PRESYSTEMIC INFLUENCES ON THIRST, SALT APPETITE, AND VASOPRESSIN SECRETION IN THE HYPOVOLEMIC RAT

by

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Bachelor of Science, University of Pittsburgh, 2002

Master of Science, University of Pittsburgh, 2006

Submitted to the Graduate Faculty of

Arts and Sciences in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2006

UNIVERSITY OF PITTSBURGH

COLLEGE OF ARTS AND SCIENCES

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June 19, 2006

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Recent studies have shown that when dehydrated rats are given access to water or various concentrations of saline solution, they consume the same volume of fluid in an initial drinking bout (Hoffmann et al., 2006). Furthermore, there was a close relation between fluid intake and distension of the stomach and small intestine when dehydrated rats drank water or saline (Hoffmann et al., 2006). These results are consistent with the hypothesis that fluid ingestion is constrained by a rapid inhibitory signal associated with GI fill. This volume-dependent early inhibition of thirst is reminiscent of the volume-dependent oropharyngeal reflex that Ramsay and colleagues described in dogs (Thrasher et al., 1981). Other studies (Huang et al., 2000) have shown that rats infused iv with hypertonic saline develop a strong motivation to consume water and show a marked increase in pVP. After water ingestion, pVP decreased rapidly before there was a change in systemic pOsm. Plasma VP remained elevated in rats that were given isotonic saline to drink. These results are consistent with the hypothesis that VP secretion is rapidly inhibited when dilute fluid enters the GI tract. The present studies sought to determine whether an early inhibition of fluid consumption by hypovolemic rats also was associated with GI fill. We imposed a 16-hr delay between the time that PEG solution was injected and the start of the drinking test. These animals have a substantial volume deficit (30-40%) as well as increased circulating levels of VP, OT, AngII, and aldosterone. Therefore, they have a pronounced thirst and salt appetite and will be eager to consume large volumes of fluid rapidly, thus allowing us to determine whether 1) distension of the stomach and small intestine provide a rapid inhibitory feedback signal for thirst and salt appetite, 2) gastric emptying of water or 0.30 M NaCl solution provide a presystemic signal that influences VP secretion, 3) changes in systemic pOsm influence ingestive behavior or VP secretion in rats with prolonged hypovolemia, and 4) GI fill continues to act as an inhibitory signal for fluid consumption after the first drinking bout.

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1.0 GENERAL INTRODUCTION

The importance of body fluid homeostasis is reflected in the mechanisms by which it is so well regulated. The maintenance of osmolality, volume, and arterial pressure are necessary for sustaining life. Animals are able to maintain stability in these parameters by controlling the concentration and volume of both fluid intake and urine output (Stricker & Verbalis, 2002). Thirst and salt appetite are motivated behaviors that influence the amount and concentration of fluid that animals are willing to ingest. For example, intraperitoneal injection of hypertonic saline causes an increase in systemic plasma osmolality (pOsm), which leads to the sensation of thirst. In addition, increased pOsm causes vasopressin (VP) and oxytocin (OT) to be secreted from the posterior pituitary. Vasopressin acts in the kidney to promote water reabsorption, whereas OT increases urinary sodium excretion indirectly (by stimulating release of atrial natriuretic peptide; Gutkowska et al., 1997). Through these combined behavioral and physiological responses, the animal is able to return elevated pOsm to normal.

Similar mechanisms are used to control fluid intake and output when an animal's volume regulatory system is challenged. Hypovolemia in rats can be produced by subcutaneous injection of a 30% solution of polyethylene glycol (PEG), a colloid solution which increases local oncotic pressure and prevents the movement of isotonic fluid back into the circulation. This action creates an edema at the site of the injection and a progressive decrease in plasma volume, which increases as a function of time until it reaches a maximal deficit of approximately 40% at about 16 hr post-injection (Stricker, 1968). At this time, equilibrium is achieved between the amount of fluid entering the local edema (constrained by the amount of subcutaneous space able to accommodate additional fluid) and fluid leaving the edema via the lymphatic circulation. The decrease in plasma volume produced by this treatment is associated with stimulation of thirst and salt appetite as well as neurohypophyseal hormone secretion (Stricker & Jalowiec, 1970; Stricker & Verbalis, 1986). The decrease in blood volume is sensed by baroreceptors located in the right

atrium and the great veins entering the heart. These stretch receptors relay information about blood volume to the nucleus of the solitary tract (NTS) in the brainstem (Kalia & Mesulam, 1980). The NTS sends projections to the median preoptic nucleus (MnPO) in the forebrain (Saper & Levisohn, 1983) to stimulate thirst and to the magnocellular neurons in the hypothalamic supraoptic nucleus (SON) and paraventricular nucleus (PVN) to stimulate VP and OT (Ter Horst et al., 1989). The renin-angiotensin system is also activated in response to the decrease in plasma volume (Stricker et al., 1979). Renin is secreted from the kidney in response to increased sympathoadrenal activity and activation of the renal baroreceptors. Angiotensin II (AngII) acts on receptors located in the subfornical organ (SFO) (Mendelsohn et al., 1984) and also stimulates thirst and neurohypophyseal hormone secretion via its neuronal connections with the MnPO and SON and PVN (Ferguson & Renaud, 1986; Simpson et al., 1978). In addition, AngII is a potent vasoconstrictor and a stimulus for aldosterone secretion. Aldosterone acts in the kidney to conserve Na^+ in urine. It also acts to stimulate salt appetite both by direct stimulation of aldosterone-sensitive cells in the NTS (Geerling et al., 2006) and by inhibiting central OT (cOT) secretion, which inhibits salt appetite in rats (Stricker & Verbalis, 1987). Salt appetite is stimulated under conditions in which AngII is elevated and cOT is inhibited (Blackburn et al., 1992). Because replacement of both water and salt are required to restore plasma volume, AngII is extremely important during volumetric challenges as it acts as a stimulus for both thirst and salt appetite. The preference for water or salt appears to depend on the presence or absence of cOT (Stricker & Verbalis, 1990).

In previous experiments, rats were given immediate access to various fluids after sc injection of PEG solution (5 ml, 30%). When animals drank water, they experienced a progressive decrease in pOsm as the ingested fluid entered the circulation and was retained by the kidneys (Stricker & Jalowiec, 1970). This decrease in systemic pOsm (i.e., osmotic dilution) inhibited further water intake (Stricker, 1969). Similarly, when rats drank hypertonic saline after injection of PEG solution, they experienced an increase in pOsm which inhibited salt appetite and thus further NaCl intake (Stricker, 1981; Stricker & Verbalis, 1987). However, when rats were given access to isotonic saline immediately after injection of PEG solution, they continued to drink saline throughout a 24-hr test period because of the absence of these inhibitory postabsorptive effects on pOsm (Stricker & Jalowiec, 1970). In fact, they drank so much fluid so quickly that they were able to avoid the development of a conspicuous volume deficit. When rats

were given access to both water and 0.5 M NaCl in a 2-bottle test after PEG treatment, they consumed water initially but further water consumption soon was inhibited by osmotic dilution. As plasma Ang II and aldosterone levels increased in response to progressive volume depletion, a salt appetite emerged and the rats began ingesting the hypertonic saline solution. The rats then switched back and forth between the two fluids to create an isotonic fluid, which restored plasma volume (Stricker et al., 1992).

Many experiments have investigated the effects of immediate access to fluid after PEG treatment on pOsm and plasma volume, and on neurohypophyseal hormone secretion. In contrast, few experiments have focused on the effects of fluid consumption in rats with an established hypovolemia. In those studies (Blackburn et al., 1992; Stricker, 1981), rats were given a sc injection of PEG solution (5 ml, 30%) but no food or fluid to consume for 24 hr. During this delay period, rats developed a volume depletion of 35-40%. Plasma levels of AngII, aldosterone, VP, and OT were elevated when fluid was returned. As a result, the stimuli for thirst and salt appetite were pronounced and rats consumed large volumes of water and then saline rapidly (Stricker, 1981). In other delayed access experiments (Stricker & Verbalis, 1986), 6 hr after PEG treatment, rats were given either no additional treatment or a water or salt load. When rats were given no additional treatment, plasma VP concentration (pVP) was exponentially related to plasma volume deficit. When rats were given a water load (ig or by access to drinking fluid), osmotic dilution of systemic blood inhibited VP secretion despite severe hypovolemia. Conversely, when rats were given a salt load (ip; 4 ml, 0.40 M NaCl), osmotic concentration increased pVP. These data indicated that changes in systemic pOsm influence pVP in hypovolemic rats 6 hr after treatment with PEG. A delayed access model was also used in this thesis in order to investigate the effects of presystemic signals on the control of thirst, salt appetite, and VP secretion in hypovolemic rats.

Twenty-five years ago, Thrasher and colleagues (1981) reported an early inhibition of VP secretion in response to ingestion of water or isotonic saline by dehydrated dogs. Subsequent experiments suggested that this inhibition was associated with the activation of oropharyngeal afferents that occurred while dogs swallowed liquids (Appelgren et al., 1991; Thrasher et al., 1987). Similar experiments attempted to identify an early inhibitory signal for VP secretion when thirsty rats consumed water. In one important experiment, Huang et al. (2000) administered an iv infusion of hypertonic saline for ~3.5 hr which led to a marked increase in

pOsm and pVP and plasma OT concentration (pOT). When these animals were then given water to drink, a rapid decrease in pVP and pOT was observed before there was a detectable decrease in systemic pOsm. In contrast, early inhibition of VP and OT secretions was not observed when these rats consumed isotonic saline. More recently, water ingestion produced by overnight water-deprivation was shown to rapidly inhibit VP secretion in rats (Stricker & Hoffmann, 2005). Because the rapid decrease in VP secretion occurred in response to ingestion of water but not of 0.15 M NaCl in dehydrated rats, the mechanism for the early inhibition of VP secretion must depend on the concentration of the ingested fluid rather than on its volume. Other experiments (Hoffmann & Stricker, 2005) also have provided some evidence for the hypothesis that putative visceral osmoreceptors are able to detect the concentration of the fluid leaving the stomach and thus facilitate the rapid inhibition of VP secretion.

