determination

Nephrectomized or sham-operated animals were cannulated, injected with 3H-inulin, and sacrificed as described above. Each brain region was homogenized in 150 μl of 50 mM sodium phosphate pH 7.0, and a 100 μl aliquot removed and solubilized with 500 μl NCS tissue solubilizer as described above. The remaining 50 μl of homogenate was diluted to 125 μl with additional buffer and centrifuged. A 100 μl sample of supernatant was adjusted to 2.3 M in ammonium sulfate by addition of 135 μl of a 4.0 M ammonium sulfate solution, and protein pellets obtained by centrifugation. The pellets were carefully resuspended in 100 μl of incubation buffer, a 50 μl aliquot taken for protein determination, and 10 μl of incubation buffer containing 5 μl of the stock rat kidney renin solution added to the remaining 50 μl. After overnight incubation at 37°C, the AI generated was quantified by radioimmunoassay.

Plasma Angiotensinogen Concentration

Plasma angiotensinogen levels were determined as previously described. Plasma (5 μl) was added to 295 μl of 100 mM sodium phosphate pH 7.0, containing 7.5 mM disodium EDTA, 0.1 mM phenylmercuric
acetate, and 5 μl of stock rat kidney renin solution. Triplicate 25 μl aliquots were then incubated 20 hours at 37°C. The AI released was quantified by radioimmunoassay and the data expressed as μg AI equivalents per ml of plasma.

Rat Kidney Renin
Renin was prepared by a modification of the methods of Skeggs et al. and Inagami et al. A homogenate of rat kidneys was extracted with toluene and purified by differential ammonium sulfate precipitation (1.2 to 2.3 M). Further purification was achieved with chromatography on DEAE cellulose, Sephadex G150, and Pepstatin-aminohexylsepharose. The final preparation had a specific activity of 46.8 ng AI/hr/mg protein (nephrectomized rat plasma as substrate = 2.2 μM angiotensinogen) and was free of angiotensinase activity as evidenced by incubation with exogenous AI. The renin preparation had a protein concentration of 10 μg/μl.

Serum Electrolytes and Urea
Serum sodium and potassium concentrations were determined with a Corning 430 flame photometer following the protocol supplied with the instrument. Plasma urea concentrations were determined by the method of Searcy et al.

Angiotensin I Radioimmunoassay
The AI was quantified by a specific radioimmunoassay using rabbit antiserum generated against [Asn¹,Val⁵] AI, which was also used as unlabeled standard. The antiserum showed < 1% crossreactivity with [Asp¹,Ile⁵] AI and [Ile⁵] AlIII. The absolute sensitivity of the assay (B/Bo = 50%) was 190 pg. [¹²⁵I]Iodo-AI was obtained from New England Nuclear and purified with Dowex AG1-X10 immediately before use. Free radio-labeled AI was separated from antibody-bound AI with dextran-coated charcoal.

Data Analysis
Unless otherwise stated, data are expressed as the means ± se. Analysis of differences between groups was accomplished by two-factor analysis of variance, and differences at particular time points by the method of Newman-Keuls.

Results
Peripheral Effects of Nephrectomy
Bilateral nephrectomy markedly altered the serum renin, urea, and electrolyte levels. Plasma angiotensinogen levels were also altered; figure 1 illustrates the time course of this well-documented effect. Nephrectomy produced a threefold increase in plasma angiotensinogen, relative to shams, which has been attributed to both a decreased plasma renin activity and an increased synthesis rate by the liver. The increase was apparent at 5 hours after surgery, was maximum by 8 hours, and was maintained through 32 hours. Plasma urea levels increased rapidly following nephrectomy (fig. 2) and were significantly elevated over controls by as early as 8 hours postoperatively. The 32-hour plasma urea levels of nephrectomized animals approached 100 mM, approximately 15 times that of sham controls. Serum electrolytes were also affected by nephrectomy. Serum sodium levels ranged from 145 to 147 mEq/liter in shams and 147 to 150 mEq/liter in nephrectomized animals. Serum potassium levels ranged from 6.7 (8 hours) to 7.1 (32 hours) mEq/liter in shams and 8.4 (8 hours) to 11.5 (32 hours) mEq/liter in nephrectomized animals.
Effect of Nephrectomy on Brain Angiotensinogen without Direct Correction for Plasma Contamination

