Roles of aldosterone and angiotensin in maturation of sodium appetite in furosemide-treated rats

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Rowland, Neil E., and Kenneth R. Morian. Roles of aldosterone and angiotensin in maturation of sodium appetite in furosemide-treated rats. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1453–R1460, 1999.—When rats are treated with furosemide, there is a rapid natriuresis. However, increased sodium appetite does not occur until some time later. One hypothesis to explain this delay is that increased circulating levels of the hormones of sodium depletion prime or sensitize the brain circuits involved in sodium appetite, perhaps by induction of target gene(s). In the present study, we describe the time course of the temporal maturation of sodium appetite after furosemide treatment and the associated changes in plasma levels of ANG II and aldosterone and in plasma volume. Sodium appetite is modest 3 h after furosemide treatment, is increased after 12 h, and is still larger after 24 h. This pattern is evident with repeated testing. Plasma levels of aldosterone and plasma renin activity are substantially increased 3 h after furosemide treatment, and so the NaCl appetite cannot result simply from progressively increasing levels of these hormones. Furthermore, activation of the subfornical organ and the ventral lamina terminalis, assessed with c-Fos immunocytochemistry, did not differ across these three times. Metyrapone, an inhibitor of adrenal steroid synthesis, was used to examine sodium appetite in the absence of elevations in aldosterone after furosemide treatment. Although metyrapone effectively blocked the increase in aldosterone, it was without effect on the appetite 3 or 4 h after furosemide treatment. Furthermore, elevations of plasma aldosterone by the use of minipumps for several days before furosemide treatment did not prime or potentiate but instead tended to inhibit the induced sodium appetite, despite achieving levels of aldosterone and plasma renin activity typically associated with a robust sodium appetite. Infusions of DOCA gave a similar result. Lastly, minipump infusions of ANG II also did not potentiate sodium appetite. Thus neither addition nor subtraction of these hormones alone influenced sodium appetite under these conditions.

circumventricular organs; c-Fos; metyrapone; deoxycorticosterone acetate

ONE OF THE MOST popular paradigms for the laboratory study of sodium appetite in rats involves acute administration of a short-acting natriuretic agent such as furosemide followed by ~24 h with salt-free food and water (7, 13, 15). At the end of this time, rats ingest concentrated (2–3× isotonic) NaCl in an amount often approaching 5 meq, which is two to three times the estimated sodium loss. It has been shown that the hormones aldosterone and ANG II, and possibly other factors, act synergistically in determining this sodium appetite (4, 6, 9, 16). One difficulty that may arise with this paradigm in regard to study of the underlying mechanism is that many sequential processes could occur during the 24 h, and so accurate targeting or measurement of critical steps is difficult. A more recently developed test paradigm (3, 9) that overcomes this problem by inducing sodium appetite with short latency (2–3 h) involves administration of furosemide along with a low dose of the converting-enzyme inhibitor captopril. This latter chemical is thought to essentially amplify the brain ANG II signal from the periphery (9), but because captopril is not specific for angiotensin peptides, it brings along a different set of problems.

We have thus reconsidered the long-term paradigm from the following perspective. We know that the loss of sodium in urine after treatment with furosemide occurs within 1 h and that very little further sodium is lost during the rest of the 24-h period (7, 13). It is assumed, but has not been shown directly, that plasma aldosterone and ANG II are elevated rapidly (7). If that is true, why do we typically wait for 24 h to test the appetite? Why does it not occur sooner? In the present study, we first characterized the time course of hormonal, neural (c-Fos immunocytochemistry), and behavioral aspects of the paradigm. We then attempted to either induce or block a short-term NaCl appetite after furosemide treatment by blockade of aldosterone production with metyrapone, a selective inhibitor of 11β-hydroxylase (5), inhibiting production of corticosterone and its conversion to aldosterone. We then examined whether elevations of either aldosterone or ANG II, achieved in the sodium-replete state by minipump infusions, are sufficient to modify intakes in either short- or long-term paradigms.

