special communication

NaCl ingestion ameliorates plasma indexes of calcium deficiency

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Tordoff, Michael G. NaCl ingestion ameliorates plasma indexes of calcium deficiency. *Am. J. Physiol.* 273 (*Regulatory Integrative Comp. Physiol.* 42): R423–R432, 1997.—Rats deprived of dietary calcium ingest large volumes of concentrated NaCl solutions. To examine why, some physiological consequences of ingesting NaCl solution were measured. Male Sprague-Dawley rats fed diet containing 150 or 25 mmol Ca^{2+}/kg were killed at 20, 40, 80, or 160 min after they started to drink solutions of 0.125% saccharin + 3% glucose (S+G), 50 mM $CaCl_2$, or 300 mM NaCl. Relative to rats fed the 25 mmol Ca^{2+}/kg diet given nothing to drink, those fed the same diet that drank NaCl or $CaCl_2$ but not S+G had elevated plasma ionized calcium concentrations and reduced plasma parathyroid hormone (PTH) concentrations. Rats fed the 150 mmol Ca^{2+}/kg diet did not show these changes. In a follow-up experiment, rats fed the 25 mmol Ca^{2+}/kg diet that drank NaCl had elevated plasma ionized calcium and reduced PTH and 1,25-dihydroxyvitamin D concentrations over the following 4–6 h. An in vitro study found that plasma ionized calcium concentrations were modulated by NaCl concentration directly. These findings indicate that NaCl ingestion can temporarily enhance the calcium status of calcium-deprived rats.

drinking; renin-angiotensin-aldosterone system; adrenocorticotropic hormone-corticosterone axis; calcium appetite; rats

RATS DEPRIVED OF CALCIUM develop a strong appetite for NaCl and other sodium salts (4, 5, 26, 27, 30–33). The physiological basis for this appetite has not been established. It does not appear to be mediated directly by the primary hormones of calcium homeostasis (30), changes in extracellular fluid volume (33), or the renin-angiotensin system (27, 31, 32). An adrenal factor may be involved because adrenalectomy decreases the NaCl intake of calcium-deprived rats (26). This is not aldosterone because neither aldosterone administration nor mineralocorticoid blockade influences the NaCl intake induced by calcium deprivation (26, 32). A role for corticosterone has been suggested (27) based on the results of adrenalectomy experiments (26) and data suggesting subtle differences between controls and calcium-deprived rats in plasma and urinary corticosterone concentrations (31). However, direct evidence for this has not been forthcoming. The central oxytocinergic system has been implicated in the control of some models of salt intake (25), but it is unlikely to play a role in the salt appetite of calcium-deprived rats because these animals have normal circulating oxytocin concentrations (31), which are indicative of central oxytocin activity (25). Thus, although the data are incomplete, it appears that none of the established physiological mechanisms controlling NaCl intake can easily account for the increased salt appetite seen during calcium deficiency.

One of the most puzzling features of the calcium-deprivation model of salt intake is that, because calcium and sodium excretion are linked (e.g., Refs. 2, 3, 9, 10, 15, 17, 18, 22, and see Ref. 27), NaCl ingestion exacerbates calcium deficiency. Why should an animal perform a behavior that makes its deficiency worse? One answer is that the benefits of drinking NaCl outweigh the disadvantages, but this leads in turn to the question of what these benefits might be. As a first step to examine this, we measured the physiological responses to NaCl ingestion by calcium-replete and -deprived rats. In the main experiment, several plasma minerals and hormones were measured at 20, 40, 80, or 160 min after rats consumed a solution containing a mixture of saccharin and glucose (S+G), NaCl, or $CaCl_2$. The rats given S+G or $CaCl_2$ were controls for the specificity of the physiological effects of drinking NaCl. Because this experiment suggested that drinking NaCl influenced the regulation of plasma calcium for longer than 160 min, a follow-up experiment was conducted in which calcium-related hormones were measured at 1, 2, 4, and 6 h after calcium-deprived rats drank NaCl. Finally, a simple in vitro study was conducted to examine the possibility that NaCl influences plasma calcium concentrations directly.

METHODS

Subjects and Diets

Both experiments used male Sprague-Dawley CD-VAF rats aged 21–23 days when purchased from Charles River Laboratories (Wilmington, MA). The main experiment was conducted in five replications involving a total of 280 rats. Groups of 56 rats arrived from the vendor at weekly intervals. The follow-up experiment involved 60 rats tested in a single replication. All the animals were maintained at 23°C under fluorescent illumination between 6:00 AM and 6:00 PM. Each rat was housed alone in a stainless steel cage, measuring 25 × 18 × 19 cm, with a mesh floor and front wall. Deionized water was available from a 300-ml glass bottle equipped with a stainless steel sipper tube that was mounted through the

front wall. Powdered diet was available from a glass jar attached to the front wall with a stainless steel spring.

In the main experiment, the diet calcium content was manipulated so that one-half the rats received diet containing 150 mmol Ca^{2+}/kg (Ca-150) and the other one-half received diet containing 25 mmol Ca^{2+}/kg (Ca-25). In the follow-up experiment, all the rats received the Ca-25 diet. The composition of the diets was based on the AIN-76A formulation, with calcium content manipulated by the addition of calcium carbonate (see Ref. 31 for formulation). The Ca-150 diet served as a "control" or "calcium-replete" diet, containing slightly more calcium than AIN-76A diet (129 mmol Ca^{2+}/kg) but less than chow (~250 mmol Ca^{2+}/kg). The two diets contained equal amounts of other macro- and micronutrients, including sodium (44 mmol/kg), potassium (92 mmol/kg), and phosphorus (129 mmol/kg). They were prepared commercially (Dyets, Bethlehem, PA), and their calcium content was verified before use.

Procedure

The rats were fed their respective diets for 3 wk to allow frank calcium deficiency to develop in the experimental groups. During this period, all the rats were handled by a technician three to five times a week. This was done to adapt each rat to the handling that occurred between leaving its cage and being decapitated, later in the procedure.