Initial investigations of local brain angiotensinogen content (BAC) in nephrectomized animals, without correction of the regions for plasma angiotensinogen content, showed a progressive increase in BAC with time (postoperative) relative to sham-operated animals (fig. 3). The increase following nephrectomy was apparent in all 12 brain regions assayed; two-factor analysis of variance indicated that the increase was highly significant ($p < 0.05$) in all regions except the medial basal hypothalamus. In the majority of the 12 regions analyzed following nephrectomy, the changes described a biphasic response with a moderate increase at 8, 16, and possibly 24 hours postoperatively, followed by a greater increase at 32 hours.

The ubiquity of the effect of nephrectomy on brain angiotensinogen and the biphasic nature of the rise in most regions suggested the possibility that the actual effect of nephrectomy was being masked by changes in the regional content of contaminating plasma angiotensinogen. Two possible mechanisms for an apparent increase in regional plasma angiotensinogen were evident. First, if the actual regional plasma space was assumed to remain constant, a portion of the increased angiotensinogen could simply reflect the increase in plasma concentrations induced by nephrectomy (fig. 1). Alternatively, in addition to the evident increase in plasma angiotensinogen, the plasma space of each region may also have increased following nephrectomy. To test these two hypotheses, the effect of nephrectomy on the regional plasma space in rat brain tissue was investigated.

Effect of Nephrectomy on Regional Brain Plasma Space

The measurement of tissue plasma space is most readily accomplished using a labeled compound that is both confined to the space occupied by the plasma and also not metabolized. Tritium-labeled inulin fulfills these criteria by being extremely stable in most tissues, commercially available and inexpensive, and, due to the restrictions imposed by the blood brain barrier, confined to the plasma compartment in most regions of the CNS. Its measured space in brain tissue has also been shown to attain a constant level in less than 5 minutes and to be maintained at that level for several hours. Further, the equilibrium space is quite similar to that obtained using large protein markers of a size similar to that of angiotensinogen.

The effect of nephrectomy on regional brain inulin space as compared to shams is given in table 2. The inulin space, expressed as (μl plasma/mg tissue) × 100, was measured at 5, 16, and 32 hours postintervention in all 12 regions in which BAC measurements were made. The central column shows that no significant differences were found between nephrectomized and control animals in the content of $^3$H-inulin (i.e., plasma) in any of the 12 regions analyzed. Similarly,
no significant time-dependent variations were observed in any region in either group (analysis not shown). These data demonstrate that nephrectomy does not change either the plasma content of any of the regions analyzed nor the vascular permeability to inulin (and presumably angiotensinogen) in these regions. Therefore, regional plasma volume alterations and/or vascular permeability changes do not seem to be responsible for the observed increases in brain angiotensinogen content.

Since the inulin space measurements were independent of time after nephrectomy or sham surgery, the results were combined and group means determined (table 3). Although no significant difference was evident between the nephrectomized and sham groups (tables 2 and 3), the group means for the different regions are kept separate because the physiological perturbation between the groups is profound. The data in table 3 agree quite well with previously published brain plasma space measurements. It is apparent from table 3 that regions known to be highly vascularized and relatively deficient as a blood-brain barrier to plasma proteins (e.g., subfornical organ, medial basal hypothalamus, area postrema) have the largest inulin contents. Similarly the regions with lower plasma spaces (septum, anterior hypothalamus, medial, and lateral dorsal hypothalamus, periaqueductal gray, ventral tegmental area) are regions known to possess a

