THE EFFECT OF BRAIN EXTRACTS ON URINARY SODIUM EXCRETION OF THE RAT AND THE INTRACELLULAR SODIUM CONCENTRATION OF RENAL TUBULE FRAGMENTS

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SUMMARY

1. Extracts were prepared from bovine hypothalamus and cerebral cortex by gel filtration on Sephadex G-25 and G-50.

2. The vasopressin in the hypothalamic extracts was inactivated with thioglycollate and the effectiveness of inactivation was tested in the alcohol-anaesthetized rat.

3. The inactivated hypothalamic extracts caused a significant rise, and the cortical extracts an insignificant fall, in the urinary sodium excretion of the conscious rat.

4. Incubation of tubule fragments in hypothalamic extracts caused a significant rise in intracellular sodium concentration of the tubules when compared with incubation in Ringer, whereas incubation in cortical extracts caused a rise which was not significant. Nevertheless the rise in intracellular sodium concentration produced by incubating the tubules in hypothalamic extracts was not significantly different from the rise produced by incubation in cortical extracts.

Key words: natriuretic hormone, brain extracts, urinary sodium excretion, sodium transport.

Expansion of the body fluids causes a rise in urinary sodium excretion. This rise is still manifest when dilutional, nervous, arterial and venous pressure changes on the kidney have been excluded, and when changes in aldosterone secretion have been nullified by the previous administration of large amounts of deoxycorticosterone acetate (DOCA) (Schrier & de Wardener, 1971; Bengele, Houttuin & Pearce, 1972; Sonnenberg, Veress & Pearce, 1972). In addition, extracts prepared from plasma and urine of individuals and animals with expanded body fluids have been shown to possess natriuretic activity (Sealey, Kirshman & Laragh, 1969; Sealey & Laragh, 1971; Viskoper, Czaczkas, Schwartz & Ullman, 1971; Brown, Koutsaimanis

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& de Wardener, 1972). Similarly blood and extracts of urine have been shown to inhibit sodium transport (Nutbourne, Howse, Schrier, Talner, Ventom, Verroust & de Wardener, 1970; Clarkson & de Wardener, 1972). These findings have led to the conclusion that urinary sodium excretion is controlled by a hormonal substance other than aldosterone. The work of Lichardus & Ponec (1972) suggests that the posterior pituitary is involved in the natriuresis of blood volume expansion, and Gitelman & Blythe (1972) have reported that extracts of posterior pituitary contain a natriuretic substance which they consider is not vasopressin. The following experiments were performed to investigate the possibility that a natriuretic substance other than vasopressin might originate in the hypothalamus.

METHODS

Preparation of brain extracts

Fragments of bovine hypothalamus and cerebral cortex (Fig. 1) were obtained from the Endocrine Research Supply Co., San Mateo, California. The animals from which the tissue was obtained were killed by a captive bolt technique. No electricity was used in the stunning or slaughter of the animals. The tissue was separated within 1 h of death and placed immediately on dry ice. Extracts were prepared from hypothalamus and cerebral cortex by a modification of the method used by Fawcett, Reed, Charlton & Harris (1968) to prepare luteinizing hormone-releasing factor. The appropriate portions of ten brains (total weight 35-45 g) were freeze-dried, kept overnight in 1500 ml of acetone and homogenized in 300 ml of acetone at room temperature. The suspension was then filtered and the insoluble residue was washed with ether and dried under vacuum. The residue was extracted twice with 60 ml of 2·0 mol/l acetic acid. The pooled extracts were filtered through a thin layer of kieselguhr on a sintered-glass funnel and freeze-dried. The freeze-dried material was extracted again with 5 ml aliquots of acetic acid so that the total volume was 40 ml. Acetone (32 ml) was then added and the

Fig. 1. Anatomical origin of hypothalamic and cortical fragments.
Brain extracts and urinary sodium excretion

The resulting solution was centrifuged. The precipitate produced by the addition of 400 ml of ether to the acetic acid-acetone supernatant was separated by centrifuging and dried in a current of air. This material was then gel filtered on columns of Sephadex G-25 (see below).