Special procedures were employed to increase the probability that rats would drink the solution given to them on the test day. On the 21st day of the dietary manipulation, all rats received 50 ml of a mixture containing 0.125% saccharin + 3% glucose (wt/vol; S+G; water was always available as well). This was done to familiarize the animals with this solution, which is avidly consumed by rats (39). The empty tubes were removed the following morning. Starting at approximately noon 2 days later, all the rats received 60-min trials with access to the S+G solution once a day for 3 days. Based on intakes of S+G solution during the third trial, the rats were assigned to one of two subreplications. The 14 rats with the highest intakes of each diet were tested the following day, whereas the 14 rats with the lowest intakes were given a fourth 60-min trial with S+G while the first one-half were being tested, and then were tested the following day.

On the test day, at approximately noon, most of the rats were given one of three solutions to drink for 20 min and were decapitated at prescribed intervals after the solutions were presented. In the main experiment, the solutions were S+G, 300 mM NaCl, or 50 mM $CaCl_2$, and the time between presentation of the solution and decapitation was 20, 40, 80, or 160 min. Generally, one rat fed each diet was represented in each solution and time condition. The two other rats fed each diet were not given a solution to drink but were killed at the 80- or 160-min time points.

Of the 280 rats tested, 16 fed Ca-150 diet and 10 fed Ca-25 diet failed to drink at least 0.5 ml of the solution given to them. These rats were not killed but instead were added to the following week's replication, such that an additional randomly chosen animal was tested in the condition the rat failed to achieve. Eight rats fed Ca-150 and one rat fed Ca-25 diet failed to drink the second time they were tested, and these animals were discarded from the experiment. Failure to drink occurred most often in the group fed Ca-150 diet given access to $CaCl_2$ (Table 1).

In the follow-up experiment, two subreplications of 30 rats each were tested. In each subreplication, 25 rats received 300 mM NaCl to drink for 20 min. Groups of five of these were decapitated at 1, 2, 3, 4, or 6 h after the beginning of the NaCl drinking period. The remaining five rats received nothing to drink, and one was killed at each time period. All the rats met the criterion of drinking >0.5 ml NaCl during the 20-min access period in this experiment.

Table 1. *Intake of S + G, NaCl, and $CaCl_2$ by rats fed Ca-150 and Ca-25 diets*

Solution	Diet	Intake, ml	No. of Rats Drinking <0.5 ml
S+G	Ca-150	9.6 ± 0.8† (38)	2
	Ca-25	7.0 ± 0.8* (39)	3
300 mM NaCl	Ca-150	4.9 ± 0.5 (40)	3
	Ca-25	7.3 ± 0.5* (40)	0
50 mM $CaCl_2$	Ca-150	1.4 ± 0.1† (33)	24
	Ca-25	5.6 ± 0.5* (41)	3

Values are means ± SE. Nos. in parentheses are group sizes. Means do not include rats that drank less than 0.5 ml, which were not used for physiological measurements. S + G, saccharin + glucose; Ca-150, Ca-25, 150 and 25 mmol Ca^{2+}/kg, respectively. *$P < 0.05$ relative to Ca-150 group; †$P < 0.05$ relative to intake of 300 mM NaCl by groups fed same diet.

Blood. At the appropriate time, each rat was removed from its cage and immediately decapitated. Truncal blood was collected into chilled polypropylene tubes containing 30 μmol EDTA. A separate 400-μl blood sample was taken in heparin-coated microhematocrit tubes for analysis of calcium and osmolarity. The EDTA-treated blood was centrifuged at 2,000 *g* for 15 min at −4°C, and aliquots were frozen at −80°C until assayed. In the main experiment, untreated plasma was analyzed for rat parathyroid hormone (PTH), corticosterone, aldosterone, adrenocorticotropic hormone (ACTH), and renin activity using ^{125}I radioimmunoassay (RIA) kits (Nichols no. 40–2230; ICN no. 07–120103, 07–108202, 07–106101; New England Nuclear no. NEA-105, respectively). Insulin was analyzed using ^{125}I RIA by the University of Pennsylvania RIA Core Facility. A 500-μl aliquot of plasma was extracted using a C_{18} SepPak column and analyzed using a ^{125}I RIA kit for rat calcitonin (Peninsula, RIK-6014). Another 500-μl aliquot of plasma was extracted with a C_{18}-OH column and analyzed using a ^{3}H RIA kit for 1,25-dihydroxyvitamin D [1,25$(OH)_2$D; Nichols no. 40–6040]. The average percent sample recovery after column chromatography was 60%. Because of the large volume of plasma required, the hormone assays were not run in duplicate.

Plasma sodium and potassium concentrations were assessed using a flame photometer (Instrumentation Laboratories, model IL943), and glucose was assessed using a colorimetric kit (Sigma, kit no. 510). The heparin-treated blood was centrifuged at 1,000 *g* for 3 min, and the plasma was frozen for later analysis of osmolarity by freezing-point depression (Advanced Instruments, 3MOplus), total calcium by colorimetry (Sigma, kit no 587), and ionized calcium by an ion-specific microelectrode (Dow Corning calcium analyzer 634).

Statistical analyses. There were seven rats in the group fed Ca-150 diet killed at 20 min, and 9, 10, or 11 rats in each of the other 27 groups. However, the number of values contributing to group means was occasionally reduced because of insufficient plasma to conduct all assays, disparate data from duplicate assays, low sample recovery, or other technical errors.

All data were analyzed by analyses of variance (ANOVAs), with separate analyses for each dependent variable. In the main experiment, fluid intakes during the test were analyzed by a two-way ANOVA with factors of drink type (S+G, NaCl, and $CaCl_2$) and diet (Ca-125 and Ca-25). The metabolic response produced by each drink was analyzed separately by two-way ANOVAs with factors of diet (Ca-125 or Ca-25) and

time [0 (no-drink control groups), 20, 40, 80, and 160 min]. Initial analyses found there were no significant differences on any measure between the no-drink control groups killed at 80 min and those killed at 160 min. Consequently, data from these groups were combined. Because the values for plasma calcium and its three primary hormones differed widely between rats fed the Ca-150 and Ca-25 diets and there was heterogeneity of variance, additional planned analyses (1-way ANOVAs) of the Ca-150 diet groups were conducted to determine whether drinking any of the solutions had subtle effects on any of these measures. However, these were all nonsignificant and thus are not presented in the text. In the follow-up experiment, intakes and plasma measures were subjected to one-way ANOVAs with time as the factor (0, 1, 2, 3, 4, and 6 h).