Thrasher and colleagues (1981) found that activation of the oropharyngeal reflex was additionally associated with an early inhibition of thirst. When dogs consumed water in response to dehydration, they consumed enough water to ultimately restore pOsm, but stopped drinking before the ingested fluid had entered the circulation. Similar experiments have attempted to identify an early inhibitory signal for thirst in rats. When water-deprived rats were given water, isotonic saline, or hypertonic saline to drink, they consumed the same amount of fluid regardless of its concentration (Hoffmann et al., 2006). Furthermore, a strong correlation between intake and the volume of fluid in the stomach and small intestine was observed in all animals. These findings led to the hypothesis that distension of the stomach and small intestine (i.e., “GI fill”) acts as an early inhibitory signal for thirst. Similar experiments have extended this observation to include the inhibition of salt appetite in adrenalectomized or DOCA-treated rats (Bykowski et al., 2005; Hoffmann et al., 2005).

2.0 INTRODUCTION

Subcutaneous injections of PEG solution in rats are known to cause progressive deficits in plasma volume (Stricker, 1968) and thereby stimulate both thirst and salt appetite (Stricker & Jalowiec, 1970). In previous studies, rats were given access to either water or isotonic NaCl solution immediately after PEG treatment. When animals drank water, they experienced systemic osmotic dilution due to renal retention of ingested water, which inhibited further intake (Stricker, 1969). In contrast, consumption of isotonic saline occurred at a rate that prevented the animals from ever becoming significantly hypovolemic (Stricker & Jalowiec, 1970).

Other studies have shown that osmotic dilution of systemic blood also inhibits VP secretion in hypovolemic rats (Stricker & Verbalis, 1986). Rats were given either a water or salt load 6 hr after PEG treatment. When these rats were killed 1 hr later, pVP had decreased to baseline levels in rats given a water load and increased in rats given a salt load. These data suggest that changes in systemic pOsm influence pVP in hypovolemic rats.

Recent studies have shown that when dehydrated rats are given access to water or various concentrations of saline solution, they consume the same volume of fluid in an initial drinking bout (Hoffmann et al., 2006). Furthermore, there was a close relation between fluid intake and distension of the stomach and small intestine when dehydrated rats drank water or saline (Hoffmann et al., 2006). These results are consistent with the hypothesis that fluid ingestion is constrained by a rapid inhibitory signal associated with GI fill. This volume-dependent early inhibition of thirst is reminiscent of the volume-dependent oropharyngeal reflex that Ramsay and colleagues described in dogs (Thrasher et al., 1981).

Other studies (Huang et al., 2000) have shown that when rats are infused iv with hypertonic saline, they develop a strong motivation to consume water and show a marked increase in pVP. After water ingestion, pVP decreased rapidly before there was a change in systemic pOsm. Plasma VP remained elevated in rats that were given isotonic saline to drink.

These results are consistent with the hypothesis that VP secretion is rapidly inhibited when dilute fluid enters the GI tract.

The present studies sought to determine whether an early inhibition of fluid consumption by hypovolemic rats also was associated with GI fill. We imposed a 16-hr delay between the time that PEG solution was injected and the start of the drinking test. These animals have a substantial volume deficit (30-40%) as well as increased circulating levels of VP, OT, AngII, and aldosterone. The advantage of using rats with established hypovolemia is that they presumably have large stimuli for thirst and salt appetite and so they will be eager to consume large volumes of fluid rapidly, thus allowing us to determine whether 1) distension of the stomach and small intestine provide a rapid inhibitory feedback signal for thirst and salt appetite, 2) gastric emptying of water or 0.30 M NaCl solution provide a presystemic signal that influences VP secretion, 3) changes in systemic pOsm influence ingestive behavior or VP secretion in rats with prolonged hypovolemia, and 4) GI fill continues to act as an inhibitory signal for fluid consumption after the first drinking bout.

3.0 METHODS

Animals. Adult male Sprague-Dawley rats (320-400 g; Harlan Laboratories) were individually housed in a temperature-controlled colony room (22-23°C) maintained on a fixed light-dark cycle (lights off from 7:00 p.m. to 7:00 a.m.). All rats had ad libitum access to tap water and pelleted laboratory chow (5001, Purina) for $\geq$1 week before experiments began.

Experimental Protocols. Rats were injected subcutaneously with 5 ml of 30% PEG solution at 5:00 p.m. on the day before the drinking test. All rats were deprived of food and water overnight. At 9:00 a.m. on the following morning (i.e., 16 hr after the injection), lights were dimmed to encourage drinking and rats were given access to water or 0.15 M NaCl solution in graduated 25-ml burettes. The drinking fluid was colored with a dark green food dye (McCormick & C., Hunt Valley, MO) that permitted the ingested fluid to be readily visible in the small intestine. The drinking test was terminated in one of three ways. Some rats (n = 30) were allowed to drink until they paused for 15 sec and moved away from the drinking tube, thereby suggesting that they were not likely to resume drinking soon. Fluid was removed at this time and animals were killed either immediately or after a 15-60 min delay. A second group of rats (n = 34) was allowed to drink for variable amounts of time (1.5-5.5 min) before being interrupted by the experimenter. These animals were used to provide information on the fate of ingested fluid before the cessation of the initial drinking bout. A third group of rats (n = 18) was allowed access to drinking fluid for 60 min and intakes were recorded every 5 min. In all cases, rats were killed by decapitation within 10 sec after the drinking test ended, and fluid intakes (±0.1 ml) and time spent drinking (±1 sec) were recorded.

Tissue Analyses. Trunk blood was collected in ice-cold heparinized tubes (143 USP sodium heparin; Becton Dickinson, Franklin Lakes, NJ) and kept on ice until stomachs and intestines were removed. The abdomen was opened and hemostats were placed at the junction of the stomach with the pylorus, at the junction of the stomach with the esophagus, and at the most

distal site of visible green dye in the small intestine, in that order. This portion of the surgical procedure took less that 2 min. Each stomach was removed from the abdominal cavity, the intestinal distance containing the dye was measured (±1.5 cm), and the intestine was removed. The excised tissue was stripped of adhering blood vessels and connective tissue, and the stomach contents and small intestines were placed in separate beakers. The beakers were covered with Parafilm until they were placed in an oven and dried to constant weight at 60°C for 2-3 days. All blood samples were centrifuged (10,000g for 10 min at 4°C), plasma was harvested, plasma Na concentration (pNa) was measured (±1 mEq/l) using a sodium-sensitive electrode (Beckman Coulter, Synchron ELISE model 4410, Brea, CA), and plasma protein concentration was measured (±0.1 g/dl) using a refractometer. The remainder of the plasma sample was stored in a -80°C freezer until it was assayed for pVP.

Radioimmunoassay. The procedures used for measuring pVP have been described previously (Schiltz et al., 1997). Briefly, duplicate 250-µl plasma samples were extracted using solid-phase columns (Sep-Pak C18 cartridges, 1 ml, 50 mg; Waters, Milford, MA), and VP was measured by radioimmunoassay in aliquots of these extracts. The assay sensitivity was 2.5 pg/ml, and the intra-assay variations were <10%.

Statistical Analysis. All data are presented in scatterplots or as means ± SE. Statistical reliability of observed differences in ingestive behavior were determined using a two-way ANOVA with repeated measures analysis. A one-way ANOVA with Tukey's Post Hoc analysis was used to determine significance at specific time points. Regression equations were calculated by the method of least squares and significance was determined using Pearson's correlation coefficients. $P < 0.05$ was considered to be statistically significant.

4.0 RESULTS

PEG-treatment (5 ml, 30%) produced marked increases in plasma protein concentration after a 16-hr delay period. These hypovolemic rats drank continuously at first when given access to water, isotonic saline, or 0.30 M NaCl. They consumed ~ 5 ml in the first 5 min of the 1-hr test (rate = ~1 ml/min) regardless of which fluid was available (Fig. 1). Rats drank 0.15 M NaCl steadily throughout the remainder of the 1-hr test, although the drinking rate (~0.3 ml/min) was not as rapid as it was during the first 5 min. In contrast, rats consumed very little water or 0.30 M NaCl after the first 10 min (4.7 ± 0.9 ml, 4.6 ± 0.7 ml, respectively, in 50 min; rate = ~0.1 ml/min). Therefore, by the end of the test, intakes of 0.15 M NaCl were much larger than those of water or 0.30 M NaCl (24.7 ± 1.2 ml, 11.2 ± 0.9 ml, 9.7 ± 0.6 ml, respectively; both *P*s <0.001).

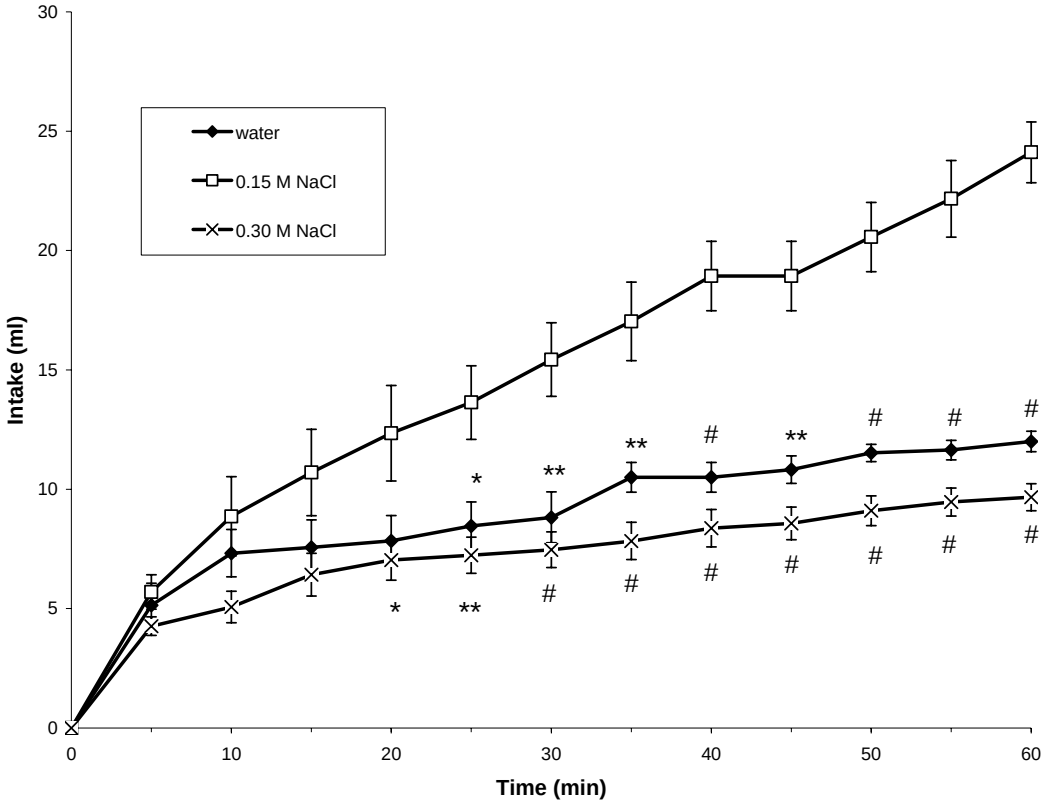

Figure 1: Cumulative intakes of water, 0.15 M NaCl, or 0.30 M NaCl solution by PEG-treated rats plotted as a function of time spent drinking (*n* = 5, 6, 7, respectively). Shown are mean ± SE values. Rats drank at a similar rate for the first 5 min of the 60-min drinking test but then slowed considerably. Mean intakes of 0.15 M NaCl were significantly greater than water or 0.30 M NaCl intakes (* = *P* <0.05; ** = *P* <0.01; # = *P* <0.001).