### Table 2. Effect of Nephrectomy on Regional Brain Inulin Space

<table>
<thead>
<tr>
<th>Area</th>
<th>Sham-operated</th>
<th>Nephrectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 hrs</td>
<td>16 hrs</td>
</tr>
<tr>
<td>Medial preoptic area†</td>
<td>2.2±0.45</td>
<td>1.4±0.27</td>
</tr>
<tr>
<td>Bed nucleus stria terminalis</td>
<td>1.4±0.9</td>
<td>1.1±0.09</td>
</tr>
<tr>
<td>Septum</td>
<td>1.1±0.22</td>
<td>0.9±0.09</td>
</tr>
<tr>
<td>Anterior hypothalamus</td>
<td>1.6±0.22</td>
<td>1.2±0.09</td>
</tr>
<tr>
<td>Subfornical organ</td>
<td>2.6±4.9</td>
<td>2.2±0.36</td>
</tr>
<tr>
<td>Medial basal hypothalamus</td>
<td>2.8±0.27</td>
<td>2.0±0.31</td>
</tr>
<tr>
<td>Lateral basal hypothalamus</td>
<td>1.6±0.04</td>
<td>1.3±0.09</td>
</tr>
<tr>
<td>Medial dorsal hypothalamus</td>
<td>1.2±0.09</td>
<td>1.0±0.13</td>
</tr>
<tr>
<td>Lateral dorsal hypothalamus</td>
<td>1.1±0.04</td>
<td>0.8±0.04</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>1.1±0.04</td>
<td>1.0±0.04</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>1.1±0.09</td>
<td>0.9±0.09</td>
</tr>
<tr>
<td>Area postrema</td>
<td>3.1±0.31</td>
<td>2.2±0.45</td>
</tr>
</tbody>
</table>

*Multiple factor analysis of variance; NS = p > 0.05.
†Determined by the equation \( \frac{\mu l \text{ plasma} \times 100}{mg \text{ area wt.}} \), \( \bar{X} \pm \text{SEM.} \)

### Table 3. Regional Brain Inulin Space Group Mean Values

<table>
<thead>
<tr>
<th>Region</th>
<th>Sham</th>
<th>Nephrectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH anterior hypothalamus*</td>
<td>1.4±0.09</td>
<td>1.3±0.09</td>
</tr>
<tr>
<td>AP area postrema</td>
<td>3.0±0.22</td>
<td>2.4±0.17</td>
</tr>
<tr>
<td>LBH lateral basal hypothalamus</td>
<td>1.5±0.06</td>
<td>1.4±0.12</td>
</tr>
<tr>
<td>LDH lateral dorsal hypothalamus</td>
<td>1.0±0.04</td>
<td>0.9±0.05</td>
</tr>
<tr>
<td>MBH medial basal hypothalamus</td>
<td>2.5±0.11</td>
<td>2.3±0.17</td>
</tr>
<tr>
<td>MDH medial dorsal hypothalamus</td>
<td>1.2±0.07</td>
<td>1.0±0.07</td>
</tr>
<tr>
<td>NST bed nucleus stria terminalis</td>
<td>1.4±0.13</td>
<td>1.2±0.12</td>
</tr>
<tr>
<td>PAG periaqueductal gray</td>
<td>1.0±0.04</td>
<td>0.9±0.07</td>
</tr>
<tr>
<td>POA medial preoptic area</td>
<td>1.8±0.13</td>
<td>2.0±0.17</td>
</tr>
<tr>
<td>SEPT septum</td>
<td>1.0±0.04</td>
<td>1.0±0.07</td>
</tr>
<tr>
<td>SFO subfornical organ</td>
<td>2.5±0.21</td>
<td>2.6±0.18</td>
</tr>
<tr>
<td>VTA ventral tegmental area</td>
<td>1.2±0.06</td>
<td>1.0±0.07</td>
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</table>

* \( \frac{\mu l \text{ plasma} \times 100}{mg \text{ area wt.}} \), \( \bar{X} \pm \text{SEM.} \)
permeability barrier to plasma proteins. These grouped plasma inulin space data provide, therefore, an average value for the quantity of plasma contained in each brain region analyzed.

**Effect of Nephrectomy on Brain Angiotensinogen Content Corrected for Plasma Angiotensinogen Content**

The group means for the individual areas provided a basis for correcting previous BAC data for plasma angiotensinogen concentration contamination. Multiplication of the plasma angiotensinogen concentration of each animal by the mean plasma volume (space) of each brain region should yield a direct measurement of the contaminating angiotensinogen for each region. Subtraction of this angiotensinogen concentration from the total angiotensinogen content of that region then would yield a net BAC. This approach was subjected to experimental test by comparing, in two separate groups of animals, direct correction of regional plasma angiotensinogen contamination through simultaneous measurement of regional plasma brain space and total BAC in the same animal with corrections employing the group mean brain space data of table 3. The latter would permit an a posteriori correction for BAC. The results (table 4) show essentially the same spectrum of significant differences between brain regions of nephrectomized and sham-operated animals by the two experimental methods; only the medial dorsal hypothalamus gave a different result by the two approaches. These results support the use of group mean plasma space data from table 3 to correct our previously obtained regional total BAC time course data for plasma angiotensinogen contamination. Further, these results also indicate the utility of this approach for future studies of components present in both brain tissue and plasma.