When a significant interaction in the main experiment or main effect of time was present, post hoc *t*-tests were used to identify significant differences between individual pairs of means. The probability criterion for ANOVAs was $P < 0.05$ and for post hoc tests was $P < 0.01$. The higher criterion for post hoc analyses was used to guard against type I errors resulting from the large number of tests conducted.

RESULTS

Solution Intake

On the day before testing, the rats fed Ca-150 diet in the main experiment weighed 226 ± 2 g and those fed Ca-25 diet weighed 201 ± 2 g (difference, $P < 0.0001$). During their last 1-h habituation test on this day, the replete animals consumed slightly, but significantly, more S+G solution than did the deplete animals [all rats fed Ca-150 = 12.1 ± 0.4, all rats fed Ca-25 = 10.1 ± 0.4, $t(265) = 2.59$, $P < 0.05$]. There were no differences in S+G intake among the 14 groups fed each diet that were subsequently tested with different drinks and killed at different times.

Intakes of S+G, NaCl, and $CaCl_2$ during the 20-min test before decapitation are shown in Table 1. The rats fed Ca-25 diet drank significantly less S+G and significantly more NaCl and $CaCl_2$ than did rats fed Ca-150 diet [fluid × diet interaction, $F(2,225) = 18.8$, $P < 0.00001$]. The groups fed Ca-150 diet drank significantly more S+G than NaCl and significantly more NaCl than $CaCl_2$. This was consistent with the marked reluctance of rats fed the Ca-150 diet to drink any $CaCl_2$ (see Table 1). The groups fed Ca-25 diet drank significantly more S+G than $CaCl_2$, with consumption of NaCl not statistically different from either of these intakes. There were no differences in intake of the solutions related to the time the rats would subsequently be killed; that is, all groups fed the same diet and given the same solution had similar intakes.

In the follow-up experiment, on the day before testing, the rats weighed 206 ± 3 g. They drank, on average, 15.6 ± 1.2 ml of the S+G solution, with no difference among the six groups in either body weight or S+G intake. On the test day, they drank on average 8.5 ± 0.5 ml 300 mM NaCl during the 20-min test, with no difference among the five groups given the solution (the 6th group had no solution to drink).

Metabolic Responses to Drinking Various Solutions

Figures 1–4 show the effects of drinking S+G, NaCl, and $CaCl_2$ on the 15 plasma measures collected. Consistent with the effects of chronic calcium deprivation, rats fed the Ca-25 diet had significantly lower plasma concentrations of ionized and total calcium and significantly elevated concentrations of PTH, $1,25(OH)_2D$, and calcitonin. There were also effects of diet on plasma glucose and insulin concentrations, with the Ca-25 groups having consistently lower concentrations of each. These effects were present whether the animals were tested after drinking no solution, S+G, $CaCl_2$, or NaCl, and so will not be discussed further.

S+G solution. Drinking S+G solution produced significant changes in plasma glucose, insulin, sodium, and renin activity that were similar whether the rats were fed Ca-150 or Ca-25 diet [respectively, $F(4,103) = 11.0$, $P < 0.0001$; $F(4,103) = 25.8$, $P < 0.0001$; $F(4,101) = 2.79$, $P = 0.03$; $F(4,103) = 10.3$, $P < 0.0001$; $F(4,103) = 6.16$, $P < 0.0002$; Fig. 1 and 2]. Plasma concentrations of glucose and insulin were significantly higher at 20 min after starting to drink S+G than at all other times. In addition, glucose concentrations of rats killed at 80 and 160 min after drinking S+G solution were significantly higher than those of controls given nothing to drink. Conversely, plasma sodium was significantly lower between 20 and 80 min, and plasma renin activity was significantly lower between 20 and 40 min after starting to drink S+G than at other times.

The only metabolic measure that was differentially affected by diet after drinking S+G was plasma corticosterone concentrations [diet × time interaction, $F(4,103) = 3.79$, $P = 0.006$]. The difference in response to S+G between the diet groups was due to rats fed the Ca-25 diet, but not the Ca-150 diet, having a marked elevation in corticosterone concentrations at 40 min after starting to drink S+G (Fig. 2). ACTH concentrations showed a similar pattern, although this was not significant.

There were no significant effects of drinking S+G on any of the other measures collected.

$CaCl_2$ solution. Plasma ionized calcium concentrations of rats fed Ca-150 diet were unaffected by drinking 50 mM $CaCl_2$; on the other hand, plasma ionized calcium concentrations of rats fed Ca-25 diet increased markedly [diet × time interaction, $F(4,98) = 2.67$, $P = 0.04$]. Without $CaCl_2$ to drink, the rats fed Ca-25 diet had plasma ionized calcium concentrations that were considerably lower than those of rats fed Ca-125 diet. After 20-min access to $CaCl_2$ solution, rats fed the Ca-25 diet had significantly increased their plasma ionized calcium concentrations, and by 40 min they had significantly increased plasma ionized calcium levels again and were now indistinguishable from the groups fed Ca-150 diet. Plasma total calcium concentrations showed a similar pattern to ionized calcium, except *1*) drinking $CaCl_2$ significantly increased plasma total calcium concentrations of rats fed Ca-25 diet but not to the range of rats fed Ca-150 diet, *2*) for both diet conditions, total calcium levels decreased between the