METHODS

Animals and housing. Adult male and female rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Some of the rats had been used in previous studies of flavor-calorie conditioning, but none involved sodium depletion, exposure to NaCl, or injections. Rats were 3–6 mo of age at the time of these studies, which were approved by the Institutional Animal Care and Use Committee. Within an experiment, only one sex and age were used. All rats were housed individually in suspended steel mesh cages in a vivarium illuminated from 0600 to 1800 with ambient temperature 23 ± 1°C. Food (Purina Chow 5001 pellets) and tap water were available ad libitum, and, during a 1-wk familiarization period, a graduated cylinder with metal sipper containing hypertonic NaCl was available. The concentrations of NaCl used in these studies were either 0.3 or 0.45 M. The higher concentration was used in two studies with ~3-mo-old female rats because we have found their spontaneous intakes

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TEMPORAL MATURATION OF SODIUM APPETITE

of 0.3 M are often quite high, and our aim was to use a concentration of NaCl that would yield very low baseline or control intakes. Aside from this, no sex differences were observed between comparable treatments in these studies.

Sodium depletion and intake test. Acute sodium depletion was produced by a single injection of furosemide (10 mg/kg, Abbott Labs, SC). In most studies, to induce a mild sodium deficiency and to maximize the induced appetite (7, 17), the rats were fed for up to 2 days beforehand with a natural ingredient, low-sodium diet (background sodium 0.01–0.02%; Teklad 90228) presented in jars inside the cages. Rats were housed in clean cages at that time with distilled water to drink. In some studies, at the time of furosemide injection, the cages were moved to an adjacent metabolic stand to allow collection of urine. At the end of the depletion period (3–24 h), two calibrated drinking tubes were attached to the front of the cage, one containing water and the other a hypertonic solution of NaCl (0.3 or 0.45 M). Intakes were recorded volumetrically after 1 h. All drinking tests were conducted between 1200 and 1500; the furosemide injections were timed in accordance with this constant test window. All rats were preexposed to the NaCl solution for several days before the furosemide treatment.

Blood sampling and plasma assays. In animals treated similarly to those used for the intake tests and at the same time of day, rats were instead first sedated (~1 min) with methoxyflurane inhalation, and a 1-ml blood sample was taken by heart puncture. Capillary tubes were filled for immediate determination of plasma protein (hand refractometer) and hematocrit ratio. The remaining sample was collected in either plain or chilled EDTA-treated tubes. Plasma was separated and frozen at ~60°C for subsequent determination with commercially available radioimmunoassay kits of aldosterone (Coat-A-Count; Diagnostic Products), plasma renin activity (PRA; ANG I kit, DuPont), and, in select cases, corticosterone (ICN). PRA increases rapidly with most anesthetic but is the least affected by brief methoxyflurane exposure (11).

Fos immunocytochemistry. In the first experiment, after the blood sample was taken as described in Blood sampling and plasma assays, the rats were deeply anesthetized with pentobarbital sodium (100 mg/kg ip) and then perfused transcardially with heparinized saline followed by 4% paraformaldehyde. The brains were postfixed overnight, and sections were then cut coronally on a Vibratome (100 µm) from just rostral to the organum vasculosum laminae terminalis (OVLT) and subfornical organ (SFO) to caudal to the paraventricular hypothalamus. Floating sections were stained for c-Fos immunoreactivity (IR) (14) with a polyclonal antibody (SC-52, 1:10,000 dilution, Santa Cruz Biotechnology) that recognizes both c-Fos and c-Fos-related antigens. Stained sections were mounted on slides and given coverslips for microscopic examination and cell counting.

Implantation of osmotic minipumps. Osmotic minipumps that deliver 1 µl/h for up to 7 days (model 2001, Alza, Palo Alto, CA) were loaded with the infusate at the desired concentration and were implanted under the skin between the scapulae. For this, the rats were sedated with methoxyflurane, a small incision was made in the skin, the pump was pushed in, and the incisions were closed with a wound clip. The animals were awake within ~1 min and were immediately returned to their home cages.

Statistical analysis. All data were treated by ANOVA with the SAS/PC programs. Significance of individual comparisons (P = 0.05) was determined with Newman-Keuls tests.
Sustained elevation of aldosterone. On the basis of the results of the first experiment, we hypothesized that sustained (>3 h) elevations in aldosterone might be responsible for sensitization of depletion-related sodium appetite. This broad hypothesis was not supported in the second experiment in which metyrapone effectively prevented elevation of aldosterone but did not attenuate either 3- or 24-h sodium appetite. However, it remains possible that metyrapone has effects other than on aldosterone and/or the blockade was not sufficient. The present study is complementary to the previous experiment: we examined whether infusions of aldosterone before sodium depletion have a sensitizing effect on sodium appetite.