As mentioned, all rats drank fluid at a similar rate during the first bout regardless of which fluid they consumed (~1.1 ml/min, Fig. 2; r = 0.76, P <0.001). Some rats drank continuously until they paused, whereas other rats were interrupted after 1.5 – 5.5 min in order to determine the fate of ingested fluid before the initial drinking bout ended.

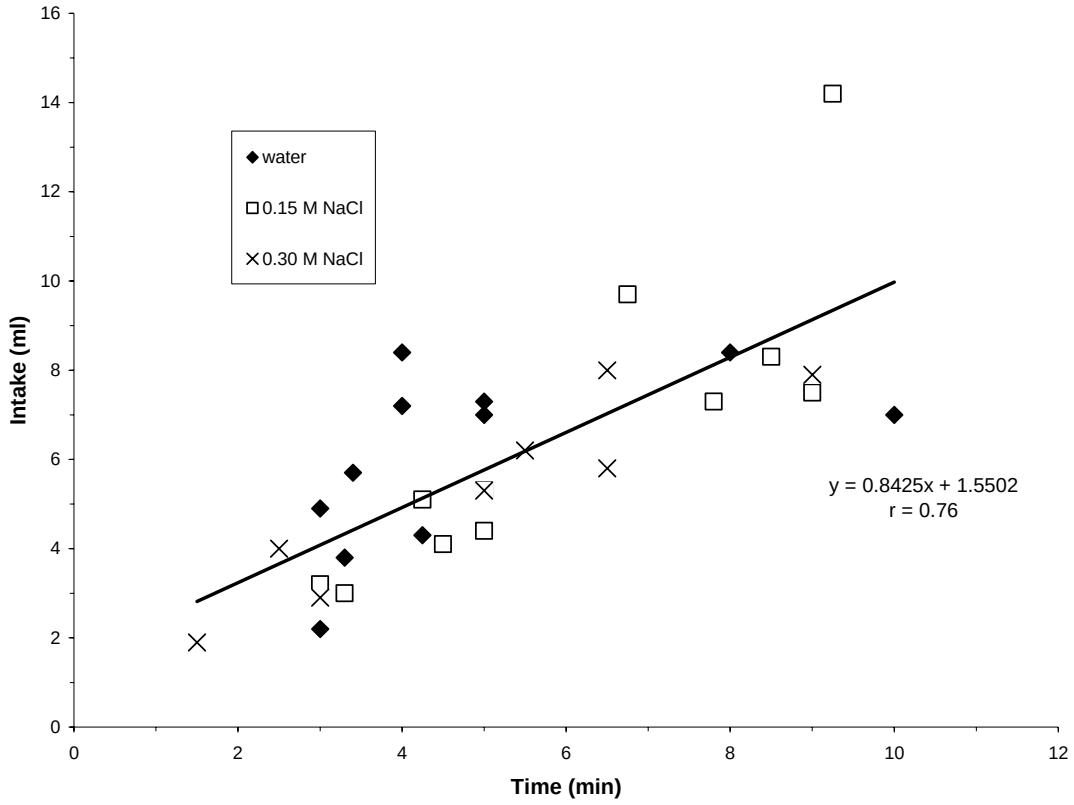

Figure 2: Intake of water, 0.15 M NaCl, or 0.30 M NaCl solution by PEG-treated rats plotted as a function of time spent drinking during the initial bout, in a test that began 16 h after the injections. Each symbol represents the intake of a single animal. Intakes were highly correlated with time spent drinking regardless of which fluid was consumed or for how long they drank (*r* = 0.76, *P* <0.001). Shown is the regression line for all data points (*y* = 0.84*x* + 1.55). According to this line, mean drinking rate slowed during the test period from 2.4 ml/min by rats killed after 1 min of drinking to 1.0 ml/min by rats killed after 10 min of drinking. This decrease reflects the increased incidence of short pauses while rats drank.

Gastric fluid volume increased as a result of fluid ingestion regardless of which solution was consumed (Fig 3). The solid line in Fig. 3 represents the volume of gastric fluid if emptying did not occur. All but one point fell to the right of that line, suggesting that animals typically emptied a portion of the ingested fluid into the small intestine. For a given intake volume >5 ml, the amount of fluid found in the stomach was dependent on the concentration of the fluid ingested. Very little fluid remained in the stomach when rats drank 0.15 M NaCl, whereas nearly all of the ingested fluid remained in the stomach when they drank 0.30 M NaCl. Rats that drank

water emptied fluid at intermediate rates. Note that these rats had very little food (<0.1 g) in their stomachs when they were killed.

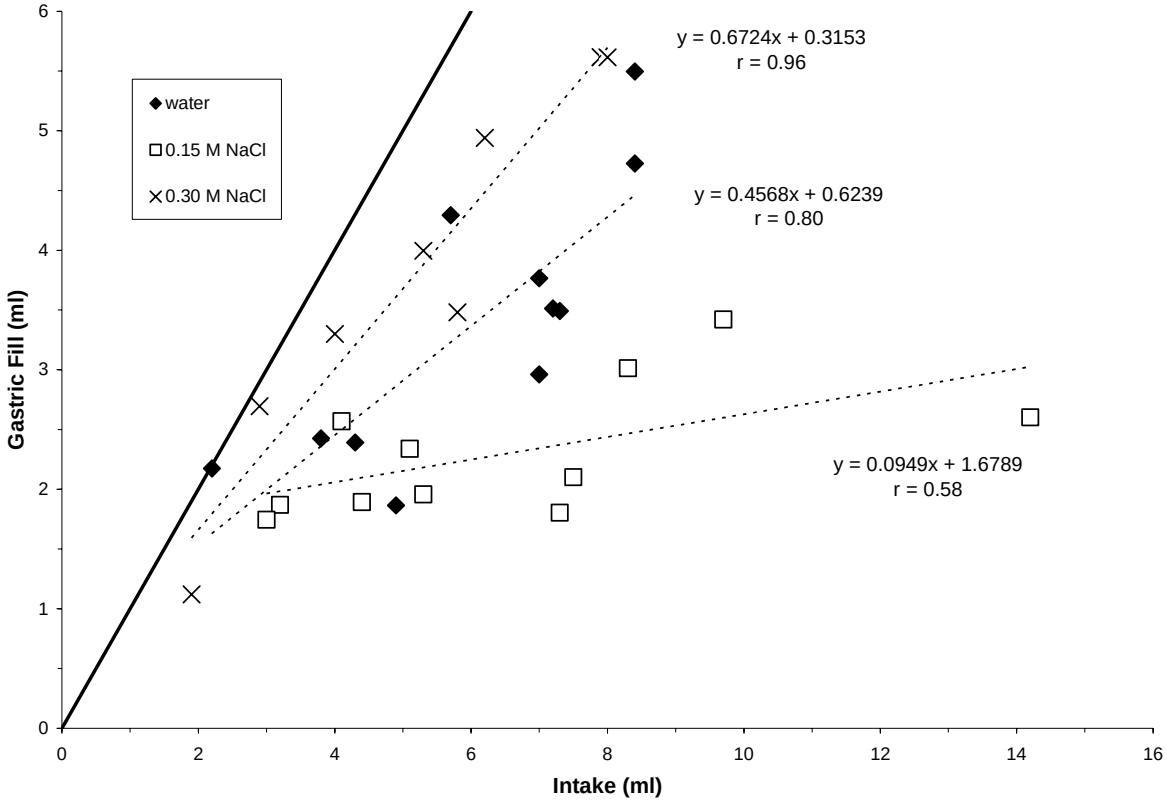

Figure 3: Gastric fluid volume plotted as a function of fluid ingested by PEG-treated rats drinking either water, 0.15 M NaCl, or 0.30 M NaCl solution. Symbols represent the same animals as in Figure 2, the regression lines and correlation coefficients are shown (*P* < 0.01 for water; *P* < 0.001 for 0.30 M NaCl). The dark solid line represents gastric fill if no fluid emptied from the stomach. The amount of fluid that remained in the stomach at the end of the first bout was dependent on the concentration of the ingested fluid. Rats that drank 0.15 M NaCl emptied fluid very rapidly and had relatively little fluid remaining, whereas rats that drank 0.30 M NaCl emptied little of the ingested fluid and rats that drank water emptied fluid at an intermediate rate.

Intestinal fill, which consists of the fluid contained in the small intestinal lumen between the pylorus and the most distal portion of the small intestine in which green dye was visible, increased exponentially as a function of the volume of fluid ingested regardless of which fluid was consumed (Fig. 4). Intestinal fluid volumes were inversely correlated with gastric fluid volumes when both were expressed as a percentage of fluid intake (not shown; $r = 0.54$, $P < 0.01$). In other words, the more fluid there was in the stomach, the less fluid there was in the

small intestine regardless of which fluid was consumed. The fluid also traveled further into the small intestine in logarithmic relation to the volume ingested regardless of which fluid was consumed (Fig. 5). Thus, intestinal distension (volume per length) was similar in all three groups (Fig. 6).