**Fig. 2.** Elution pattern obtained from desalting the protein material derived from ten hypothalamic or cortical fragments on Sephadex G-25. The hatched area represents the fraction which was refiltered on Sephadex G-50. ———, Absorption at 254 nm.

**Fig. 3.** Elution pattern obtained from gel filtration on Sephadex G-50 of the accumulated first fractions from three separate Sephadex G-25 separations. The hatched area represents the fraction tested for natriuretic and antidiuretic activity. ———, Absorption at 254 nm.

and subsequently certain fractions were refiltered on Sephadex G-50 (see below). All fractionations were carried out at +5°C. The samples and eluent were pumped through the columns by the use of LKB standard Perspex pumps. The eluent was 0.3 mol/l acetic acid and timed collections of 10 min duration were used throughout. The absorption of the fractions was
measured at 254 nm and the fractions were collected into an automatic fraction collector (LKB Ultrorac). The presence of salt was detected manually in an Eppendorf flame photometer.

Gel filtration on Sephadex G-25. This was carried out on columns 2.5 cm x 90 cm. The material prepared from ten brain fragments was dissolved in 10 ml of 0.3 mol/l acetic acid. The solution which contained large quantities of various salts, was applied to the columns and eluted at a flow rate of 40 ml/h. Three fractions appeared before and three after that which contained the salts (Fig. 2). The first fraction was freeze-dried and kept at −20°C.

Gel filtration on Sephadex G-50. This was carried out on columns 2.2 cm x 170 cm. The flow rate was 20 ml/h. The accumulated first fractions from three separate Sephadex G-25 separations were dissolved in 3 ml of 0.3 mol/l acetic acid. A portion (0.03 ml) of 1 mol/l sodium chloride was added to the now desalted sample before it was applied to the columns so that the point at which salts emerged could be traced. Three fractions appeared before and one after that which contained the added sodium chloride (Fig. 3). The third fraction, which was found to be the most active, was freeze-dried and kept at −20°C until assayed for antidiuretic and natriuretic activity, or for its effect on the intracellular sodium concentration of suspensions of renal tubule fragments.

Preparation of extracts for assay procedures

These preparations were carried out on the day of the assay. Extracts which were tested in vivo by rat assay were dissolved in 0.278 mol/l dextrose solution containing 10 mmol/l NaCl (referred to subsequently as dextrose-saline) and the pH was adjusted to 7.4 with 0.5 mol/l NaOH. Extracts tested for an effect on the intracellular sodium concentration of renal tubule fragments incubated in vitro were dissolved and adjusted by a method previously described (Clarkson & de Wardener, 1972) so that the pH and electrolyte concentration were in the physiological range. In the final solution, whether it was prepared for in vivo or in vitro assay, 1 ml contained material derived from ten brains. In most experiments 0.1 ml of the solution was set aside for the measurement of protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

Assay and inactivation of vasopressin

Hypothalamic extracts were assayed for vasopressin in the alcohol-anaesthetized water-loaded rat by a technique previously described (Forsling, Jones & Lee, 1968). Before testing for natriuretic activity, extracts prepared both from hypothalamus and cortex were treated with thioglycollate. A portion (0.1 ml) of 0.2 mol/l thioglycollic acid was added to 1 ml of dissolved extract and the pH was adjusted to between 7.4 and 7.5 with 1 mol/l sodium carbonate. The solution was allowed to stand at 25°C for 1 h. All of the hypothalamic extracts were tested for completeness of inactivation with the vasopressin assay technique described above (Forsling et al., 1968). It was assumed that all the remaining antidiuretic activity after treatment with thioglycollate was due to vasopressin; in this way the calculation of the amount of vasopressin contained in the sample was the maximum possible.

Vasopressin does not influence the intracellular sodium concentration of renal tubule fragments (Clarkson & de Wardener, 1972). Extracts in which tubule fragments were incubated were therefore not treated with thioglycollate.
Brain extracts and urinary sodium excretion

Natriuretic effect of vasopressin

Experiments were carried out to determine whether the small amount of vasopressin remaining in some of the extracts after treatment with thioglycollate (see below) were sufficient to cause a rise in the urinary sodium excretion of the conscious rat preparation used for assessing the natriuretic activity of the extracts. Dextrose–saline (0.3 ml) containing 20 and 30 μunits of synthetic arginine vasopressin (Sigma) was injected into the tail vein. The difference in the urinary sodium excretion of the rats due to an injection of vasopressin was compared with control rats which received 0.3 ml of dextrose–saline alone.