Five groups of six male rats (400 ± 35 g) were used in the behavioral part of this experiment. Four groups were to be furosemide treated, whereas the fifth was a control. Three days before the intake test, two groups were implanted with an osmotic minipump loaded with aldosterone (0.75 µg/µl; Sigma) in 20% propylene glycol-saline vehicle. This infusion was designed to achieve in sodium-replete rats plasma aldosterone levels that were comparable to those seen after furosemide treatment. These rats were injected with furosemide after 2 or 3 days of infusion and 24 or 3 h before the salt intake test, respectively. These rats were fed Purina Chow until the time of furosemide treatment and then received the low-sodium diet. The other three groups were fed the low-sodium diet for 3 days (starting the same day that minipumps were implanted in the other groups) but had no implant. These rats were injected with furosemide after 2 or 3 days of low-sodium diet and 24 or 3 h before the salt intake test, respectively. The fifth group was a control, fed the low-sodium diet and injected with water either 24 or 3 h after the salt intake test. About 3 wk later, additional rats of the same sex and age were added to the study for physiological measures, and 12 groups of 6 rats each matched for prior experience were formed. The design had three background treatments (4 groups each): either aldosterone pumps in rats fed Purina Chow (as described above), low-sodium diet (3 days), or Purina-Chow-fed controls (a sham implant procedure was performed). The acute injections for each background treatment were designed to mimic the behavioral study: either furosemide or vehicle was given 24 or 3 h before blood was sampled. These samples, taken as before, were used to determine plasma aldosterone and protein concentrations and PRA. As in the behavioral study, low-sodium diet was given to Purina-fed rats after their furosemide injection. For simplicity of data presentation, the plasma parameters for the 3- and 24-h groups within a background treatment have been combined because the differences between the data at these times were generally quite small.

Because DOCA is more effective in stimulating sodium appetite than aldosterone alone (6), we repeated the behavioral study in the same male rats (now ~500 g, but excluding those treated with aldosterone, and with random assignment to new groups) with minipumps loaded with DOCA (0.75 µg/h). A DOCA-treated, no-furosemide control group was included to assess whether this dose of DOCA would itself produce a salt appetite, but it did not (mean NaCl intake 1.2 ml; data not shown). No plasma work was done in association with the DOCA study.

Sustained elevations in angiotensin. The results of the above studies did not support our hypothesis concerning aldosterone as the likely “priming” hormone. In the final study, we examined another likely candidate, ANG II, in the paradigm used above for aldosterone or DOCA pretreatment. Thirty female rats (228 ± 15 g) were divided into five groups. Four groups were implanted with osmotic minipumps loaded with ANG II (Sigma) to deliver 12 µg·kg⁻¹·h⁻¹, a dosage that Caputo et al. (1) showed previously increases spontaneous 0.3 M NaCl intake by ~20 ml/day and blood pressure by ~30 mmHg in male Harlan Sprague-Dawley rats. Neither fluid intake nor blood pressure was measured in the present female rats. Two of the groups were fed Purina Chow and two groups were fed the low-sodium diet for 3 days. The fifth group was fed Purina Chow and received no pump. Only water was available to drink during these 3 days. At the end of this time, one of the pump groups in each diet condition was injected with furosemide, and the other groups were injected with water. Food and water were removed at the time of injection, and 3 h later water and 0.45 M NaCl were presented for a 1-h intake test.

RESULTS

Time course of behavioral, physiological, and neural aspects of furosemide. The intakes of 0.3 M NaCl are shown in Fig. 1. There was no difference across the 3 wk of repeated testing. NaCl intake increased with the delay between furosemide injection and the test, and at each week the intake in the 24-h group was greater than that of the 3-h group (P < 0.05). On week 2, the 12-h group drank reliably more than the 3-h group. The control rats that did not receive furosemide drank <1 ml of NaCl at each test; all of the furosemide intakes were higher than this baseline. Not shown are concurrent water intakes that averaged (across all weeks) 4.6, 0.8, and 0.4 ml in the 24-, 12-, and 3-h groups, respectively.