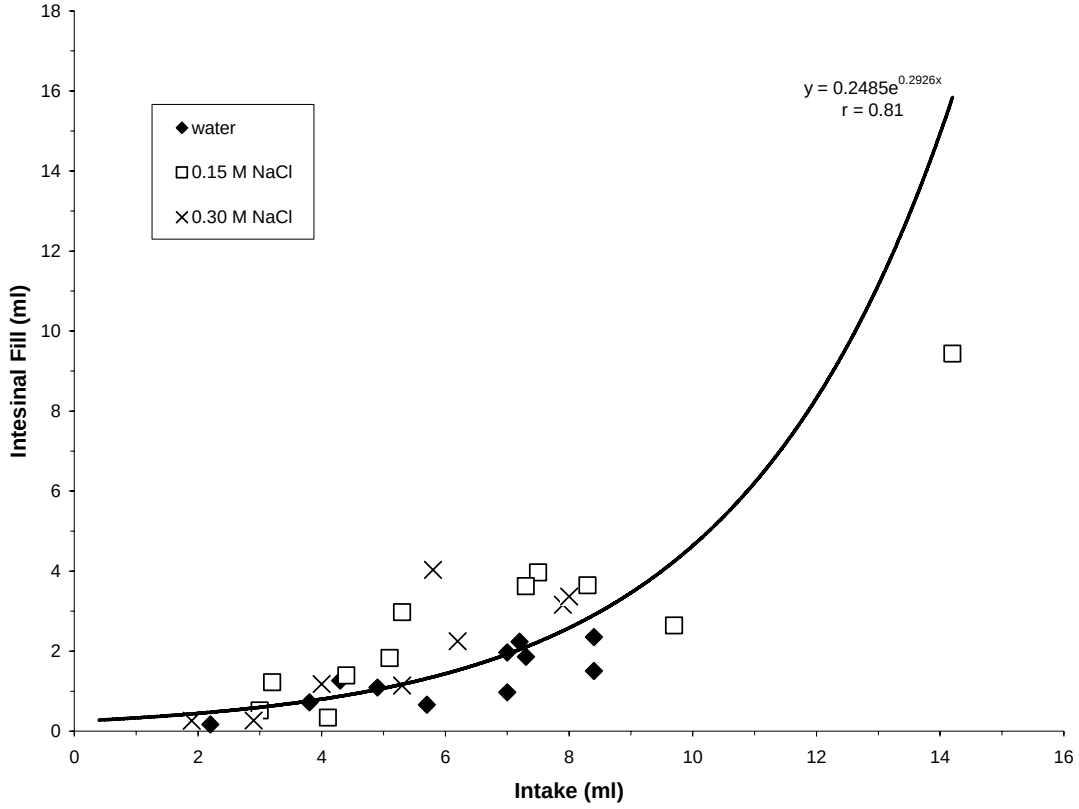

Figure 4: The volume of fluid in the dye-colored segment of the small intestine, plotted as a function of fluid intake. Symbols represent the same rats shown in previous figures. Intestinal fluid volume did not begin to accumulate until rats had ingested >2 ml of fluid, at which point it increased exponentially in proportion to fluid intake (r = 0.81, P <0.001).

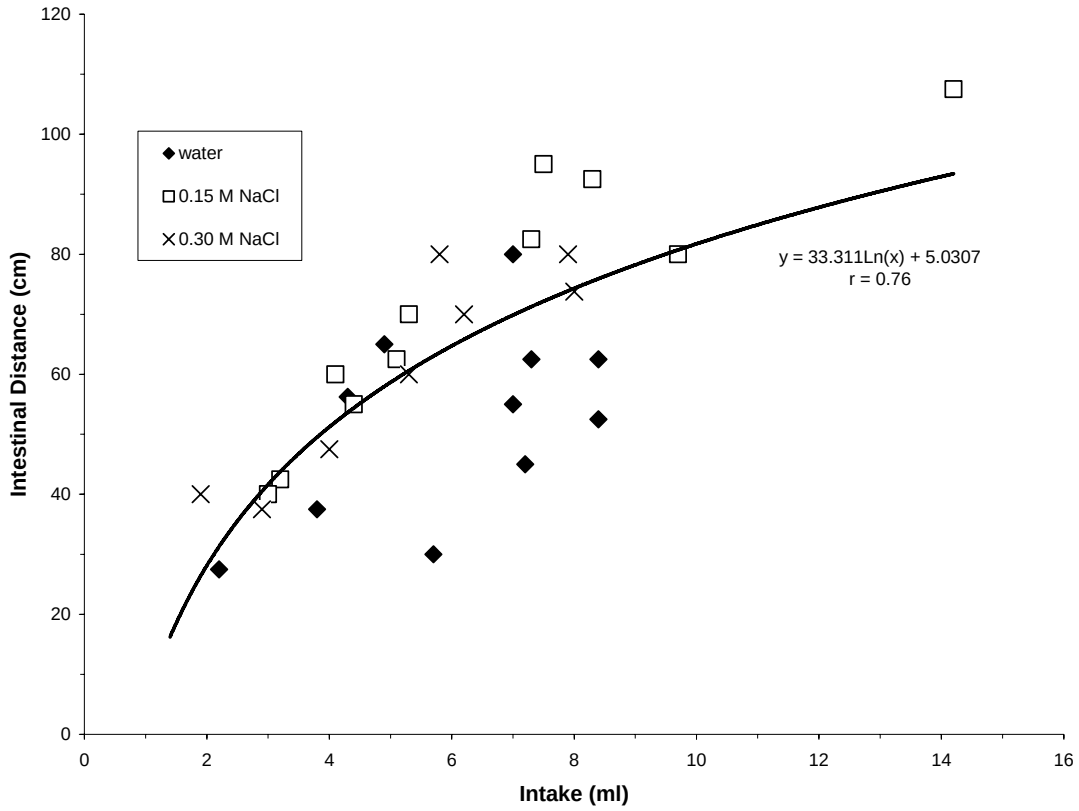

Figure 5: Distance measured from the pylorus to the most distal point in the small intestine at which green dye was visible, plotted as a function of fluid intake. Symbols represent the same animals as in the previous figures. The intestinal distance increased as the volume of ingested fluid increased. Note that green dye was not found in the cecum of any rat.

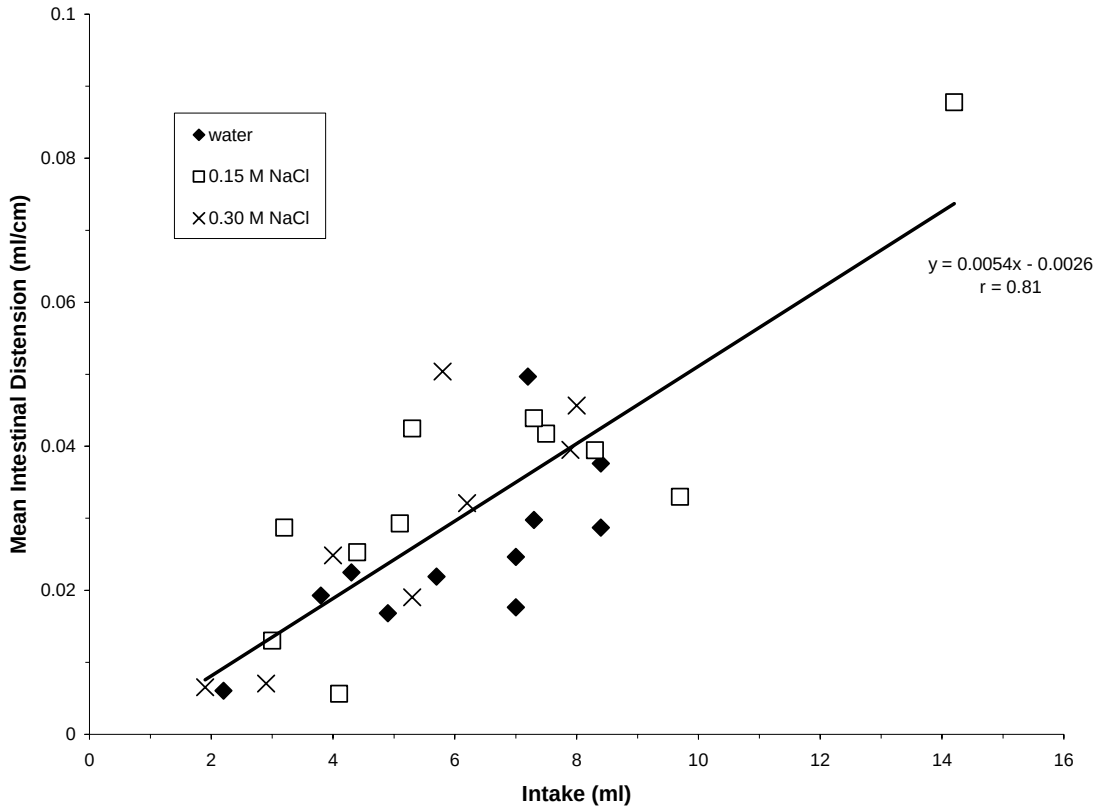

Figure 6: Calculated mean distension of the dye-colored segment of the small intestine, plotted as a function of ingested fluid. Symbols represent the same animals as in the previous figures. Intestinal distension increased progressively as the volume of ingested fluid increased. Note that the dye-colored fluid was not actually distributed uniformly throughout the segment; rather, intervals of intestine containing little or no fluid punctuated the segment.

The sum of the fluid in the stomach and small intestine increased in proportion to fluid intake, and the two variables were highly correlated regardless of which fluid was consumed (Fig. 7; $r = 0.97$, $P < 0.001$). The solid line in Fig. 7 represents the volume of fluid in the stomach and small intestine if no absorption had occurred. When rats drank water or 0.15 M NaCl, all points fell to the right of this "no absorption" line except when very small volumes were consumed; since no green dye was found in the cecum, these results indicate that some fluid usually was absorbed from the small intestine into the systemic circulation. More specifically, the absorbed volume of fluid increased as a function of intake, ranging from 5 - 25% of the fluid ingested during this initial bout. In contrast, when rats consumed 0.30 M NaCl, GI fill usually was close to or to the left of the "no absorption" line, the latter points indicating that fluid actually had moved into the small intestine from the systemic circulation.