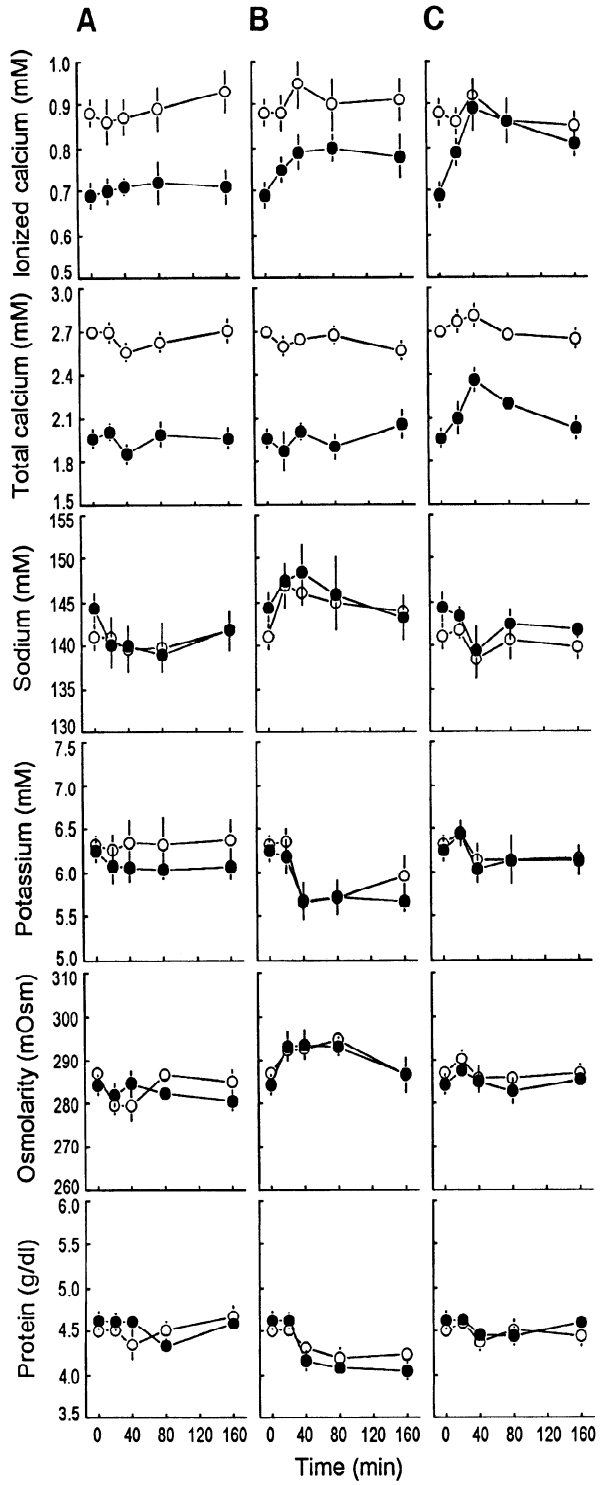

Fig. 1. Plasma mineral concentrations and other measures collected from rats fed diet containing 150 (○) or 25 (●) mmol Ca^{2+}/kg (Ca-150 or Ca-25, respectively) and given to drink solutions of 0.125 saccharin + 3% glucose (S+G; *A*), 300 mM NaCl (*B*), or 50 mM $CaCl_2$ (*C*) for 20 min, starting at *time 0*. Table 1 gives solution intakes.

40 and 160 min after drinking $CaCl_2$, and *3*) the interaction of diet with $CaCl_2$ intake was marginally nonsignificant.

Plasma PTH concentrations showed the opposite pattern to plasma calcium [diet × time interaction, $F(4,99) = 3.05$, $P = 0.02$]. Drinking $CaCl_2$ had no effect on PTH levels of rats fed Ca-150 diet but significantly reduced PTH levels of rats fed Ca-25 diet at 20 and 40 min after starting to drink. There was a marginally nonsignificant interaction between diet and time on plasma calcitonin concentrations [$F(4,98) = 2.39$, $P = 0.056$], which was due to the group fed Ca-25 diet killed

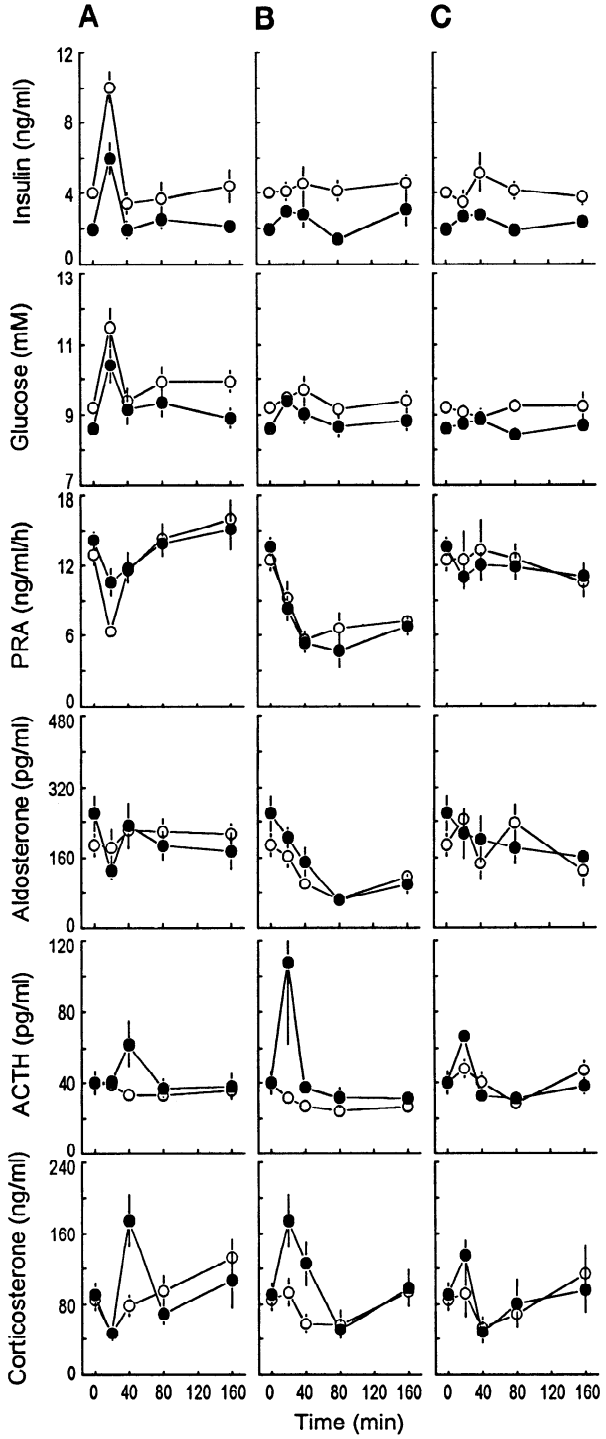

Fig. 2. Plasma hormone and glucose concentrations collected from rats fed Ca-150 (○) or Ca-25 (●) diet and given to drink solutions of S+G (*A*), 300 mM NaCl (*B*), or 50 mM $CaCl_2$ (*C*) for 20 min, starting at *time 0*. PRA, plasma renin activity; ACTH, adrenocorticotropic hormone.

at 40 min having elevated plasma calcitonin concentrations (see Fig. 3).