To test whether inactivation of the antidiuretic property of vasopressin completely removed its natriuretic activity, the urinary sodium excretion of the conscious rat preparation used for assessing natriuretic activity was measured after injection of 0.3 ml of dextrose–saline containing 15 μunits of thioglycollate-treated arginine vasopressin.

Assay for natriuretic activity

Vasopressin-inactivated hypothalamic extracts and thioglycollate-treated cortical extracts were tested for natriuretic activity by a technique which has been described previously (Brown et al., 1972). A portion (0.3 ml) of the final solution of dissolved extracts was injected into the tail vein of the conscious Wistar rat. Urine was collected during 10 min periods. The mean urinary sodium excretion during four control periods was compared with the mean urinary sodium excretion during the eight periods after the injection of the extracts. The difference in urinary sodium excretion due to the extract was compared with the difference in urinary sodium excretion of control rats which received 0.3 ml of thioglycollate-treated dextrose–saline alone. Hypothalamic and cortical extracts 1–8 (Tables 2 and 3) were each assayed on two rats. One rat was injected with one of the extracts and the other with dextrose–saline. Hypothalamic extracts 9–11 and cortical extract 9 were each assayed on six rats. Three rats were injected with an extract and three with dextrose–saline. In these assays the results shown in Tables 2 and 3 represent the means obtained in each group of three rats.

Test for an effect of the brain extracts on intracellular sodium concentration of tubule fragments

This test was performed with a modification of Burg & Orloff's (1962) technique, which has been described previously (Clarkson, Talner & de Wardener, 1970). The intracellular sodium concentration of tubule fragments incubated in seven extracts obtained from hypothalamus and nine extracts obtained from cortex was compared with that of tubules incubated in Krebs–Ringer bicarbonate alone.

Statistical analysis

The data did not follow a normal distribution. The non-parametric Mann-Whitney U test and Wilcoxon matched pairs signed-ranks test (Siegel, 1956) were therefore used. The level of significance for a two-tailed test was estimated throughout.

RESULTS

Natriuretic effect of small quantities of vasopressin (Table 1)

Small amounts (20 and 30 μunits) of vasopressin were tested in three and four rats respec-
tively. The mean change in urinary sodium excretion due to the injection of vasopressin less the change due to the injection of dextrose-saline into control rats was 0.06 and 0.07 μmol/min. Greater amounts of vasopressin caused a natriuresis. Therefore extracts inactivated with thioglycollate which contained more than 25 μunits of vasopressin in 0.3 ml of final solution were rejected.

Vasopressin content of cortical and hypothalamic extracts and the effect of inactivated vasopressin on urinary sodium excretion

Cortical extracts were found to possess no antidiuretic activity. The hypothalamic extracts contained up to 8 μunits of vasopressin in 0.3 ml of the dissolved extracts prepared for assay. After treatment with thioglycollate six of the hypothalamic extracts contained no antidiuretic activity, four contained less than 10 μunits and one contained 24 μunits in 0.3 ml of final solution of dissolved extract.

TABLE 1. Effect of small amounts of arginine vasopressin on the urinary sodium excretion of the conscious rat

AVP–DS values show the difference between arginine vasopressin and dextrose–saline results.

<table>
<thead>
<tr>
<th>Urinary sodium excretion (μmol/min)</th>
<th>(\text{Arginine vasopressin} )</th>
<th>(\text{Dextrose–saline} )</th>
<th>AVP–DS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Δ</td>
</tr>
<tr>
<td>(μunits/0.3 ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3.16</td>
<td>2.67</td>
<td>-0.49</td>
</tr>
<tr>
<td>30</td>
<td>2.21</td>
<td>2.35</td>
<td>+0.14</td>
</tr>
</tbody>
</table>

The mean urinary sodium excretion in the four control periods before the injection of 15 μunits of inactivated arginine vasopressin into six rats was 2.67 μmol/min and in the eight periods after the injection was 2.82 μmol/min. The mean urinary sodium excretion of four control rats which received thioglycollate-treated dextrose–saline at the same time as the six rats given the inactivated arginine vasopressin rose from a mean of 2.98 to 3.58 μmol/min.

