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Research Article

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It also has been reported that dialysates of natriuretic plasma, but not of hydropenic plasma, when placed directly into the tubule lumen, inhibit proximal sodium reabsorption. In double blind studies carried out independently in Bethesda, London, and Cologne, we failed to detect the presence of a dialyzable inhibitor in natriuretic plasma. Finally, in contrast to other recent reports, we were unable to detect inhibitory activity in plasma obtained from dogs during the "escape" phase of chronic deoxycorticosterone acetate administration.

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Failure to Demonstrate a Hormonal Inhibitor of Proximal Sodium Reabsorption

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ABSTRACT Recently, it has been reported that a humoral inhibitor of proximal sodium reabsorption could be detected in plasma, and dialysates of plasma, of rats and dogs undergoing saline diuresis. We have repeated these studies using similar techniques and protocols. Fractional sodium reabsorption by the proximal tubule (as estimated in free-flow micropuncture studies from tubule fluid-to-plasma inulin ratios) was found not to be lower during infusion of "natriuretic" plasma than during subsequent infusion of "hydropenic" plasma. Similarly, infusion of natriuretic plasma failed to prolong reabsorptive half-time of the shrinking drop beyond that seen during hydropenic plasma infusion. No increase in urine volume or rate of sodium excretion was observed during the period of natriuretic plasma infusion, nor did natriuretic plasma result in an increase in these measures in rats undergoing water diuresis.

It also has been reported that dialysates of natriuretic plasma, but not of hydropenic plasma, when placed directly into the tubule lumen, inhibit proximal sodium reabsorption. In double blind studies carried out independently in Bethesda, London, and Cologne, we failed to detect the presence of a dialyzable inhibitor in natriuretic plasma. Finally, in contrast to other recent reports, we were unable to detect inhibitory activity in plasma obtained from dogs during the "escape" phase of chronic deoxycorticosterone acetate administration.

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INTRODUCTION

The natriuresis that accompanies the expansion of extracellular fluid volume by isotonic saline is mediated in part by inhibition of tubule sodium reabsorption (1, 2). Of the several mechanisms that have been proposed to explain this inhibition, considerable interest has followed the suggestion (1, 3) that a humoral substance is released into the blood stream in response to volume expansion. Two laboratories recently have reported that such a humoral inhibitor could be demonstrated using micropuncture and clearance techniques (4, 5). In these studies dialysates of plasma from saline-loaded dogs and rats ("natriuretic plasma"), when placed into the lumen of proximal tubules of antidiuretic assay rats, produced an inhibitory effect on intrinsic reabsorptive capacity as measured by the shrinking drop technique. In contrast, dialysates of plasma from these animals before they had been expanded with saline ("hydropenic plasma") had no such effect on sodium reabsorption. Similarly, it was reported that infusions of natriuretic plasma, but not hydropenic plasma, decreased sodium reabsorption by the proximal tubule as measured either by the shrinking drop technique, or in free-flow studies by changes in the tubule fluid-to-plasma inulin ratio. Finally, in experiments using assay rats with hereditary diabetes insipidus, increased rates of urine flow were seen after infusions of plasma from saline-loaded dogs but not after infusion of plasma from hydropenic dogs.

Experiments were undertaken independently in Bethesda, and in London and Cologne to investigate further these four assay methods. The results obtained in the three laboratories, reported together here, fail to con-

firm the previous conclusion that a humoral inhibitor of sodium reabsorption can be demonstrated by these methods.

METHODS

The following experimental protocols were used:

Bethesda

Studies were performed using Sprague-Dawley rats weighing 200–320 g. They were anesthetized with Inactin (100 mg/kg) and prepared for micropuncture as described previously (6). Samples of jugular venous blood were obtained for assay from 15 anesthetized dogs before (hydropenic plasma) and after (natriuretic plasma) the intravenous infusion of isotonic saline solution (100 ml/kg in 1 hr). This volume of saline was found by clearance measurements in seven of the dogs to result in the excretion of at least 10% of filtered sodium (range 10.4–18.8%). Blood also was obtained from four rats before and after the infusion of 30 ml of isotonic saline solution. Blood was centrifuged immediately after collection and the plasma was stored at 4°C for 1–7 days.