Drinking $CaCl_2$ influenced plasma ACTH and corticosterone concentrations in the same manner in rats fed Ca-25 and Ca-150 diets [$F(4,100) = 2.74$, $P = 0.03$; $F(4,99) = 3.86$, $P = 0.006$, respectively]. Relative to rats given nothing to drink, both groups killed at 20 min showed a significant increase in plasma ACTH and corticosterone, and both groups killed at 40 min showed a significant decrease in corticosterone.

Drinking $CaCl_2$ had no effect on any of the other measures collected [sodium, potassium, osmolarity, protein, insulin, glucose, plasma renin activity (PRA), aldosterone, $1,25(OH)_2D$].

NaCl solution. Irrespective of diet, drinking NaCl influenced plasma osmolarity and concentrations of sodium, protein, potassium, ionized calcium, PTH, ACTH, corticosterone, PRA, and aldosterone [$F(4,105) = 4.81$, $P = 0,001$; $F(4,105) = 4.85$, $P = 0,001$; $F(4,104) = 11.8$, $P < 0.0001$; $F(4, 104) = 7.62$, $P < 0.0001$; $F(4,104) = 2.62$, $P = 0.039$; $F(4,106) = 4.64$, $P = 0.002$; $F(4,105) = 2.89$, $P = 0.025$; $F(4,106) = 4.77$, $P = 0.001$; $F(4,106) = 27.9$, $P < 0.0001$; $F(4,102) = 12.0$, $P < 0.0001$; respectively]. Relative to the groups fed the same diet and given nothing to drink, plasma osmolarity was significantly elevated at 20–80 min, sodium at 20–40 min, ionized calcium at 20–160 min, and ACTH and corticosterone at 20 min only. Plasma protein, potassium, PTH, and aldosterone concentrations were significantly reduced at 40–160 min. PRA was significantly reduced at 20–160 min.

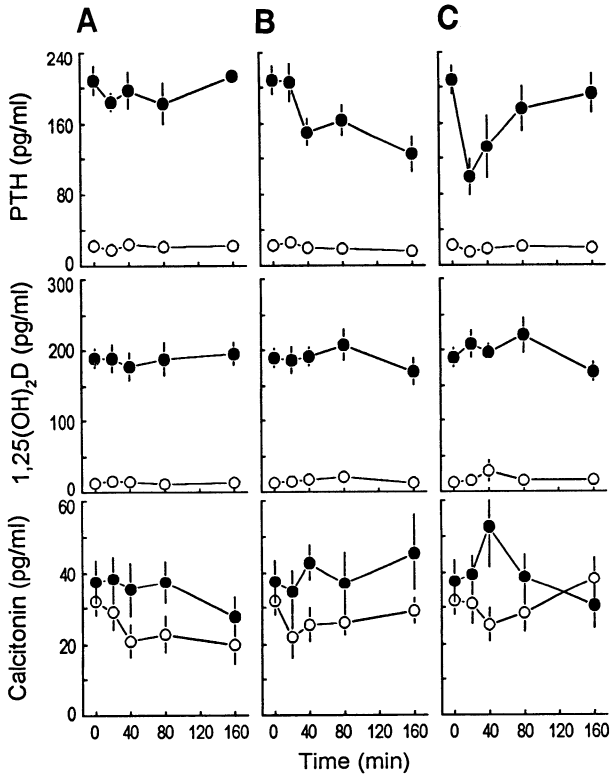

Fig. 3. Plasma concentrations of the 3 primary calcium regulatory hormones collected from rats fed Ca-150 (○) or Ca-25 (●) diet and given to drink solutions of S+G (*A*), 300 mM NaCl (*B*), or 50 mM $CaCl_2$ (*C*) for 20 min, starting at *time 0*. PTH, parathyroid hormone; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D.

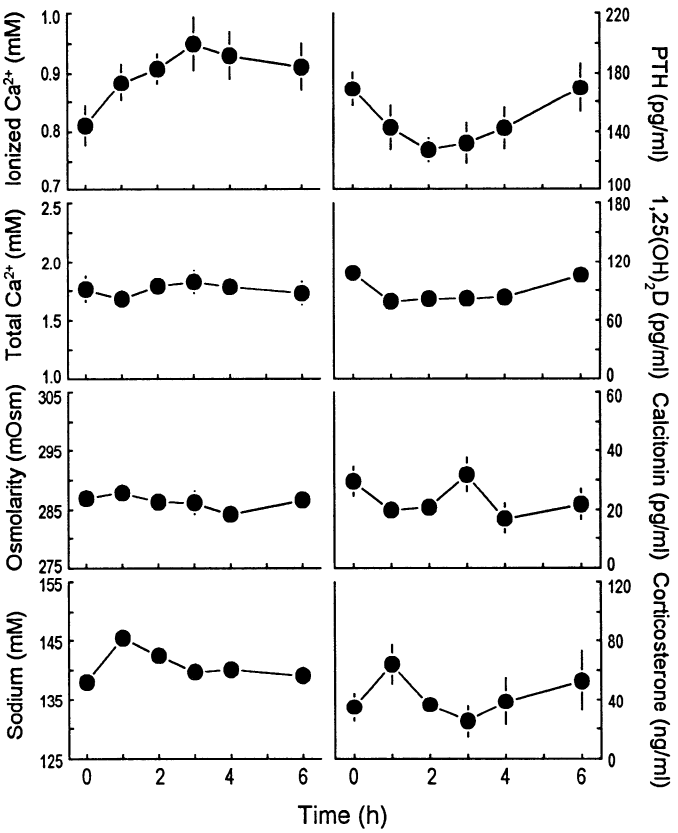

Fig. 4. Plasma minerals, hormones, and other measures collected from rats fed Ca-25 and given 300 mM NaCl to drink for 20 min, starting at *time 0*. Vertical lines show SE. SE was smaller than the symbol size where these are not shown.

For PTH, ACTH, and corticosterone, but not the other measures, the effect of drinking NaCl was modulated by diet [diet × time interactions, $F(4,106) = 3.27$, $P = 0.014$; $F(4,105) = 2.60$, $P = 0.04$; $F(4,106) = 2.59$; $P = 0.04$, respectively]. Drinking NaCl significantly decreased PTH levels of rats fed Ca-25 diet at 40–160 min after starting to drink. The Ca-25 group killed at 160 min had PTH levels that were also lower than those of Ca-25 groups killed at 20 or 80 min. Drinking NaCl had no effect on PTH concentrations of rats fed Ca-150 diet; analysis of data from animals fed the Ca-150 diet only showed that drinking NaCl produced a marginally nonsignificant reduction in PTH concentrations in these animals [$F(4,53) = 2.53$, $P = 0.053$].