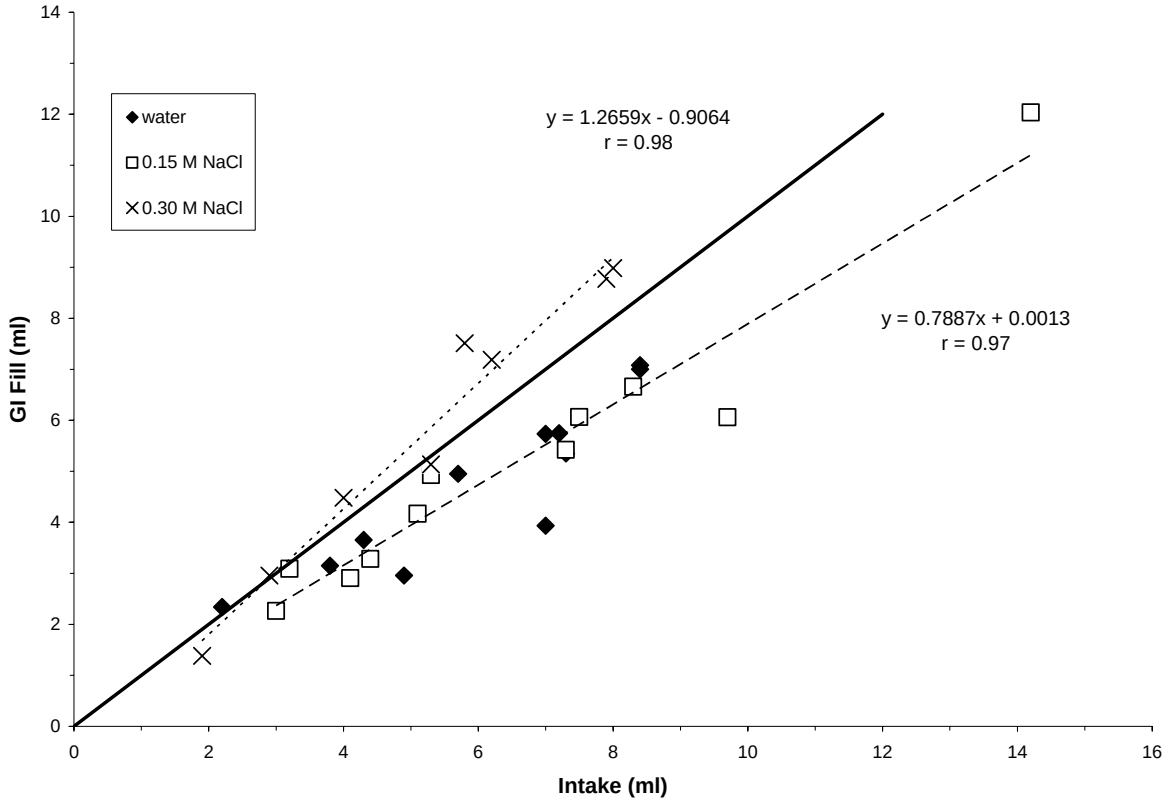

Figure 7: The sum of the measured fluid in the stomach and small intestine ("GI fill") of individual rats, plotted as a function of their intake. Symbols represent the same animals as in the previous figures; correlation coefficients and the equations of linear trendlines are shown. The solid line represents GI fill if "no net absorption" had occurred. In each group, GI fill was highly correlated with fluid intake (both *P*s <0.001). The upper regression line represents rats drinking 0.30 M NaCl and the lower regression line represents rats drinking either water or 0.15 M NaCl.

Other PEG-treated rats were given continuous access to water or 0.30 M NaCl for 60 min. When rats were given access to water, they did not ingest much additional fluid after the initial drinking bout. When these rats were killed at the end of the 60-min test period, they had considerably less fluid in their stomachs and intestines (2-3 ml) than rats that were killed at the end of the first bout of water ingestion (6-8 ml). When rats were given access to 0.30 M NaCl, they also did not ingest much additional fluid after the initial drinking bout and they had amounts of fluid in their stomachs and intestines (6-8 ml) that were similar to those of rats drinking 0.30 M NaCl that were killed at the end of the first bout.

When a separate group of rats was given continuous access to isotonic saline for 20-60 min, they drank rapidly during the initial bout and more slowly for the remainder of the test

period similar to the animals presented in Fig. 1. GI fill increased in proportion to fluid intake when rats were killed immediately after the first bout, and the two variables were highly correlated (Fig. 8; r = 0.97, P <0.001). When rats were allowed to drink for longer periods of time, greater variability was observed in the relation between GI fill and intake (r = 0.62). After 60 min, rats had significantly more fluid in their stomachs and intestines (11-13 ml) than rats drinking 0.15 M NaCl that were killed at the end of the first bout (6-8 ml).

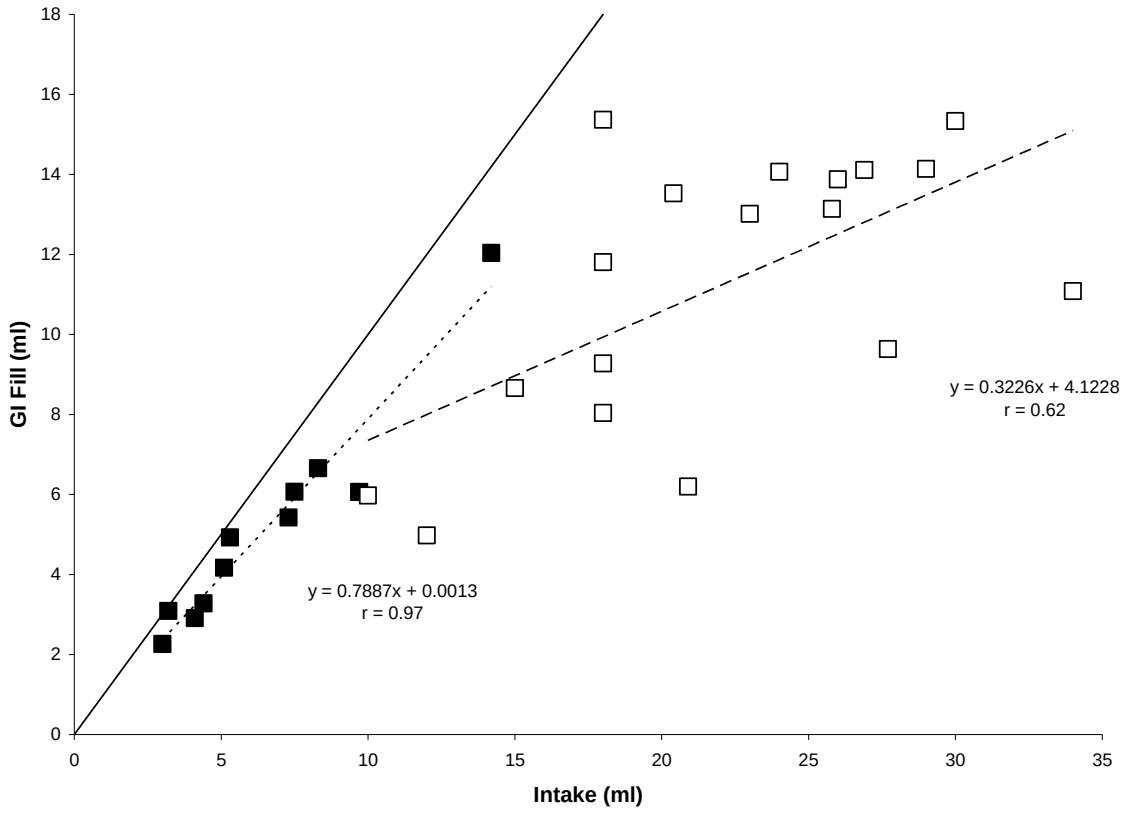

Figure 8: The sum of the measured fluid in the stomach and small intestine ("GI fill") of individual rats, plotted as a function of their intake. Rats were given continuous access to isotonic saline for 3-60 min. Symbols represent individual animals; correlation coefficients and the equations of linear trendlines are shown. The solid line represents GI fill if "no net absorption" had occurred. GI fill was highly correlated with fluid intake (r = 0.97, P <0.001) when rats were killed immediately after the first drinking bout. There was greater variability in GI fill when rats were allowed to drink for longer periods of time.

The percentage of ingested fluid that emptied from the stomach (i.e., gastric emptying) increased logarithmically as a function of time after the initial drinking bout (Fig. 9). Regardless of which fluid rats consumed, fluid emptied rapidly during the first 10 min, while the animals

were still drinking, and much more slowly during the remainder of the 60-min test. Water and isotonic saline emptied from the stomach at similar rates, while 0.30 M NaCl emptied more slowly. Rats that consumed water or 0.15 M NaCl emptied 60-80% of the ingested fluid by the end of the first drinking bout, while rats that consumed 0.30 M NaCl emptied only 30-40% of the ingested fluid by then. All rats emptied >80% of the ingested fluid by 60 min.