Group I. Paired samples of plasma obtained from five dogs before and after saline loading were infused into seven antidiuretic rats. During these infusions fractional sodium reabsorption by the proximal tubule was determined by the recollection technique. A solution containing 10% inulin in 0.5% NaCl was infused at the rate of 0.02 ml/min. After a 30 min equilibration period an infusion of plasma from expanded dogs was begun at the rate of 0.02 ml/min. 30 min after the start of the natriuretic plasma infusion samples of tubule fluid were collected from three to five late proximal convolutions. This period of tubule fluid collection lasted 30 min. The infusion of expanded donor plasma then was stopped and was replaced with an infusion of hydropenic plasma obtained from these same dogs before saline loading. 30 min after the onset of this infusion, samples of tubule fluid were recollected from the same tubule segments, again in a 30 min period. The transit time of Lissamine green was measured during each collection. The concentration of inulin in tubule fluid was measured by the microfluorometric method (7). Inulin concentration in plasma was measured by the anthrone method (8). Sodium was measured by flame photometry.

Group II. In four antidiuretic rats the above protocol was followed except that during the infusions of sample pairs of natriuretic and hydropenic plasma, sodium reabsorption by the proximal tubule was measured by the shrinking drop technique of Gertz (9). The half-time for reabsorption of a drop of Ringer's solution between columns of castor oil was determined from photographs taken at 4-sec intervals.

Group III. Dialysates of sample pairs of jugular venous plasma obtained from five dogs before and after saline loading were assayed in eight antidiuretic rats using the shrinking drop technique. Dialysates of pooled plasma samples obtained before and after saline loading in four rats were assayed in two antidiuretic rats. These dialysates, as well as Ringer's solution, were injected directly into the lumen of proximal tubules to serve as the aqueous drop. The rats and two of five of the dogs from which the plasma samples were obtained had been pretreated with deoxycorticosterone acetate (DOCA) for 7 days. Plasma was dialyzed for 16–24 hr at 4°C against an equal volume of saline-bicarbonate solution as described by Rector et al. (4). Sample pairs from the same dog were coded and tested in

the same rat in varying sequence in periods of 30–45 min each. The identities of the dialysates remained unknown to the experimenters until completion of the experiment. Shrinking drop measurements, usually repeated two to three times in each tubule, were made in two to five tubules with each dialysate of hydropenic and natriuretic plasma and Ringer's solution.

London and Cologne

Group I. Wistar and Brattleboro rats weighing 200–300 g and undergoing water diuresis were used in experiments designed to test the effect of infusion of plasma on the delivery of sodium and water out of the proximal tubule. Samples of peripheral or jugular venous blood were obtained from eight conscious dogs before and after the infusion of isotonic saline (100 ml/kg in 1 hr). The blood was centrifuged and the plasma kept at 4°C until it was assayed in London within 24–48 hr. These plasma samples were infused into 13 water-loaded Wistar rats and three Brattleboro rats with hereditary diabetes insipidus. The rats were anesthetized by intragastric administration of 10% ethanol in a volume of water equal to 8% of body weight. An intravenous infusion of 3% ethanol in 50 mM NaCl solution was begun approximately 60 min later at a rate of 0.1 ml/min and continued for the duration of the experiment. The assay of plasma samples was begun only after a stable rate of urine flow was achieved. Hydropenic and natriuretic plasma samples were infused in alternating sequence at 0.025 ml/min for 20–40 min. The cystostomy tube for urine collection and the jugular catheter for infusion were placed on the day before the study. Urine volume was estimated by weighing and sodium concentration was measured by flame photometry.