The interactions affecting ACTH and corticosterone concentrations were due to these hormones increasing at 20 min after rats fed Ca-25, but not Ca-150, diet started to drink. For corticosterone, this diet-related difference was also present in the groups killed at 40 min (Fig. 2).

Drinking NaCl had no significant effects on plasma total calcium, glucose, insulin, calcitonin, or $1,25(OH)_2D$.

Longer-term effects of drinking NaCl. The results of the follow-up experiment were similar to the main experiment (Fig. 4). Relative to the values of controls given no 300 mM NaCl to drink, plasma ionized cal-

cium concentrations were significantly elevated in rats killed at 1–6 h after the start of ingestion [$F(5,54) = 2.44, P = 0.046$]. There were also significant reductions in plasma PTH concentrations in rats killed at 2–5 h [$F(5,54) = 2.42, P = 0.047$] and small but significant reductions in plasma $1,25(OH)_2D$ concentrations at 1–3 h after drinking NaCl [$F(5,52) = 3.15, P = 0.015$]. Drinking NaCl significantly increased plasma osmolarity [$F(5,54) = 5.99, P = 0.0002$], with the groups killed at 1 and 2 h having higher osmolarity than did controls. There were also significant changes in plasma potassium concentrations [$F(5,54) = 2.65, P = 0.033$], with reductions in plasma potassium at 2–4 h after drinking NaCl. There were no effects of drinking NaCl on plasma sodium, total calcium, calcitonin, or corticosterone concentrations.

Effect of Sodium on Plasma Calcium In Vitro

The studies cited above demonstrated that plasma ionized calcium concentrations were elevated by drinking NaCl solution. To test the hypothesis that the increase in plasma ionized calcium was due to the interaction of sodium with calcium or calcium-binding proteins in plasma, we measured ionized and total calcium concentrations of calcium solutions and plasma with sodium content manipulated by adding NaCl.

Method. Separate "stock" pools of heparin-treated plasma were collected by decapitation from rats fed either Ca-150 or Ca-25 diet. Triplicate 180-μl aliquots were mixed with 20-μl aliquots of various concentrations of NaCl solution. These were then analyzed for ionized and total calcium concentrations using the methods outlined above. Sodium concentrations were measured by both flame photometry (as above) and by an ion-specific electrode (Ciba Corning sodium/potassium analyzer model 614). However, the results of these methods were very similar, so the sodium results were combined. The experiment reported here used fresh rat plasma, but similar results have been found with rat and human plasma that was frozen at −70°C and then thawed (unpublished observations).

To examine whether changes in ionized calcium concentrations produced by adding NaCl to plasma were due to physiochemical interactions with the Ciba Corning 634 ionized calcium analyzer, a parallel experiment was performed using $CaCl_2$-NaCl mixtures. Triplicate 180-μl aliquots of stock solutions of $CaCl_2$ with the same concentration of calcium as seen in plasma (i.e., Ca-150 diet = 1.38 mM; Ca-25 diet = 1.16 mM) were mixed with 20-μl aliquots of various concentrations of NaCl, chosen to match the values seen in plasma.

Results. The sodium concentration of plasma from rats fed Ca-150 diet was 130 ± 2 mM, and from rats fed Ca-25 diet it was 129 ± 1 mM. Adding NaCl to plasma or $CaCl_2$ solutions produced equivalent increases in sodium concentration (Fig. 5). Plasma ionized calcium increased linearly with sodium concentration [$F(8,36) = 384.0, P < 0.00001$]. Regression equations found that ionized calcium increased ~0.04 mM for each 10-mM increase in NaCl concentration. This effect of sodium on ionized calcium was similar whether it involved plasma from rats fed Ca-150 diet or Ca-25 diet. In marked contrast, with $CaCl_2$ solutions, ionized calcium concentrations were inversely related to sodium concentrations [$F(5,24) = 11.6, P < 0.00001$], although this inverse effect was very small (see Fig. 5). Sodium concentration had no effect on plasma or solution total calcium concentrations.

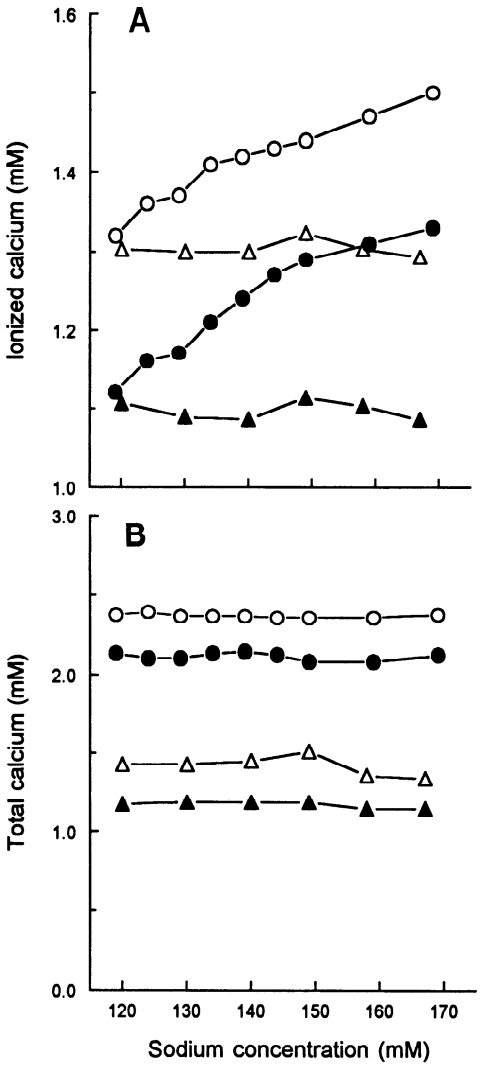

Fig. 5. Effects of sodium concentration on solution and plasma ionized (*A*) and total (*B*) calcium concentrations. △, 1.38 mM $CaCl_2$ solution; ▲, 1.16 mM $CaCl_2$ solution; ○, plasma of rats fed Ca-150; ●, plasma of rats fed Ca-25. In all cases, the SE of each set of triplicate values was smaller than the symbol sizes.