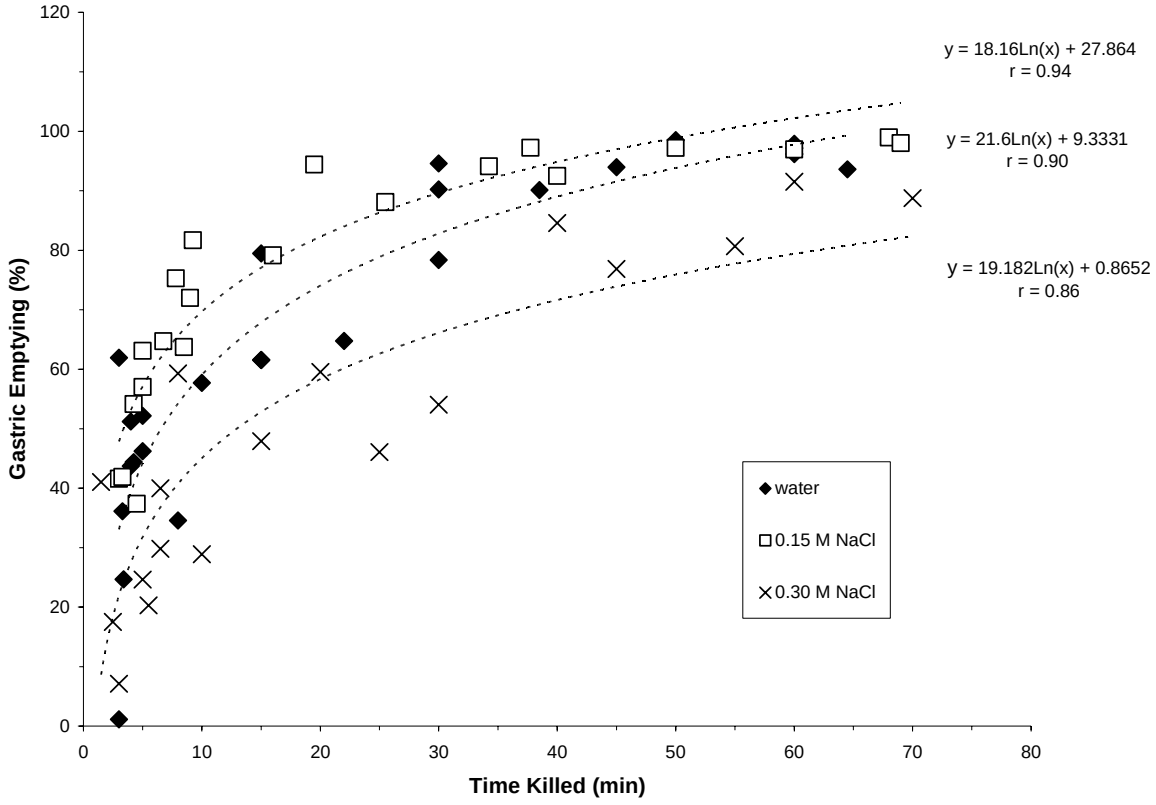

Figure 9: Percentage of fluid ingested by PEG-treated rats in an initial drinking bout that emptied from the stomach into the small intestine, plotted as a function of time. Rats drank water, 0.15 M NaCl, or 0.30 M NaCl solution. Symbols represent data from individual animals, correlation coefficients and the equations of logarithmic trendlines are shown (*P*s < 0.001). Fluid emptied rapidly during the initial drinking bout and much more slowly afterward. Water and 0.15 M NaCl emptied more rapidly than 0.30 M NaCl (*P* <0.05).

When rats consumed water or 0.15 M NaCl, nearly all of the fluid ingested in the initial bout was present in the stomach and small intestine during the first 10 min of the test period (Fig. 10). Subsequently, GI fill decreased similarly as fluid was absorbed from the small intestine. Less than 20% of the ingested fluid remained in the stomach and small intestine after 45 min. However, when rats consumed 0.30 M NaCl, the amount of fluid in the stomach and small

intestine actually was greater than the volume of ingested fluid during the first 30 min of the test period. Thereafter, GI fill decreased rapidly as fluid was absorbed into the circulation, and by the end of the 60-min test it had fallen to levels similar to those of rats that had consumed water or 0.15 M NaCl.

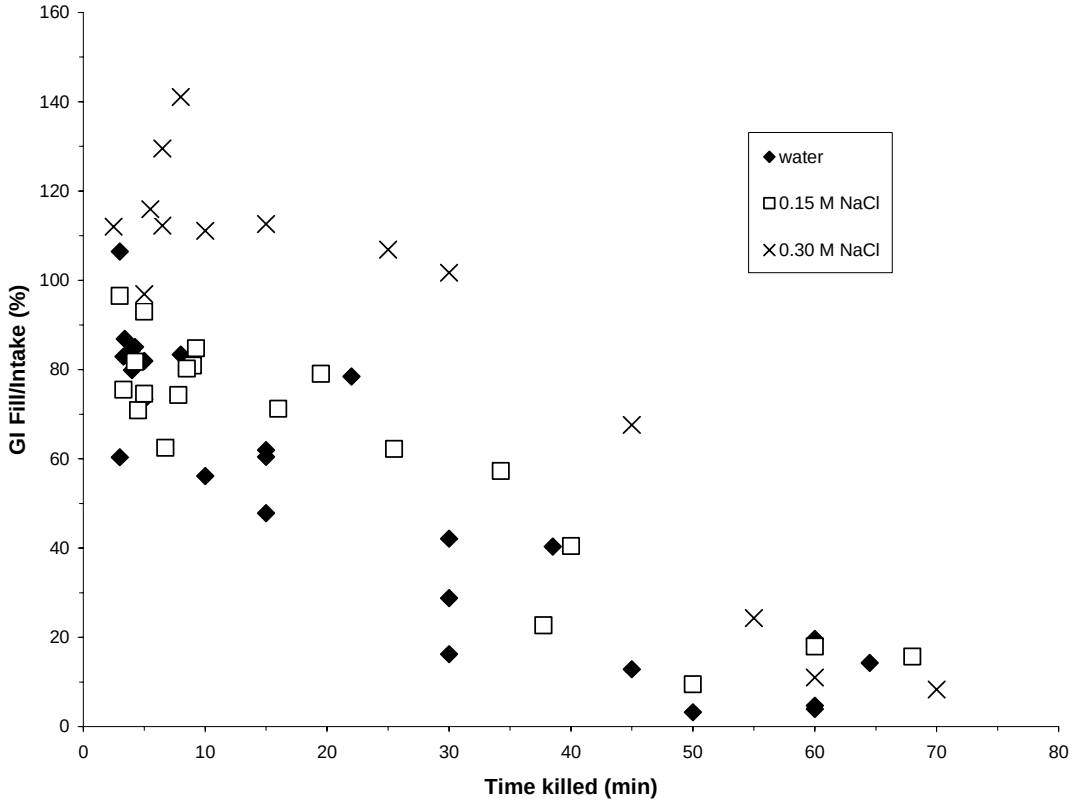

Figure 10: Volume of ingested fluid in the stomach and small intestine ("GI Fill"), expressed as a percent of the volume consumed in an initial bout and plotted as a function of time. PEG-treated rats drank water, 0.15 M NaCl, or 0.30 M NaCl solution. Symbols represent individual animals. GI Fill decreased rapidly when rats ingested water or 0.15 M NaCl, and much more slowly when 0.30 M NaCl was consumed.

As expected (Stricker & Verbalis, 1986), there was an exponential increase in plasma VP levels as a function of increasing plasma volume deficits in PEG-treated rats deprived of drinking fluid (Fig. 11). PEG-treated rats given water, 0.15 M NaCl, or 0.30 M NaCl to drink and then killed after an initial bout always showed the same exponential increase in pVP as did the animals with no access to fluid (Fig. 12). Rats that were given no treatment had baseline

pVP of 5-10 pg/ml. As shown in Table 1, pNa of control rats were not significantly different from those of rats that consumed one of the three fluids when measured after 10 min of drinking.

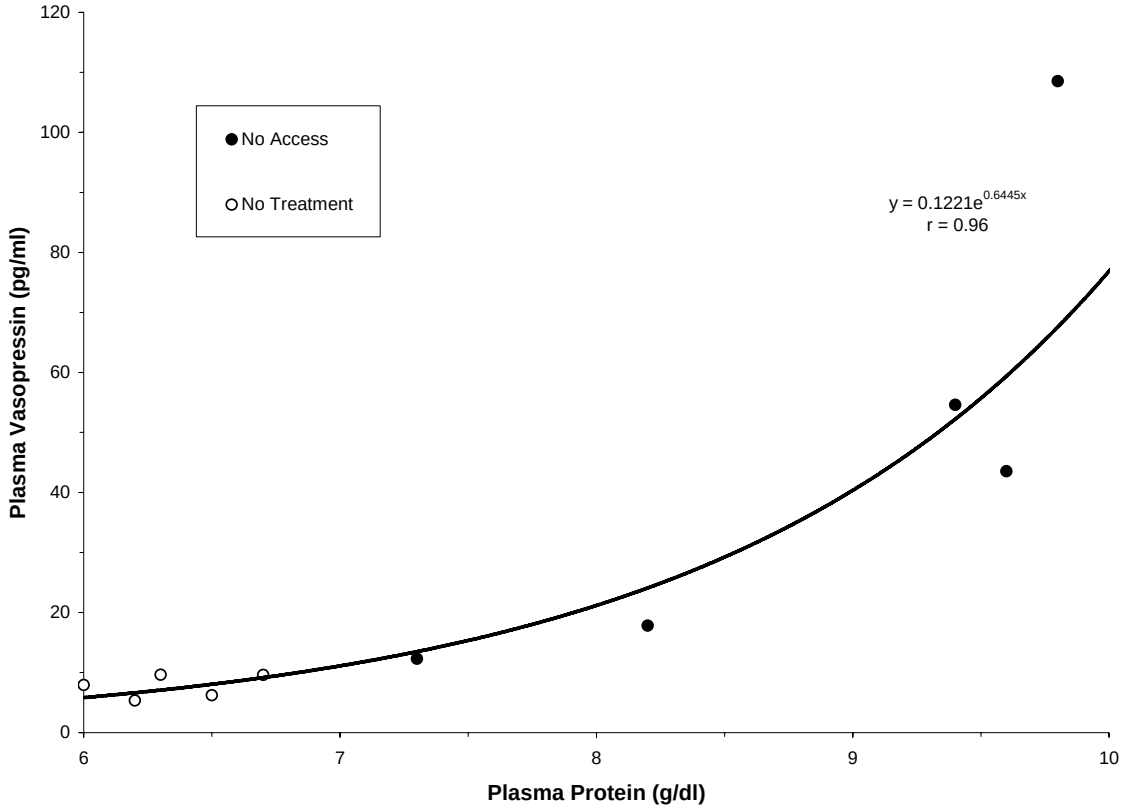

Figure 11: Plasma vasopressin levels (pg/ml), plotted as a function of plasma protein concentration. PEG-treated rats were fluid-deprived for used for baseline measurements. Symbols represent individual animals. Plasma VP increased exponentially in proportion to plasma protein concentration (*r* = 0.96, *P* <0.001).