Group II. Dialysates of jugular venous plasma obtained from three dogs on three separate occasions were assayed in 10 antidiuretic Wistar rats using the shrinking drop technique. The dialysates were injected directly into the lumen of the proximal tubule to serve as the aqueous drop. Three conscious male collies were infused with saline (100 ml/kg in 1 hr) on 3 consecutive wk. The nine pairs of plasmas drawn from these animals before and after saline infusion were placed into Visking cellophane bags and dialyzed in a plastic container against an equal volume of bicarbonate-saline solution at 4°C for a period of 20–24 hr. Each pair of plasma dialysate obtained from the same dog was coded and taken to Cologne where shrinking drop assays were performed within 1–5 days. The identity of the samples remained unknown to the experimenter until completion of the study. Each sample pair was assayed in the same rat; one pair of samples was reassayed in a second rat (sample 8). The assays of these nine pairs of dialysates were performed in 10 male Wistar rats weighing 200–300 g and anesthetized with an intraperitoneal injection of Inactin (90–125 mg/kg). Surgical losses of extracellular fluid were replaced by 1–4 ml of either isotonic saline (150 mM NaCl) or bicarbonate-saline (Na, 135 mEq/liter, Cl, 110 mEq/liter, and HCO₃, 25 mEq/liter). When the operative procedures were finished, a constant infusion of isotonic saline or bicarbonate-saline was given at the rate of 0.016 ml/min for replacement of any continued fluid loss. The reabsorptive half-time was measured both by serial photographs taken at 5-sec intervals and visually by means of an eyepiece micrometer. To avoid contamination of the dialysate with fluid from tubules or from the surface of the kidney the tip of the capillary containing the dialysate was blocked with a small amount of oil until the dialysate was injected into the tubule lumen.

RESULTS

Bethesda

Group I. Fractional reabsorption of sodium by the proximal tubule was measured in antidiuretic rats during infusions of natriuretic and hydropenic plasma. Natriuretic plasma was infused first and was followed by plasma from hydropenic dogs. The tubule fluid-to-plasma (TF/P) inulin ratios were found to be lower during the infusion of hydropenic plasma (the second infusion) than during the infusion of natriuretic plasma (Fig. 1). Fractional reabsorption fell in nearly every tubule studied; in none was there a significant increase during the infusion of hydropenic plasma. Transit time shortened from 11.3 sec \pm 0.65 SE during the initial infusion period of 9.0 sec \pm 0.56 SE during the second infusion period indicating increased linear velocity of flow. Plasma volume increased progressively during these experiments; the average change after the infusion of both plasma samples, as calculated from the hematocrit, was +26%. The per cent of filtered sodium that was excreted remained the same during each period, averaging 0.42% \pm 0.22 SE for the initial period and 0.42% \pm 0.23 SE for the recollection period.

Group II. Infusion of natriuretic plasma into four rats was found not to prolong the reabsorptive half-time of the shrinking drop (Fig. 2). In two of the four rats the average values for reabsorptive half-time during the period of the second infusion were greater than during the infusion of natriuretic plasma. The changes

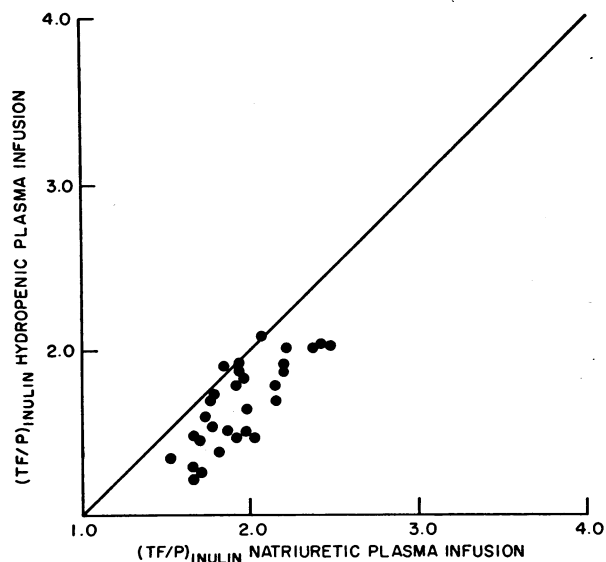


FIGURE 1 (Bethesda) Tubule fluid-to-plasma inulin ratios measured during infusion of plasma from dogs undergoing saline diuresis (first collections) and during infusion of hydropenic plasma from the same dogs (recollections).