Figure 5 shows ionized calcium concentrations uncorrected for pH. There was no effect of NaCl concentration on pH (data not shown), although plasma had a lower pH than did the solutions (e.g., Ca-150 plasma = 7.61 ± 0.01; 1.38 mM $CaCl_2$ solution = 7.23 ± 0.02). Thus the effect of sodium on ionized calcium concentrations could not be explained by a change in pH.

DISCUSSION

After calcium-deprived rats drank ~2 mmol of a 300 mM NaCl solution their plasma ionized calcium levels

increased significantly and remained elevated for at least 6 h. Plasma PTH concentrations decreased in a reciprocal manner to the increases in ionized calcium, and in one experiment there were also small but significant decreases in plasma $1,25(OH)_2D$ concentrations. The relationship between sodium ingestion and calcium homeostasis was seen only in calcium-deprived animals; although there were trends for calcium-replete rats to show increases in plasma ionized calcium and decreases in PTH concentrations, these were not significant. Taken together, these findings suggest that drinking NaCl acutely reduces the severity of the calcium deficiency experienced by calcium-deprived rats.

The amelioration of plasma indexes of calcium status seen here appears contrary to the effects of NaCl on calcium homeostasis reported by most others, although this literature is not entirely consistent. For example, "albino" or Wistar-Kyoto rats loaded with NaCl by feeding a high-sodium diet had increased calcium absorption and loss, increased parathyroid function, increased $1,25(OH)_2D$ concentrations, and lowered bone and total body calcium content (7, 9). On the other hand, salt-loaded Dahl salt-sensitive (Dahl-S) and Dahl salt-resistant (Dahl-R) rats had normal plasma ionized calcium and $1,25(OH)_2D$ concentrations, and the Dahl-S but not Dahl-R rats had lowered PTH concentrations (14). Humans loaded with dietary NaCl for 5 or more days had the expected increases in urinary calcium (2, 12, 15, 17, 22, 23) as well as increases in calcium absorption (2, 17). Serum calcium decreased (22, 23) or did not change (12, 15, 22); serum PTH decreased (15, 22), did not change (12, 22, 23), or increased (2); and serum $1,25(OH)_2D$ either decreased (22) or increased (2, 12, 22). Even without the inconsistencies, these studies are difficult to interpret because the control condition is generally a low-salt diet. Changes in calcium metabolism could be due to the alleviation of sodium restriction rather than to excess NaCl per se (but see Ref. 12).

More similar to the methods used here are experiments involving measurement of changes in calcium status induced by acute intravenous infusions of NaCl. During volume expansion with calcium-free Ringer solution jejunal calcium absorption reversed (i.e., from blood to lumen), plasma ionized calcium concentrations decreased, and plasma PTH concentrations increased (3). Similarly, in humans, large (e.g., 2.25 mmol Na^+/kg body wt) isotonic saline infusions caused a significant drop in serum total calcium concentrations (1, but see Ref. 6) and a significant increase in serum PTH concentrations (35). On the other hand, infusion of 500 ml 150 mM NaCl (~5.5 mmol Na^+/kg body wt) into the femoral vein of dogs had no effect on plasma ionized calcium (19), and infusion of 1,000 ml 340 mM NaCl into normotensive and hypertensive humans had no effect on serum ionized calcium or PTH concentrations (18).

The disparity between our results and findings that NaCl exacerbates calcium homeostasis can be accounted for by several methodological differences. One is that the effects seen here were in calcium-deficient animals, which, to our knowledge, have not been tested by others with respect to sodium-induced changes in calcium metabolism. Calcium deficiency appeared to magnify the effect of NaCl on calcium regulatory hormones, probably because concentrations (and presumably turnover) of these hormones were already high. Second, in this study, the rats voluntarily ingested NaCl solution in a single bout. This allows expression of any sodium-mediated influence on oral, gastric, or intestinal controls of calcium metabolism that would not be seen using intravenous infusions and that may dissipate or be masked by other food components during chronic dietary NaCl manipulation. Third, unlike most of the acute infusion studies previously conducted, the NaCl load was hypertonic.

The mechanism by which NaCl ingestion influences plasma ionized calcium concentrations is unknown. Our demonstration that plasma ionized calcium is modulated by sodium concentration in vitro raises the possibility that the increase in plasma ionized calcium produced by drinking NaCl is due to a direct interaction of calcium with sodium in plasma. Plasma, presumably protein, was important because the results could not be reproduced by adding NaCl to $CaCl_2$ solutions. They were not a function of the Ciba Corning 634 calcium analyzer used here because similar findings have been reported with five commercial calcium electrodes (20). We suspect that high concentrations of sodium (or perhaps chloride) ions reduce the binding of calcium to plasma proteins. This is likely to be relevant for the detection of calcium in vivo because concentrations of PTH, which is controlled by ionized calcium concentrations, were influenced by NaCl ingestion.

Another possible explanation for why NaCl consumption increases plasma calcium concentrations is based on the interaction of angiotensin II and PTH. In humans, acute infusions of angiotensin II decrease serum ionized calcium and increase serum PTH concentrations (10, 13). The mechanism for this is unknown but it may involve angiotensin-stimulated aldosterone release because patients with primary aldosteronism have low serum calcium and high serum PTH concentrations (21). On the assumption that a similar relationship holds for low as well as high levels of angiotensin II and aldosterone, the marked reduction in these hormones produced by drinking NaCl would be expected to increase plasma calcium concentrations and reduce plasma PTH concentrations. Neither this hormonal mechanism nor direct physiochemical mechanisms can provide a ready explanation for why the effects of drinking NaCl on calcium metabolism were seen most prominently in calcium-deprived animals. It may be, however, that replete animals already have basal hormone levels and have more capacity to buffer fluctuations in calcium homeostasis.

The results from rats given access to S+G or 50 mM $CaCl_2$ solution eliminate the possibility that the effects of NaCl on calcium metabolism are due to the act of drinking or to the volume of fluid ingested. Rats fed Ca-25 diet drank similar volumes of NaCl and $CaCl_2$ or S+G solution. However, differences in intake may

contribute to the diet-related differences in hormonal response because rats fed Ca-25 and Ca-150 diets drank different amounts of the three solutions.