The data in table 3 represent a subset of the more extensive data presented in figure 3 and 4. Although several areas exhibit minor disagreements between the table and figures, this reflects experimental limitations associated with the simultaneous measurement of inulin and brain angiotensinogen in small brain tissue samples. This problem does not affect the data in the figures and will be discussed in greater detail elsewhere. Our final conclusions and analyses are based on the more extensive data presented in the figures and represent a conservative approach to the identification of regional differences in brain angiotensinogen following nephrectomy.

**Time Course of Regional Net Brain Angiotensinogen Content Following Nephrectomy**

The time course of the effect of nephrectomy on net regional BAC is illustrated in figure 4. In this experiment the total BAC data described above was corrected for plasma angiotensinogen content using the plasma

**Table 4. Regional Brain Angiotensinogen at 32 Hours after Intervention**

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Brain region</th>
<th>AH</th>
<th>AP</th>
<th>LBH</th>
<th>LDH</th>
<th>MBH</th>
<th>MDH</th>
<th>NST</th>
<th>PAG</th>
<th>POA</th>
<th>SEPT</th>
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<tr>
<td>Areas individually corrected for plasma contamination</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td>X</td>
<td>13.1</td>
<td>15.7</td>
<td>13.6</td>
<td>13.9</td>
<td>12.1</td>
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<td>0.7</td>
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<td>0.5</td>
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Data are expressed as ng angiotensin I equivalents/mg protein (means ± SEM). p values were determined by single-factor analysis of variance.
measurements from each animal and the regional plasma space data from table 3. This correction eliminated the moderate rise previously observed in BAC following nephrectomy but had little effect on the marked increases in some areas seen at 32 hours postoperatively. This indicates that the "moderate" increases evident in all regions (fig. 3) were due solely to the increase in plasma concentration produced by nephrectomy. To test this hypothesis, we measured the BAC, 3H-inulin brain space, and plasma angiotensinogen simultaneously in a group of animals 16 hours following nephrectomy, a time prior to any specific BAC changes but after plasma angiotensinogen levels have reached their maximum. We found that, on the average, plasma angiotensinogen contamination accounted for 16.4% (16.4% ± 1.7%) of the total BAC in shams and 32.2% (32.2% ± 3.2%) in nephrectomized animals at 16 hours postoperatively. Similarly, plasma angiotensinogen concentration rose from 1.56 ± 0.27 μg Al/ml in shams to 3.04 ± 0.19 μg Al/ml in nephrectomized animals. This represented a 1.95-fold increase in mean PAC and a 1.96-fold increase in mean BAC. This analysis confirmed our assumption that early, moderate elevations of uncorrected BAC could reflect plasma contamination and increases in plasma angiotensinogen.

It was apparent from an analysis of figure 4 that measurable angiotensinogen remained in all 12 regions after subtraction of contaminating plasma angiotensinogen and that sham nephrectomy had no effect on BAC in any region. Statistical comparison of the BAC at various times after nephrectomy or sham operation showed that nephrectomy produced no significant difference in the septum, preoptic area, subforminal organ, area postrema, or medial basal hypothalamus. A markedly significant difference was produced by nephrectomy, however, in the nucleus of the stria terminalis, anterior hypothalamus, medial and lateral dorsal hypothalamus, lateral basal hypothalamus, central tegmental area, and area postrema. A posteriori statistical analysis (see legend to fig. 4) of the data from these regions showed that the increase in BAC after nephrectomy was significantly different from control at 24 hours only in the medial dorsal hypothalamus but in all these regions at 32 hours. Since the changes in BAC remaining after correction for plasma angiotensinogen were seen in specific regions and not in others, it may be concluded that we are measuring actual increases in the brain angiotensinogen content of these regions.