Effect of hypothalamic extracts on the urinary sodium excretion of the rat (Table 2)

The protein content of the extracts varied between 1.9 and 7.3 mg in 0.3 ml of the final solution which was injected into the rat. The mean urinary sodium excretion in the four control periods before the injection of the eleven hypothalamic extracts into seventeen rats was 2.7 μmol/min and it rose to 3.52 μmol/min in the eight periods after the injection (2P = 0.01). The mean urinary sodium excretion of the seventeen control rats which received thioglycollate-treated dextrose–saline solution rose from a mean of 2.11 μmol/min to 2.32 μmol/min; this change was not significant. The rise in urinary sodium excretion due to the extracts was significantly greater than that due to the injection of the dextrose–saline (2P<0.05). The urine flow showed no consistent change during the eight periods which followed the injection of the extract.
**TABLE 2.** Protein content of hypothalamic extracts and the urinary sodium excretion of the conscious rat before and after injection of 0.3 ml of vasopressin-inactivated extract and thioglycollate-treated dextrose–saline

ΔHE—ΔDS values show the difference between results for vasopressin-inactivated extract and thioglycollate-treated dextrose–saline.

NS = not significant.

<table>
<thead>
<tr>
<th>Extract no.</th>
<th>Injected Lowry protein (mg)</th>
<th>Urinary sodium excretion (µmol/min)</th>
<th>Vasopressin-inactivated hypothalamic extract</th>
<th>Thioglycollate-treated dextrose–saline</th>
<th>ΔHE—ΔDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Δ</td>
<td>Before</td>
</tr>
<tr>
<td>1</td>
<td>5.1</td>
<td>1.47</td>
<td>2.00</td>
<td>+0.53</td>
<td>1.12</td>
</tr>
<tr>
<td>2</td>
<td>7.3</td>
<td>2.90</td>
<td>3.78</td>
<td>+0.88</td>
<td>2.58</td>
</tr>
<tr>
<td>3</td>
<td>7.6</td>
<td>3.29</td>
<td>2.68</td>
<td>−0.61</td>
<td>2.75</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>2.26</td>
<td>4.51</td>
<td>+2.25</td>
<td>2.10</td>
</tr>
<tr>
<td>5</td>
<td>6.1</td>
<td>1.36</td>
<td>2.55</td>
<td>+1.19</td>
<td>3.16</td>
</tr>
<tr>
<td>6</td>
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<td>4.81</td>
<td>6.69</td>
<td>+1.88</td>
<td>3.16</td>
</tr>
<tr>
<td>7</td>
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<td>4.18</td>
<td>4.26</td>
<td>+0.08</td>
<td>1.97</td>
</tr>
<tr>
<td>8</td>
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<td>1.42</td>
<td>2.05</td>
<td>+0.63</td>
<td>1.67</td>
</tr>
<tr>
<td>9</td>
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<td>3.09</td>
<td>+0.56</td>
<td>2.44</td>
</tr>
<tr>
<td>10</td>
<td>3.7</td>
<td>3.52</td>
<td>4.00</td>
<td>+0.48</td>
<td>1.93</td>
</tr>
<tr>
<td>11</td>
<td>2.3</td>
<td>1.97</td>
<td>3.13</td>
<td>+1.16</td>
<td>2.17</td>
</tr>
<tr>
<td>Mean±sd</td>
<td>2.70</td>
<td>3.52</td>
<td>+0.82</td>
<td>±1.16</td>
<td>2.11</td>
</tr>
</tbody>
</table>

(2P = 0.01) (NS) (2P < 0.05)
TABLE 3. Protein content of cortical extracts and the urinary sodium excretion of the conscious rat before and after injection of 0.3 ml of thioglycollate-treated extract and dextrose-saline

$\Delta$CE-$\Delta$DS values show the difference between the results for thioglycollate-treated extract and dextrose-saline. NS = not significant.