As expected, the rats fed Ca-25 diet drank significantly more 50 mM $CaCl_2$ during the 20-min test than did rats fed Ca-150 diet; indeed, the rats fed Ca-150 diet were reluctant to ingest any 50 mM $CaCl_2$ (Table 1). These findings confirm that concentrated $CaCl_2$ is unpalatable to rats when calcium replete (see Ref. 28) but consumed avidly when calcium deficient. The test here, involving over 100 ad libitum-fed and -watered rats, adds strength to recent work showing that small groups of thirsty calcium-deprived rats lick more than do controls for solutions of 25, 75, 150, or 300 mM $CaCl_2$ (5). Because the tests used in these experiments were relatively short (20 min) and the rats had not previously ingested $CaCl_2$ solution, the results argue for an innate response to calcium and thus against the generally held supposition that calcium appetite is entirely learned (e.g., Ref. 23).

Although calcium deprivation increased intake of $CaCl_2$ and NaCl it decreased intake of S+G solution, both during 1-h training trials and the 20-min test. This finding extends earlier work showing that calcium deficiency reduces the intake of cola beverage, sucrose, and saccharin in both long (24 h)- and short-term tests (4, 16, 33). There has been no attempt to determine why this avoidance of sweet compounds occurs, although it is not due to a general reduction in calorie intake (33). It is tempting to link the reduced intake of sweet solutions to the calcium-deprived rats' lower plasma insulin concentrations, although a link between insulin and sweet solution intake is controversial.

This is the first study to examine the physiological consequences of drinking calcium by calcium-deprived rats, and as such it addresses the question of what satiates the appetite for calcium. Our previous work has shown that calcium-deprived rats ingest an initial large bout of calcium, but it takes 2–3 days before calcium intakes return to levels of calcium-replete controls (5, 32; Tordoff, unpublished results). The results found here indicate that the initial ingestive bout is sufficient to normalize plasma ionized calcium concentrations but not plasma total calcium, PTH, $1,25(OH)_2D$, or calcitonin concentrations. It seems highly unlikely that the return of plasma ionized calcium to concentrations seen in replete rats is responsible for terminating the initial ingestive bout because this did not occur until at least 20 min after the rats had stopped drinking. It also seems unlikely that volume or osmotic constraints were critical because the calcium-deprived rats had the capacity to drink more [they ingested significantly more S+G than $CaCl_2$ (Table 1)] and ingest a 10-fold greater osmotic load (7.3 ml of 300 mosmol NaCl vs. 5.6 ml of 35 mosmol $CaCl_2$). Perhaps taste factors or changes in the rate of calcium absorption from the gut are responsible for terminating the initial ingestive bout, as is the case for sodium-deficient rats given NaCl to drink (29). Beginning 40 min after the rats started to drink $CaCl_2$, plasma calcium concentrations declined and PTH concentrations increased, with total calcium and PTH concentrations regaining levels of rats given no drink within 160 min. It would be interesting to know whether these changes trigger subsequent calcium ingestion.

An interesting ancillary finding was that ingestion of all three solutions produced transient increases in plasma ACTH and corticosterone concentrations, particularly in calcium-deprived rats. It is unlikely that these changes were involved in the changes in PTH, $1,25(OH)_2D$, or ionized calcium concentrations because *1*) rats that drank S+G had no changes in these measures despite increased ACTH and corticosterone concentrations, and *2*) glucocorticoid administration increases serum PTH concentrations (8), which is the opposite of the results found here. Previous studies have shown that corticosterone concentrations rise immediately after ad libitum-fed rats eat or drink glucose solution, but not after they drink water (11, 24). The finding of the present study that this occurred after drinking NaCl or $CaCl_2$, as well as S+G solution, argues that the increase is not a function of calorie intake. Instead, it may represent a response to rapid ingestion, a discernible taste, or stimulation of gastrointestinal volume and osmotic mechanisms. It is unclear why the ACTH-corticosterone axis of calcium-deprived but not control rats should be activated after drinking. There is evidence that daily corticosterone excretion, and thus corticosterone production, is higher in calcium-deprived than control rats (31). Perhaps corticosterone production in response to ingestion is enhanced under these conditions. The more easily stimulated release of corticosterone may be secondary to the calcium-deprived groups' lower plasma glucose and insulin levels, which are intimately related to the control of glucocorticoid secretion. Lower insulin and higher corticosterone also point to greater rates of catabolism, which is consistent with the calcium-deprived animals' lower body weights.

Perspectives

The most intriguing finding of this study was that drinking NaCl temporarily improved plasma indexes of calcium status in calcium-deprived rats. This was not a trivial effect; the effects of drinking NaCl on PTH concentrations were of equal magnitude and longer duration than those of drinking $CaCl_2$. It was also not expected, based on the many demonstrations that NaCl consumption increases calcium excretion, (e.g., Refs. 2, 3, 9, 10, 15, 17, 18, 22).

The finding that drinking sodium acutely improves indexes of calcium status is noteworthy because one of these might reinforce the NaCl drinking behavior. We speculate that rats associate drinking NaCl with the amelioration of their calcium deficiency, leading to an increased intake and preference for NaCl. However, the consumption of sodium produces only a temporary and incomplete improvement in calcium status and, because of the linked excretion of calcium and sodium, in fact accelerates deterioration. The animal is thus caught in a vicious cycle driven by the short-term improvement in calcium status produced by drinking NaCl. Such an

explanation is consistent with experiments using brief exposure tests that suggest there is no difference between control and calcium-deprived rats in their initial response to NaCl solutions (5), although such a difference is present for $CaCl_2$ and other calcium salts. It might also explain why calcium-deprived animals ingest several diverse compounds (4, 5, 33) if these too improve calcium status.

It will require additional work to characterize the extent to which learning can account for the elevated salt drinking of calcium-deprived rats. In particular, it will be necessary to identify the precise nature and locus of the unconditioned stimulus. These studies seem important given that, similar to calcium-deprived rats, most humans in Westernized societies ingest less calcium than requirements. It is possible that improvements in calcium status produced by ingesting NaCl reinforce, and are therefore a cause of, the high salt preferences of humans.

I thank Rebecca Hughes and Diane Pilchak for excellent technical assistance and Drs. Gary Beauchamp and Karen Teff for insightful comments on earlier versions of the manuscript.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-40099 and DK-46791. Insulin was assayed by the University of Pennsylvania Diabetes Research Center Radioimmunoassay Core Facility (DK-19525).

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Received 24 July 1996; accepted in final form 17 February 1997.

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