The plasma variables are shown in Fig. 2. Aldosterone concentrations were increased above Purina Chow-fed control levels in the low-sodium diet, no-furosemide group and were further increased in all of the furosemide-treated groups, being highest at the 12- and 24-h delays. PRA was not reliably elevated above control levels in the low-sodium diet, no-furosemide group but showed a large increase by 3 h after furosemide treatment, followed by a gradual decline over 24 h. The
hematocrit ratio and plasma protein concentrations were elevated above control levels by the low-sodium diet alone. Plasma protein was elevated at 3 and 12 h after furosemide relative to the levels of the low-sodium diet, no-furosemide group. c-Fos IR was induced by furosemide in only the SFO and around the OVLT. c-Fos-positive cells were counted in the sections of the SFO and OVLT that on gross inspection appeared to contain the most IR cells in each rat; the results are shown in Fig. 3. There was no statistical difference between the number of cells in the 3-, 12-, and 24-h groups, although the 12-h group tended to be lowest. Although not formally quantified, there was also no obvious difference in the intensity of staining between the groups. c-Fos IR was absent in both SFO and OVLT of Purina Chow-fed controls and low-sodium diet, no-furosemide rats.

Blockade of aldosterone synthesis with metyrapone. In the first study, plasma aldosterone was greatly elevated by furosemide and the low-sodium diet (Fig. 4). Injection of 100 mg metyrapone/kg completely reversed this increase within 1 h and for at least 24 h. Corticosterone levels were increased by furosemide and the low-sodium diet (to a mean of 318 ng/ml) and were reduced significantly at 3, 12, and 24 h after metyrapone treatment to mean values of 47–109 ng/ml (full data not shown). PRA exhibited a more complex time course (Fig. 4). As in the first experiment, PRA was only modestly increased above the control range 24 h after furosemide, but this value was increased about threefold within 1 h of metyrapone and then was suppressed significantly after 3 and 12 h.

In the second study, the mean intakes were not affected by metyrapone (Fig. 5). Thus acute metyrapone neither potentiated nor inhibited significantly the small NaCl appetite 3 h after furosemide treatment, nor did it affect the more robust NaCl appetite 24 h after furosemide treatment. Because the intakes in the 3-h groups were small and there was a suggestion of metyrapone inhibition, this part of the study was repeated with heavier (≈300 g) rats, and metyrapone again had no effect on NaCl intake (4.1 ± 0.5 ml for furosemide only and 5.0 ± 1.1 ml for furosemide plus metyrapone).

Plasma aldosterone levels were greatly elevated in furosemide groups but were reduced to the control

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**Fig. 2.** Plasma aldosterone concentration (A), plasma renin activity (PRA; B), hematocrit ratio (C), and plasma protein concentration (D) of control rats and rats given low-sodium diet for 48 h and either no furosemide (No Furo) or a single injection 3, 12, or 24 h before sampling. Data are means ± SE. *Significantly greater than No Furo group (P < 0.05).

**Fig. 3.** Number of c-Fos immunoreactive (Fos-ir) cells in maximally stained section of subfornical organ (SFO, left) or organum vasculosum laminae terminalis (OVLT, right) in controls and rats given low-sodium diet for 48 h and either no furosemide (No Furo) or a single injection 3, 12, or 24 h before sampling. Data are means ± SE and are from same rats as in Fig. 2. All Furo groups differ significantly from control and No Furo groups.
range (100–200 pg/ml) in all of the metyrapone-treated groups (Fig. 5). Corticosterone levels were significantly elevated by furosemide (477 ± 101 ng/ml at 3 h and 526 ± 90 ng/ml at 24 h) and reduced to control levels (200 ng/ml) in all of the metyrapone-treated groups. PRA was elevated above values in the furosemide-only group by two injections of metyrapone and reduced by one injection in the 24-h groups (Fig. 5), although all levels were elevated above typical nondepleted control levels (not run in this study). As in the first experiment, plasma protein concentration was higher 3 h after furosemide treatment (8.8 ± 0.1 g/dl) than 24 h (8.4 ± 0.1 g/dl); these values were unaffected by treatment with metyrapone (data not shown). Hematocrit ratios varied in parallel with protein.