Discussion

Before correction for plasma contamination, all brain regions examined following bilateral nephrectomy (except the medial basal hypothalamus) demonstrated a significant increase in BAC (fig. 3). Some of the regions showed moderate increases that tended to plateau after 8 to 16 hours while others exhibited a biphasic pattern consisting of an early plateau phase followed by a marked increase after 24 hours. In addition, the variability (standard deviation) of the data was relatively large. The early increases in BAC (prior to correction) seen in all areas had a similar time course.
to that of the plasma angiotensinogen and indicated that these moderate (and ubiquitous) increases could simply reflect changes in plasma levels and plasma contamination due to residual plasma and/or to an altered blood brain barrier resulting from elevated plasma urea and hyperkalemia which develops following nephrectomy.

Two possible experimental approaches existed to the problem of plasma contamination of brain regions — perfusion of the blood from the brain vasculature or the use of a marker substance in the plasma. Simple perfusion of the cerebral vascular bed with buffer solutions was found to be inadequate for the rapid removal of plasma constituents and actually increased the variability of BAC measurements through its inconsistent removal of plasma angiotensinogen.21 In addition, perfusion yielded no information about the state of the blood-brain barrier, thus necessitating additional experimentation. Use of a marker substance was deemed a superior approach. Tritiated inulin is confined largely to the vascular space in the brain,21,22 does not undergo metabolism, exhibits a similar brain space as albumin,21 and in addition is inexpensive and relatively safe to use. Further, it has been shown to be an excellent marker for changes in the blood-brain barrier and plasma volume in brain tissue.21 An increase in the inulin space would reflect either a breakdown of the barrier to inulin (inulin loss from the plasma to the brain extracellular fluid) or an increase in the vascular volume of the tissue. We first demonstrated that the regional brain inulin space did not change following nephrectomy. This indicated that nephrectomy does not result in a nonspecific increase in the rate of passage of plasma angiotensinogen from the vascular space into the extracellular fluid of the brain and it permitted us to use the inulin space data as a quantitative measure of the plasma volume of each region.

Once the group mean plasma volume of each brain region was known from studies of many animals, the combination of these values with the experimentally determined plasma angiotensinogen content permitted direct computation of the vascular angiotensinogen content of each brain region for a given animal, thus yielding the net BAC. This procedure allowed us to distinguish the effect of nephrectomy on net BAC. The moderate increases in total BAC prior to 16 hours were then shown to be attributable to changes in the plasma angiotensinogen concentration. The variability of the data was also greatly decreased23 and, most significantly, a specific pattern was unmasked of regions responding to nephrectomy with increases in net BAC.

The regions that exhibit increases in net BAC following nephrectomy show a close correspondence to sites that mediate cardiovascular and drinking responses to exogenous All. The brain areas dissected and studied in the present investigation are shown in the Appendix in figure A-1. The anterior hypothalamus, periaqueductal gray, and ventral tegmental area are all sites where exogenous All stimulates drinking and/or vasopressor responses1,21 and that exhibit elevation of BAC following nephrectomy. The preoptic area—anterior hypothalamus border, a very sensitive All drinking site,2 is included within our anterior hypothalamus section. The lateral (basal and dorsal) and medial dorsal hypothalamus also respond to nephrectomy and are regions through which descending drinking and vasopressor pathways pass.2,23 Further, the medial basal hypothalamus exhibits a high density of All immunofluorescence staining.4 Areas that we find do not respond to nephrectomy either are distant to known sites of All actions or are circumventricular organs that appear to respond to plasma All.

Blood pressure was unaffected following nephrectomy over the time frame of this investigation. It has been reported, however, that nephrectomized animals that are maintained longer than 48 hours by special diet or dialysis exhibit hypertension to levels of the renoprival hypertensive syndrome.25 If a brain angiotensin system were involved in the latter hypertensive process, changes in the peptidergic system, as reflected in regional BAC, might be evident by 32 hours. To examine this possibility we may compare brain regions where changes in net BAC are found with their proximity to known neuron pressor pathways. There is some correlation between areas that exhibit elevated BAC and the location of the vasopressor pathways of Enoch and Kerr;25 however, discrepancies exist at the preoptic area (where the BAC does not change but where the pathway is thought to originate) and at the medial dorsal hypothalamus (where the BAC increases greatly but where the pathways do not traverse). It is premature to exclude the possibility that selected areas exhibit changes in net BAC that could translate (in the intact animal) into hypertension at a later time.