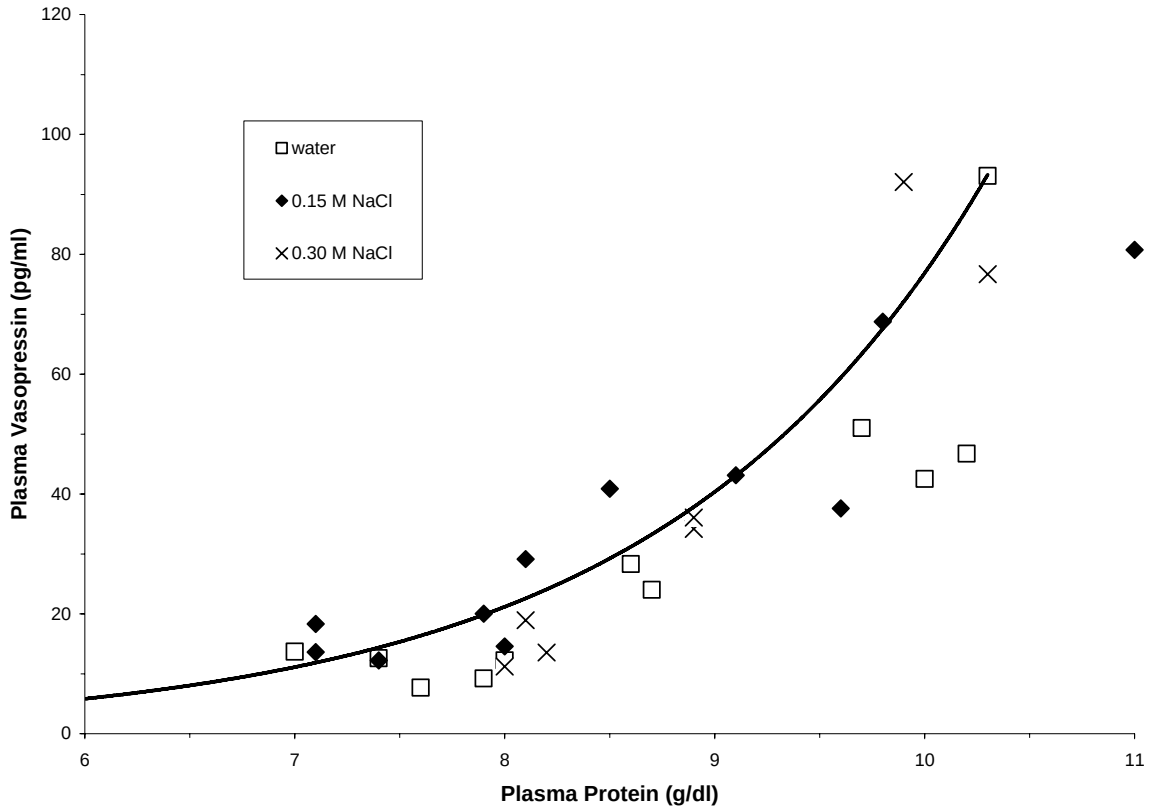

Figure 12: Plasma VP levels (pg/ml), plotted as a function of plasma protein concentration, in PEG-treated rats. The animals either were fluid-deprived or given access to water, 0.15 M NaCl, or 0.30 M NaCl solution to drink and then killed shortly after an initial bout. Symbols represent data from individual animals. Plasma VP increased exponentially as plasma protein concentration increased, regardless of which fluid was consumed. The data fell close to the exponential regression line, which was taken from PEG-treated animals not given access to fluid (from Fig. 11).

When rats were given access to water, 0.15 M NaCl, or 0.30 M NaCl for 60 min and then killed, they also showed the same exponential increase in pVP as did the animals with no access to fluid (Fig. 13). This occurred despite the fact that pNa was decreased when rats drank water, unchanged when they drank 0.15 M NaCl, and increased when they drank 0.30 M NaCl (Table 1).

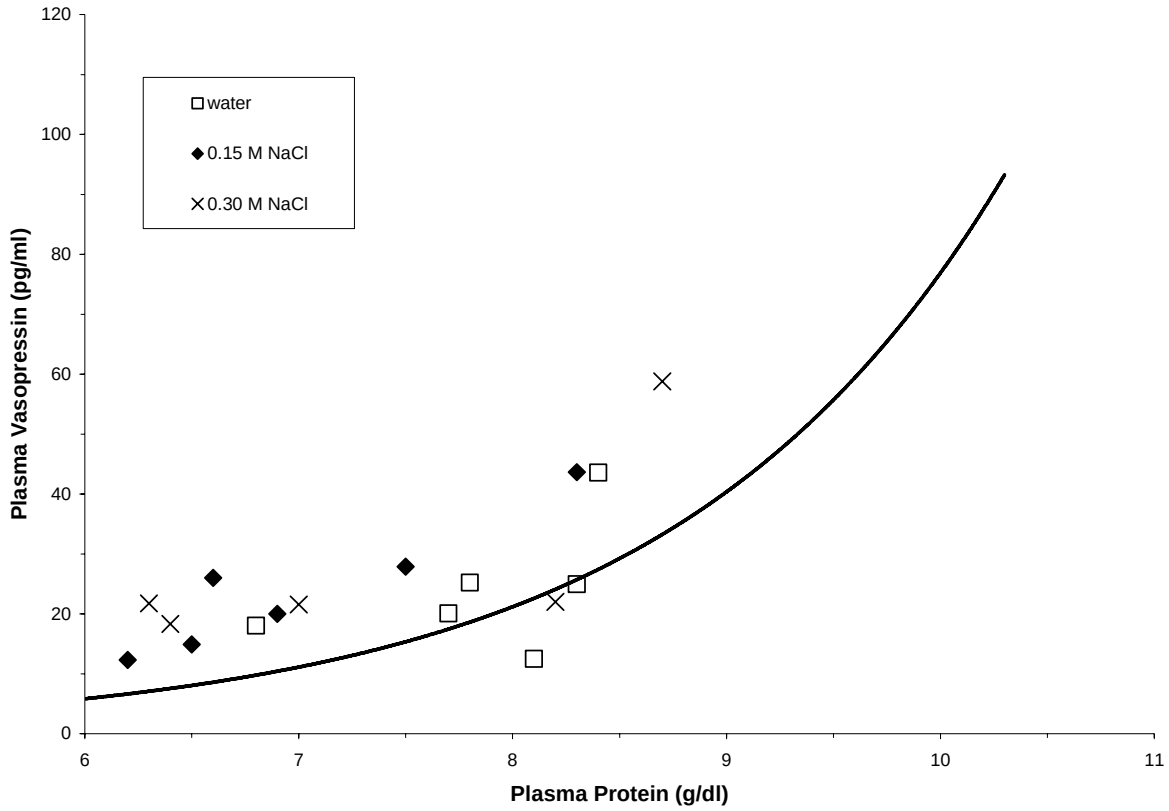

Figure 13: Plasma vasopressin levels (pg/ml), plotted as a function of plasma protein concentration. PEG-treated rats were either fluid-deprived or given access to water, 0.15 M NaCl, or 0.30 M NaCl solution during a 60-min drinking test. The exponential regression line represents those animals that were not given access to fluid (*r* = 0.96). Symbols represent individual animals. Plasma VP increased exponentially in proportion to plasma protein concentration, regardless of whether rats had access to fluid or which fluid was consumed.

Table 1: Plasma Na^+ concentrations (pNa, in mEq/L) in PEG-treated rats after 10 or 60 min of access to water, 0.15 M NaCl, or 0.30 M NaCl. Regardless of the concentration of ingested fluid, plasma Na^+ was not significantly different from control values (142.7 ± 0.4 mEq/L, *n* = 6) after 10 min of drinking. After 60 min, however, pNa decreased in rats that had consumed water (*P* <0.001) and increased in rats that had consumed 0.30 M NaCl (*P* <0.001).

Group	*n*	10 min	*n*	60 min
Water	11	141.6 ± 0.4	5	138.4 ± 0.5
0.15 M NaCl	11	143.2 ± 0.3	6	144.7 ± 0.6
0.30 M NaCl	8	142.8 ± 0.8	7	149.7 ± 0.4

5.0 DISCUSSION

Recent experiments have made great progress in understanding the early signals that inhibit or stimulate thirst and neurohypophyseal VP secretion in response to various treatments that elicit thirst *or* salt appetite (Stricker & Hoffmann, 2005, 2006). By studying hypovolemic rats, it is possible to investigate the effects of these early signals in animals that experience both thirst *and* salt appetite, although the two phenomena were investigated separately with one-bottle tests. When salt appetite is elicited by bilateral adrenalectomy or daily DOCA treatment, rats will not consume water. Similarly, when thirst is elicited by iv infusion of hypertonic saline or overnight water deprivation, rats will not consume very concentrated saline solution. Thus, in hypovolemic rats, the motivation to consume water likely results from thirst and the motivation to consume 0.30 M NaCl likely results from salt appetite. However, the motivation to consume isotonic saline is less clear; rats may be drinking in response to thirst or salt appetite.

The goals of this experiment were 1) to determine whether GI fill provides an early inhibitory signal for thirst and salt appetite in hypovolemic rats, 2) to examine the contribution of GI fill as an inhibitory signal for fluid consumption after the first drinking bout, 3) to determine whether a presystemic signal influences VP secretion when hypovolemic rats consume water or 0.30 M NaCl, and 4) to determine whether changes in systemic pOsm influence VP secretion when rats with prolonged hypovolemia consume water or 0.30 M NaCl.

5.1 THIRST AND SALT APPETITE

5.1.1 Does GI Fill provide an early inhibitory signal for thirst and salt appetite which leads to the termination of the initial drinking bout?

Previous studies with hypovolemic rats have shown that osmotic dilution in systemic blood inhibits thirst and that increases in systemic pOsm inhibit salt appetite (Stricker, 1969; Stricker & Verbalis, 1987). However, in the present delayed access drinking test, hypovolemic rats stopped drinking water long before there was a detectable drop in systemic pOsm. Therefore, systemic osmotic dilution could not have led to the termination of the initial drinking bout. Rather, it is likely that a presystemic signal contributed to the inhibition of fluid intake in these animals. Two plausible explanations for the observed inhibition of water intake include mediation by a concentration-dependent signal or mediation by a volume-related signal. When hypovolemic rats consumed concentrated saline they also stopped drinking before there was a detectable increase in systemic pOsm, again eliminating the possibility that increased systemic pOsm led to the termination of the initial drinking bout. Observations that PEG-treated rats consumed similar amounts of 0.30 M NaCl and water in initial drinking bouts are consistent with the hypothesis that the inhibition of fluid intake results from a volume-related signal, but they do not provide evidence against an alternative hypothesis that inhibition of fluid intake results from a concentration-dependent signal. In order to distinguish between these two hypotheses, hypovolemic rats were given 0.15 M NaCl to drink. These rats drank volumes in an initial bout that were similar to the amounts of water and 0.30 M NaCl that were consumed. Because isotonic saline could not have led to the generation of a concentration-dependent signal, these observations suggest that both thirst and salt appetite are inhibited by a volume-dependent signal in hypovolemic rats.