In a similar (3 h) study in additional rats, metyrapone did not affect the diuretic action of furosemide despite the fact that it completely prevented an elevation in aldosterone. The urinary volume in the first hour after injection did not differ between a group receiving furosemide only (7.7 ± 0.4 ml) and a group receiving furosemide and at the same time metyrapone (7.4 ± 0.4 ml). Furthermore, no group excreted >1 ml in the next 2 h.

Sustained elevation of aldosterone. The behavioral results are shown in Fig. 6. In the aldosterone study, both of the aldosterone pump groups consumed significantly less NaCl (P < 0.05) than the time-matched low-sodium diet groups. This result is the converse of the sensitization predicted for the 3-h furosemide group. The results were similar in the DOCA study: both of the DOCA pump groups consumed less NaCl than the time-matched low-sodium groups, but in this case these reductions were not significant.

The plasma measures from the aldosterone study are summarized in Fig. 7. Aldosterone levels were significantly elevated in the pump-treated groups to a level comparable to that in the furosemide-treated, low-sodium diet group. Furosemide raised aldosterone levels and especially PRA in both low-sodium diet and Purina Chow-fed groups. Furosemide had no significant effect on either of these measures in the aldosterone pump rats, and PRA was suppressed by the aldosterone infusion. The hematocrit ratio and plasma protein varied in parallel, with the highest levels in the low-sodium diet group and with significant elevations by furosemide in all groups. In the 3-h groups in each of the three background treatments, urinary volume was also measured for 1 h after furosemide and did not differ between groups.

Sustained elevation of angiotensin. The no-pump group fed the low-sodium diet consumed 3.5 ± 0.9 ml...
NaCl and 1.2 ± 0.4 ml water in the test 3 h after furosemide. These intakes are close to those reported for 3-h groups in the foregoing experiments. The angiotensin pump group fed the low-sodium diet but not treated with furosemide consumed significantly less NaCl (1.8 ± 0.7 ml; P < 0.05) and a negligible amount of water (<0.2 ml). The other three groups consumed only small amounts (<0.4 ml) of NaCl and water, indicating that this dose of ANG II alone does not induce sodium appetite. Furthermore, in Purina Chow-fed groups, this dosage of ANG II was not sufficient to induce water and NaCl intake in this paradigm.

DISCUSSION

We showed that sodium appetite matures in a more or less linear fashion with time for 24 h after furosemide treatment. As first described by Jalowiec (7), we used a background of 48 h of low-sodium diet. This diet alone produces a modest increase in aldosterone and a decrease in plasma volume as indicated by the plasma protein but is not associated with either sodium appetite or c-Fos IR in the SFO or OVLT. We surmised that this diet might amplify short-term (3 h) effects. For example, Stricker (17) successfully used this strategy to accelerate hypovolemia-related sodium appetite. The intakes were, however, only modest after 3 h. In other studies (7, 13), it has been shown that within 3 h of furosemide injection, the urinary sodium loss is 1.5–2 meq/rat, and it is worth noting that even the mean 4-ml intake of 0.3 M NaCl by the 3-h group is 1.2 meq, close to the deficit. Rather, it is the excessive consumption by the 12-h and especially the 24-h groups that characterizes this paradigm (7, 13). There was no trend for the appetite to increase with repeated treatment in any of the groups. Although one study (15) has reported...
increases with repeated furosemide, it should be noted that the present rats were “primed” by 2–3 days of low-sodium diet and so do not replicate the conditions of those studies.