Regions in which net BAC increases following nephrectomy correlate extremely well with the two drinking pathways described by Swanson et al.2,23 Since All produces drinking in the preoptic anterior hypothalamus and periaqueductal gray areas2 but does not produce drinking in the lateral or medial dorsal hypothalamus (MDH), two possible models for brain angiotensinogen-containing and All-producing pathways are tentatively suggested: first, the drinking pathways may be All-producing cells themselves, responding to All and releasing All in the midbrain; or, second, the increases in BAC may reflect All cells that originate along the pathways (perhaps in the lateral hypothalamus or midbrain) and project rostrally to the preoptic area—anterior hypothalamus to modulate (stimulate) the drinking pathway. Both models are compatible with the known circuitry of the lateral hypothalamus,27 but no model can at present be more than speculation.

Definitive data on the site(s) of synthesis of brain angiotensinogen are not yet available; however, several aspects of the current investigation as well as results from this and other laboratories strongly indicate that brain angiotensinogen is not of plasma origin. In the present study, it was found that changes in net BAC induced by nephrectomy are observed only in specific regions while other regions exhibit no changes. In addition, the time course of the BAC changes in those
areas are similar to each other and very different from that of the plasma angiotensinogen. One obvious explanation of our observations could be that selective changes in the permeability to plasma angiotensinogen occurred 24 hours after nephrectomy in those regions that exhibited increases; however, this interpretation was not consistent with the inulin space experiments where no changes were found in inulin permeability.

Another possible explanation of the net BAC increase relative to the plasma is that activation of a specific plasma angiotensinogen uptake system occurred in those regions where net BAC increased. The observation that the net BAC increase occurs only after 24 hours argued against this explanation, as such a hypothetical uptake system would need to have a long latency (> 24 hours) before exerting an influence on the BAC. In addition, Morris and Reid\cite{28} and Printz et al.\cite{10} have shown that plasma angiotensinogen does not cross into the CNS in the dog and rabbit, respectively.

A third possible explanation relating plasma to brain angiotensinogen in these experiments is that the level of CSF angiotensinogen is increased following nephrectomy and contaminates the brain regions in which net BAC is seen to increase. This argument is not supported by the independent report that CSF angiotensinogen does not increase following nephrectomy.\cite{29} Also, there is no correlation between the locations of the areas studied relative to the cerebral ventricles and whether or not their levels of net BAC increased. Finally, results from several laboratories including our own indicate that biochemical differences exist between plasma and brain angiotensinogen (Printz et al., Reid et al.,\cite{30} Morris and Reid\cite{11}).

The arguments presented here, while indicating that net brain angiotensinogen is not of plasma origin, do not provide direct information as to a specific site of origin for it. Correction of the total BAC for plasma contamination does not reduce the net BAC levels to zero in any region, even those that do not respond to nephrectomy. The low levels of net BAC found in some regions (septum, hippocampus, striatum, cortex, cerebellum\cite{11},\cite{23} (also T.J. Gregory, unpublished observations, 1980) may be accountable to CSF angiotensinogen contamination; however, the relatively high levels in the preoptic area and medial basal hypothalamus (areas that do not respond to nephrectomy) cannot be similarly explained. This indicates that regions that respond to nephrectomy are doing so specifically and that those with high levels of angiotensinogen that do not respond to nephrectomy are also doing so specifically. This argument, taken with the observation that the majority of regions with high BAC are within the blood-brain barrier, further indicates that brain angiotensinogen is of brain origin. This interpretation is also consistent with the recently reported release of angiotensinogen from brain slices.\cite{32}