In contrast to the similar amounts of water, 0.15 M NaCl, and 0.30 M NaCl that rats consumed in initial drinking bouts, gastric emptying depended on the concentration of the fluid consumed. Specifically, isotonic saline empted more rapidly from the stomach than did water or 0.30 M NaCl. This concentration-dependent emptying may be controlled by a local circuit involving visceral osmoreceptors located in the duodenum. Although the distribution of fluid in the gastrointestinal tract depended on the concentration of the fluid ingested, the total amount of

fluid in the stomach and small intestine was tightly correlated with the volume of fluid ingested and was very similar in all three groups of animals. These results are consistent with the hypothesis that GI fill acts as an early inhibitory signal for thirst, which was first proposed to explain similar observations in rats made thirsty by overnight water-deprivation (Hoffmann et al., 2006). More recent data have suggested that GI fill also acts as an early inhibitory signal for salt appetite elicited by daily DOCA treatment (Stricker et al., ms submitted) or bilateral adrenalectomy (Bykowski et al., 2005). Thus, the present data suggest that GI fill acts as an early inhibitory signal in hypovolemic rats, which experience both thirst and salt appetite.

It is plausible that stretch receptors located on the outer walls of the stomach and small intestine are responsible for sensing the distension that occurs in these organs in response to fluid intake and for providing a signal that inhibits further intake. This pre-systemic signal would allow the animals to avoid large changes in systemic pOsm, which would result from consuming and emptying large quantities of water or concentrated saline. This signal would be especially valuable in hypovolemic animals, which are anuric due to the marked decrease in plasma volume and therefore are unable to buffer changes in systemic pOsm by adjusting the concentration of excreted urine.

5.1.2 What is the contribution of GI Fill after the first bout?

In order to explore the contribution of GI fill as an inhibitory signal after the first bout, rats were given access to water, 0.15 M NaCl, or 0.30 M NaCl for 60 min. The contents of the stomach and small intestine were analyzed in order to understand what happens to GI fill after the first bout. If GI fill remained elevated, we expected that the rats would not return to drink due to the persistence of that inhibitory signal. Alternatively, GI fill might decrease as fluid is absorbed from the small intestine, in which case we expected that rats would return to drink. In this case, we expected that fluid ingestion might occur at a rate similar to the rate at which fluid was absorbed from the small intestine such that rats were drinking to replace the absorbed fluid.

PEG-treated rats given access to water did not ingest much additional fluid after the initial drinking bout. When these rats were killed at the end of the 60-min test period, they had considerably less fluid in their stomachs and intestines (2-3 ml) than rats that were killed at the end of the first bout of water ingestion (6-8 ml). The majority of the fluid was consumed in the

first 10 min of the test and evidently it had emptied from the stomach and been absorbed into the circulation by 60 min. These animals also had significantly lower pNa than did control rats (Table 1). Previous data have shown that osmotic dilution of systemic blood is a strong inhibitory signal for thirst during hypovolemia (Stricker, 1969). Thus, it seems likely that water consumption by the present hypovolemic rats was being inhibited by GI fill initially and by osmotic dilution later in the 60-min drinking test.

PEG-treated rats given access to 0.30 M NaCl also did not ingest much additional fluid after the initial drinking bout. When these rats were killed at the end of the 60-min test period, they had amounts of fluid in their stomachs and intestines (6-8 ml) that were similar to those of rats drinking 0.30 M NaCl that were killed at the end of the first bout. The majority of the fluid was consumed in the first 10 min of the test and therefore no net absorption occurred during the remainder of the 60-min test. These animals also had significantly higher pNa than did control rats (Table 1). Previous studies have shown that increased pOsm in systemic blood provides a strong inhibitory signal for salt appetite (Stricker & Verbalis, 1987). Thus, it appears that consumption of 0.30 M NaCl by the present hypovolemic animals was inhibited by GI fill initially and by increased systemic pOsm (and perhaps by GI fill as well, though see below) later in the 60-min drinking test.

After the initial drinking bout, rats given access to isotonic saline paused for only ~5 min before continuing to ingest fluid (though at a slower rate). When these rats were killed at the end of the 60-min test period, they had significantly more fluid in their stomachs and intestines (11-13 ml) than rats drinking 0.15 M NaCl that were killed at the end of the first bout (6-8 ml). Of the 24 ml of fluid consumed, approximately 50% remained in the stomach and small intestine. Plasma Na concentrations were not significantly different from those of control rats (Table 1). Thus, it appears that consumption of isotonic saline by the present hypovolemic animals was being inhibited by GI fill initially but much less so later in the 60-min drinking test, in spite of the marked increase in GI fill.

One possible explanation for this observation is that there is an adaptation of the stretch receptors in the GI tract that causes the inhibitory signal associated with GI fill to decrease during the drinking test. Although the rats did continue to drink isotonic saline throughout the 1-hr test, they consumed the fluid at a much slower rate (0.3 ml/min) after the initial drinking bout than they had at first (0.9 ml/min). Thus, although GI fill was not sufficient to inhibit drinking

during the last 50 min of the test, it may have played a role in slowing down the rate at which rats consumed fluid.

5.2 VASOPRESSIN SECRETION

5.2.1 Does a presystemic signal influence VP secretion when hypovolemic rats consume water or 0.3 M NaCl?

The present data show that pVP increases exponentially as a function of plasma protein concentration 16 hr after injection of PEG. This same relationship between pVP and plasma protein concentration was observed in previous studies in which rats were killed 6 hr after injection of PEG (Stricker & Verbalis, 1986). Stricker and Verbalis (1986) also showed that systemic osmotic dilution inhibits pVP secretion and that an ip injection of hypertonic saline stimulates VP secretion further in hypovolemic rats. Although the early inhibition of VP secretion has not been studied using the colloid model, other experiments (Huang et al., 2000) have shown that water intake provides an inhibitory signal for VP secretion in rats with elevated systemic pOsm, which occurred before any change in systemic pOsm was detected. In contrast, VP remained elevated when rats ingested a comparable volume of isotonic saline. These data suggest that some presystemic consequence of the concentration of ingested water, not its volume, provides an early inhibitory signal for VP secretion in rats. These findings were recently extended to water-deprived rats; when dehydrated rats drank water, there was a rapid decrease in pVP that did not occur when they drink isotonic saline. In addition, it has been shown that an ig NaCl load (4 ml, 0.40 M solution) in a water-deprived rat causes an increase in VP secretion that precedes the increase in systemic pOsm (Stricker et al., 2002).

The present studies sought to determine whether 1) water ingestion provides an early signal for the inhibition of VP secretion, and 2) ingestion of concentrated saline provides an early signal for the stimulation of VP secretion in hypovolemic rats. Although the pVP of rats infused iv with hypertonic saline were elevated to pressor ranges (Huang et al., 2000), the nature of the stimulus was osmotic. In water-deprived rats, there was a mixed stimulus for VP secretion with osmotic and volumetric components, although the elevated pVP was subpressor. In both of these

models, the primary action of VP is as an antidiuretic agent. In the colloid model, however, thirst is elicited with a large and purely volumetric stimulus, pVP levels are very elevated, and the primary action of VP is as a pressor agent.

PEG-treated rats given water, 0.15 M NaCl, or 0.30 M NaCl to drink for an initial bout and then killed always showed the same exponential increase in pVP as did the animals denied access to drinking fluid (Fig. 12). Thus, it appears that ingestion of 0.30 M NaCl does not cause a presystemic increase in pVP, as was expected. Similarly, water ingestion does not provide an early signal that inhibits VP secretion when the excitatory stimulus for its secretion is volumetric. In considering why water ingestion inhibits VP secretion stimulated by increased systemic pOsm but not by decreased plasma volume, one possibility is that the hypovolemia interfered with the generation of the peripheral inhibitory signal (i.e., the animals might not have detected the local osmotic dilution). Alternatively, water ingestion may not have inhibited VP secretion in hypovolemic rats because the excitatory stimulus is much larger than that of the rats studied in previous experiments (i.e., rats that were infused with iv saline or water-deprived), and it is therefore more difficult to inhibit. However, it should be noted that there is a lack of inhibition of VP secretion throughout a wide range of plasma volume deficits (Fig. 12), not just when hypvolemia is extreme. Furthermore, pVP in the PEG-treated rats was similar to values in rats infused iv with hypertonic saline, suggesting that the excitatory stimulus of VP secretion was similar in the two studies. Perhaps it is not the current magnitude of stimulation but the duration of stimulation that is critical (i.e., a few hours versus 16 hr). Additional investigations are required to evaluate these possibilities.

5.2.2 Do changes in systemic plasma osmolality influence VP secretion when rats with prolonged hypovolemia consume water or 0.3 M NaCl?

When rats were given access to water or 0.30 M NaCl for 60 min and then killed, they also showed the same exponential increase in pVP as the animals with no access to fluid (Fig. 13) in spite of the marked decrease and increase (respectively) in systemic pNa. These observations were surprising given the previous observations of Stricker and Verbalis (1986), which showed that systemic osmotic dilution suppresses VP secretion and an ip injection of hypertonic saline stimulates further VP secretion in hypovolemic rats. In both the previous experiment and the

present study, water ingestion resulted in a 3-6% decrease in pNa concentration. The range of volume deficits also was similar in both experiments. However, the previous drinking test was administered 6 hr after injection of PEG solution, while the current drinking test was administered 16 hr after PEG treatment. As mentioned in the previous paragraph, the more prolonged stimulus may have made it more difficult to inhibit VP secretion. If so, it seems important to determine at what point between 6 hr and 16 hr a 3-6% osmotic dilution of systemic blood becomes no longer effective in inhibiting VP secretion in hypovolemic rats. Similarly, it remains to be determined whether greater osmotic dilution can inhibit VP secretion 16 hr after injection of PEG, and whether water ingestion provides an early inhibitory signal for VP secretion 6 hr after injection of PEG. These residual issues also require further investigation.

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