<table>
<thead>
<tr>
<th>Extract no</th>
<th>Injected Lowry protein (mg)</th>
<th>Urinary sodium excretion ((\mu)mol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thioglycollate-treated cortical extracts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>1</td>
<td>3.6</td>
<td>2.15</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>2.16</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>2.91</td>
</tr>
<tr>
<td>4</td>
<td>3.1</td>
<td>4.66</td>
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<td>5</td>
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<tr>
<td>8</td>
<td>4.5</td>
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<td>9</td>
<td>8.6</td>
<td>4.86</td>
</tr>
<tr>
<td>Mean $\pm$ SD</td>
<td></td>
<td>3.58 $\pm$ 0.76</td>
</tr>
</tbody>
</table>

(\(2P < 0.01\)) (\(2P < 0.05\)) (NS)
Effect of cortical extracts on the urinary sodium excretion of the rat (Table 3)

The protein content of the extracts varied between 2.2 and 8.6 mg in 0.3 ml of the final solution injected into the rat. The mean urinary sodium excretion in the four control periods before the injection of the nine cortical extracts into eleven rats was 3.58 μmol/min and in the eight periods after the injection it was 2.93 μmol/min (2P<0.01). The mean urinary sodium excretion in the eleven control rats which received thioglycollate-treated dextrose–saline fell from 3.35 to 2.61 μmol/min (2P<0.05). The fall in urinary sodium excretion due to the in-
Injection of the cortical extracts was not significantly different from that due to the dextrose-saline.

Comparison of the effect of hypothalamic and cortical extracts on the urinary sodium excretion (Tables 2 and 3)

The change in urinary sodium due to the injection of hypothalamic extracts less the change due to an injection of dextrose-saline (HE-DS) was significantly greater ($2P<0.02$) than the change in urinary sodium excretion due to the injection of cortical extracts less the change due to an injection of dextrose saline (CE-DS).

Intracellular sodium concentration of tubule fragments incubated in extracts of hypothalamus and cortex

The intracellular sodium concentration of tubule fragments incubated in hypothalamic extracts was significantly higher than that of tubules incubated in control buffer. The mean difference was $+7.2$ mmol/l of cells ($2P<0.02$). The mean intracellular sodium concentration of tubule fragments incubated in cortical extracts was also higher than that of tubules incubated in control buffer, but the mean difference, which was $+3.1$ mmol/l of cells, was not significant. The increase in intracellular sodium concentration of the tubules incubated in hypothalamic extracts, however, was not significantly greater than the increase due to incubation in cortical extracts.

DISCUSSION

Smith (1957) was the first to suggest that urinary sodium excretion in man might be controlled by a circulating substance originating in the hypothalamus. He proposed that it might be an antinatriuretic hormone. There are several examples of neurosecretory substances which influence urinary sodium excretion. In mammals vasopressin and oxytocin may increase urinary sodium excretion in the rat, dog, camel and sheep, but not in man (Bentley, 1971). But vasopressin and oxytocin are released in response to a contraction of body fluids volume, whereas blood and urine become natriuretic upon expansion of body fluids. Nevertheless, since non-mammalian neurohypophyseal hormones and analogues of these hormones have a mainly natriuretic action (Jard & Morel, 1963; Chan & du Vigneaud, 1970; Cort, Strub, Häusler & Rudinger, 1973; Gillessen, Studer & Rudinger, 1973; Plíška, Vašák, Ruter & Rudinger, 1973), it is possible that a naturally occurring natriuretic substance may exist in the neurohypophyseal region which is released by an increase in body fluid volumes. This possibility gains some support from the evidence that electrolytic lesions of the posterior hypothalamus diminishes the natriuretic response to volume expansion (Cort & Lichardus, 1963; Lichardus, Joneč & Strážovcová, 1969).