A physiological meaning to the increases in net BAC induced by nephrectomy would be of foremost interest. In this regard, we have shown that it is of significance that areas that respond to adrenalectomy and corticosterone replacement\cite{33} are not the same as those that respond to nephrectomy. We have proposed that this may reflect a specific steroid hormone-mediated process.\cite{10},\cite{23} The finding that nephrectomy and adrenalectomy show a different pattern of responding areas indicates that the "stimulus" of the brain peptidergic system is different in the two physiological perturbations. Angiotensinogen is the biosynthetic precursor of All and has no other known function. Therefore, the underlying assumption is that the localization of brain angiotensinogen is related to the functions of All in the CNS. An increase in regional BAC could reflect an increase in brain angiotensinogen synthesis secondary to an increased turnover of angiotensinogen to All; alternatively, an increased BAC may reflect a "back-up" of precursor secondary to a decreased turnover to All. In the absence of actual measurements of brain angiotensinogen turnover, changes in All-mediated responses induced by nephrectomy may suggest one mechanism over the other. Blood pressure is not markedly affected by nephrectomy; however, we have found that All-induced drinking is markedly elevated at 24 hours after nephrectomy.\cite{33} This increase in induced drinking favors the second mechanism because it may reflect an up-regulation of putative All-drinking receptors in response to a decrease in All formation. Therefore, we speculate that the alterations in net BAC could reflect the animal's response to a severe interruption of peripheral salt and volume homeostasis.

**Appendix**

**Description of Brain Dissection**
The brains were removed and frozen on dry ice in less than 3 minutes. The freezing was done on precooled aluminum foil with the dorsal surface of the brain down, and the tissue was stored at −70°C for up to 1 week prior to dissection. The brains were dissected free-hand with a razor blade on a precooled glass plate while frozen. Regions dissected are shown in figures A-1 and A-2. Fine tissue slices were taken by making coronal sections as follows:

**Slice 1.** The widest portion of the olfactory tubercle to the posterior edge of the olfactory tubercle.

**Slice 2.** The posterior edge of the olfactory tubercle to the posterior edge of the optic chiasm.

**Slice 3.** The posterior edge of the optic chiasm to the anterior edge of the mamillary bodies.

**Slice 4.** The medial point of origin of the cerebral peduncles to the anterior edge of the pons.

**Slice 5.** Approximately 1 mm rostral to the obex to the caudal edge of the obex.

Blocks of tissue were then removed from each slice as follows. While viewing the posterior surface of Slice 1, the block of tissue bounded by the fornices and lateral edges of
Rostral regions of the rat brain dissected and assayed for angiotensinogen content for comparison with reported hypothalamic drinking and blood pressure pathways. The regions assayed for angiotensinogen content (from table 1) are enclosed in boxes.
FIGURE A-2. Caudal regions of the rat brain dissected and assayed for angiotensinogen content for comparison with reported hypothalamic drinking and blood pressure pathways. The regions assayed for angiotensinogen content (from table 1) are enclosed in boxes.
the optic nerves was designated POA, the block of tissue bounded by the lateral ventricles, corpus callosum, and striae medularis was designated SEPT, and the block of tissue remaining between the POA and SEPT containing the fornices and the bed nucleus of the stria terminalis was designated NST. While viewing the posterior surface of Slice 3, cuts were made horizontally through the fornices and along a line connecting the ventral edges of the optic tracts and vertically (perpendicular to the horizontal cuts) through the most medial edge of the internal capsule and the fornices. The dorsal lateral blocks were combined and designated LDH, the ventral lateral blocks were combined and designated LBH, the medial dorsal block was designated MDH, and the medial ventral block was designated MBH.

While viewing the anterior surface of Slice 5, a block of tissue bounded by the dorsal, ventral, and lateral edges of the mid-brain periaqueductal gray was designated PAG, and a block of tissue bounded laterally by the medial margin of the substantia nigra and dorsally by a horizontal cut through the ventral margin of the central gray (including the interpeduncular nucleus) was designated VTA. While viewing the rostral surface of Slice 5, a block of tissue bounded by the lateral and ventral margins of the gray tissue surrounding the area postrema was designated AP. Anatomical reference was to the stereotaxic atlas of König and Klippel and the major constituents of each tissue block are described in table 1.

Each tissue block was weighed to the nearest 0.1 mg on a Mettler analytical balance. In the cases in which any tissue weight varied by more than two standard deviations from the mean of all those in a particular experiment the data from that entire brain were not included.

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