To our knowledge, this is the first description of the temporal evolution of the hormonal responses in this paradigm. It is evident that the low-sodium diet induces a small but significant rise in aldosterone above levels in Purina Chow-fed controls, but not in PRA. This contrasts with the observation that when rats are acutely switched from a high-sodium to a low-sodium diet, PRA tends to rise sooner than aldosterone. Within 3 h of treatment with furosemide in the low-sodium-primed rats, both aldosterone and PRA are greatly elevated, with the greatest fractional increment being in PRA. Jalowiec (7) deduced that aldosterone must be rapidly elevated from the observed urinary K⁺/Na⁺ ratios. After 3 h, aldosterone levels continued to rise while PRA declined, despite the lack of ambient sodium for replacement. Both hematocrit and plasma protein data indicated that hypovolemia was greater at 12 h after furosemide treatment than at either 3 or 24 h. Two points should be made. First, at the time of the 3-h intake test described above in which the intakes were modest, aldosterone was still rising, but PRA and the inferred ANG II levels were very high. Second, at the time of the 24-h intake test in which the intakes were large, aldosterone was high but PRA had declined substantially from the 3-h level. Thus if aldosterone and ANG II (PRA) are in fact the principal two hormones of sodium appetite, then NaCl appetite is driven as a consequence of their past elevation rather than current concentrations. A more detailed temporal analysis of hormone levels remains to be done to ascertain when the true peaks occurred in this paradigm. The data are consistent with the view that the temporal delay in the maturation of sodium appetite is because the transcription and translation of specific genes are involved (4). Because steroid hormones in general are well known to activate genomic mechanisms, aldosterone is one candidate trigger. c-Fos IR is one index of functional neuronal activation (12) and ultimately induces target gene expression, so it might change with time in the sodium-depletion paradigm. In the 24-h version, it has been shown that furosemide induces c-Fos in the SFO and OVLT (4, 12), but it is not known whether this pattern either accumulates or changes with time. We report that c-Fos IR cell numbers were similar both 3 and 24 h after furosemide treatment. This suggests that c-Fos expression may be sustained for long periods of time, although it must be cautioned that the present data do not prove that the antigen is the same at each time point or that the same cells are involved. However, the spatial distribution throughout the SFO and OVLT was similar in all groups. The low-sodium diet alone, which elevated aldosterone but not PRA, did not induce c-Fos IR in the SFO. This is consistent with other data showing that a high level of circulating ANG II, elevated by either physiological or pharmacological treatments, gains access to the SFO and is the primary inducer of c-Fos (4, 12, 14).

If aldosterone were a sufficient trigger mechanism for the appetite, then blockade of aldosterone synthesis should attenuate and exogenous administration should potentiate or accelerate sodium appetite. Our results do not support either aspect of this prediction. The 24-h data are strongest in the metyrapone study because the full duration of normal aldosterone action has been blocked, whereas the 3-h paradigm is the strongest in the minipump paradigm because the acute depletion occurs within this time frame. Metyrapone seems to be a useful agent for studying the hormonal basis of sodium appetite. One previous study (5) reported a small increase in 24-h intake of hypotonic NaCl after metyrapone treatment, but we found no evidence for a behavioral effect either in the present paradigms or in pilot studies. Infusion of ANG II also did not affect the induction of a rapid salt appetite. The role of peripheral ANG II in sodium appetite is still unclear (3, 4, 9), in particular because sodium depletion induces angiotensin-related genes in brain (8).

Perspectives

A preponderance of previous studies has shown it is likely that both aldosterone and angiotensin and perhaps other hormones work synergistically to produce sodium appetite. The present study shows that neither aldosterone nor angiotensin alone is sufficient as trigger or priming mechanisms, at least in this paradigm. In a slightly different paradigm, it was shown that blockade of either aldosterone or ANG II action in the fully matured (24 h), furosemide-induced NaCl appetite is sufficient to attenuate the intake, but both are necessary to fully block the behavior (16). Further studies remain to be done to determine what aspects of these procedures, including, for example, the 2- to 3-day low-sodium diet priming, might be responsible for these apparently different conclusions.

Several pieces of evidence point toward the OVLT and surrounding region and the central nucleus of the amygdala as crucial sites for sodium appetite (4, 9). The apparent lack of change in c-Fos IR in the OVLT between 3 and 24 h after furosemide treatment, coupled with the lack of induction in the amygdala by this treatment (data not shown), suggests this metric will not be particularly useful in elucidating the neural circuitry involved in this behavior. Lastly, we raise the logical alternative, reviewed elsewhere (4, 9), that in addition to excitatory mechanisms for sodium appetite there seem to be several inhibitory systems. Within the furosemide paradigm, then, we may not be waiting for the temporal development of excitation, but instead for the temporal dissipation of inhibition. Our data neither support nor refute this suggestion but perhaps provide a useful framework within which to examine the question.

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