The results reported here suggest that in the hypothalamus there is a natriuretic substance other than vasopressin or oxytocin, which is not present in the cerebral cortex. It was imperative that the extracts injected into the assay rats should contain either no vasopressin or an amount of vasopressin which did not cause a natriuresis, and that they should contain only small quantities of salt. There may be some residual antidiuretic activity after inactivation with thioglycollate, and small residual amounts of vasopressin ($<25$ μunits) may be sufficient to cause a natriuresis. The initial separation was therefore carried out on Sephadex G-25 to remove most of the vasopressin, and all the salt from the extract. The first fraction eluted from
the Sephadex G-25 column, which contained relatively small amounts of vasopressin and no salt, was therefore separated further on Sephadex G-50. The natriuretic fraction eluted from the Sephadex G-50 column overlapped the area where the salts appeared, which suggests that the active substance has a molecular weight of approximately 1500. As only the first fraction from the G-25 column was used, it is probable that much of the natriuretic substance present in the nervous tissue was not extracted.

Application of the thioglycollate-inactivation test in the manner described eliminates the possibility that vasopressin is responsible for the natriuretic response. The content of oxytocin in the hypothalamus is much less than that of vasopressin (Van Dyke, Adamson & Engel, 1955; Bisset, Errington & Richards, 1973), and as thioglycollate is equally effective in inactivating oxytocin it is unlikely that the natriuretic effect of the extract was due to this hormone.

Other active substances of low molecular weight (100-200) are known to be present in the hypothalamus, e.g. acetylcholine, serotonin, dopamine, noradrenaline and polyamines such as spermidine, and angiotensin (Piezzi, Larin & Wurtman, 1970; Myers & Beleslin, 1970; Bertler & Rosengren, 1959; Bertler, 1961; Philippu, Heyd & Burger, 1970; White, Cohen, Rippel, Story & Schally, 1968; Fischer-Ferraro, Nahmod, Goldstein & Finkielman, 1971). However, the initial extraction procedures should have oxidized dopamine and noradrenaline, and the remainder would have been discarded with the salts during the first fractionation on Sephadex G-25. Unpublished work (P. R. Brown & K. G. Koutsaimanis) has also shown that spermidine and serotonin do not cause the type of natriuresis observed. The possibility that the extracts might contain some angiotensin cannot be eliminated. Barraclough, Jones & Marsden (1967) have shown that in conscious rats a continuous intravenous infusion of low concentrations of angiotensin causes a moderate but persistent fall in urine flow and sodium excretion, whereas higher concentrations cause a profound initial fall and then a rise in urine flow and sodium excretion. The natriuretic hypothalamic extracts did not cause any consistent change in urine flow. It is therefore unlikely that angiotensin could have been responsible for the natriuretic effect of these extracts.

It has been shown that the natriuretic substance in the plasma (Clarkson et al., 1970) and the urine (Clarkson & de Wardener, 1972) from animals and man in whom the extracellular fluid volume has been expanded inhibits sodium transport of renal tubule fragments. It was for this reason that the effect of the brain extracts on the renal tubule fragments was studied. The intracellular sodium concentration of tubule fragments incubated in hypothalamic extracts was significantly higher than that of tubules incubated in the control Ringer solution, and the higher intracellular sodium concentration of tubules incubated in cortical extracts was not significant. Nevertheless the effect of hypothalamic extracts on intracellular sodium concentration of tubule fragments was not significantly different from that due to cortical extracts. Unfortunately it is therefore impossible to draw any definitive conclusion from this part of the work.

There are several peptides other than vasopressin and oxytocin, bound to neurophysin in the posterior pituitary, the physiological functions of which have not yet been defined (Lande, Lerner & Upton, 1965; Preddie & Saffran, 1965; Shin, Labella & Queen, 1970; Labella, Shin, Vivian & Dular, 1971; Sachs, Goodman, Osinchak & McKeloy, 1971). Since oxytocin and vasopressin originate in the hypothalamus, it is possible that these unidentified peptides are secreted in a similar fashion and could be present in the hypothalamus. The extraction procedures described here suggest that the natriuretic material present in the hypothalamic
extracts is attached to protein. It is possible therefore that the natriuretic activity of the material is due to one of these unidentified peptides.

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Brain extracts and urinary sodium excretion