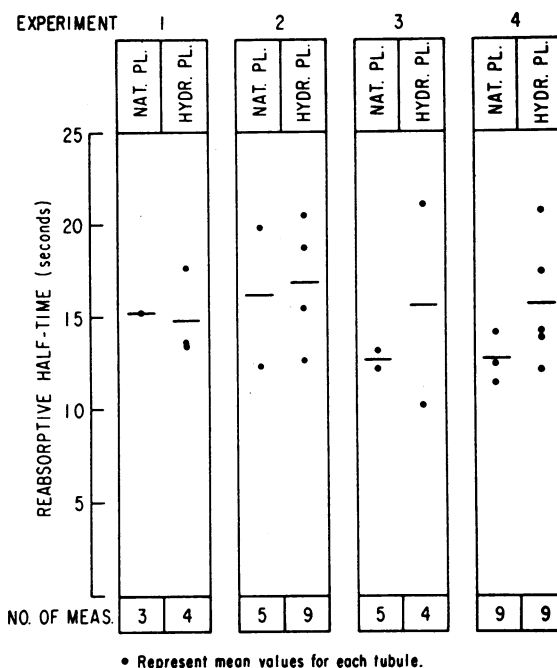


FIGURE 2 (Bethesda) Reabsorptive half-times of shrinking drops of Ringer's solution measured in four antidiuretic rats during infusions of natriuretic plasma (nat. pl.) and hydropenic plasma (hydr. pl.) from dogs. Measurements made in the same tubule are averaged for that tubule. Horizontal bar indicates the mean of values from different tubules.

in transit time and plasma volume were similar to those measured in group I.

Group III. The results using dialysates of natriuretic plasma and hydropenic plasma are summarized in Fig. 3. Samples from dog Nos. 1, 2, and 3 were tested in each of two rats. Samples from dog Nos. 4 and 5 were tested in one rat each. Pooled samples of plasma from four rats obtained before and after saline loading were tested in each of two rats. In eight of these ten assay rats Ringer's solution was tested as an unknown. In each experiment the sequence of the samples was varied. No difference in reabsorptive half-time was seen with natriuretic plasma, hydropenic plasma, or Ringer's solution.

It has recently been reported that after chronic DOCA administration to dog (10) and man (11), increased levels in plasma of a dialyzable inhibitor of proximal sodium reabsorption could be detected. As can be seen in Fig. 3, dialysates of plasma from DOCA-escaped hydropenic donor animals were found not to prolong reabsorptive half-time beyond that seen with dialysates from non-DOCA-treated animals.

London and Cologne

Group I. In these studies antidiuretic hormone was absent because of inhibition by ethanol anesthesia and

water loading (Wistar rats) or because of heredity (Brattleboro rats). The rate of urine flow in these rats therefore was taken as an index of the fraction of filtered sodium and water delivered out of the proximal tubule. As shown in Fig. 4, values for urine flow rate during the infusion of hydropenic plasma (mean = $117 \mu\text{l}/\text{min} \pm 0.04 \text{ SD}$) were not less than during the infusion of an equal volume of plasma from saline-loaded dogs (mean = $113 \mu\text{l}/\text{min} \pm 0.04 \text{ SD}$). Similarly the rate of urinary sodium excretion did not differ in the two experimental periods. Sodium excretion averaged $0.49 \mu\text{Eq}/\text{min} \pm 0.06 \text{ SD}$ during infusion of hydropenic plasma and $0.45 \mu\text{Eq}/\text{min} \pm 0.05 \text{ SD}$ during infusion of natriuretic plasma. Prior treatment of the assay rats with DOCA did not influence the results.

Group II. The results of shrinking drop experiments performed to evaluate the effect of dialysates of hydropenic and natriuretic plasma on reabsorptive half-time are summarized in Fig. 5. Since the identity of the sample was unknown at the time of the experiment the sequence of study varied in different experiments. Measurements of reabsorptive half-time by photographic and visual methods gave similar results. As

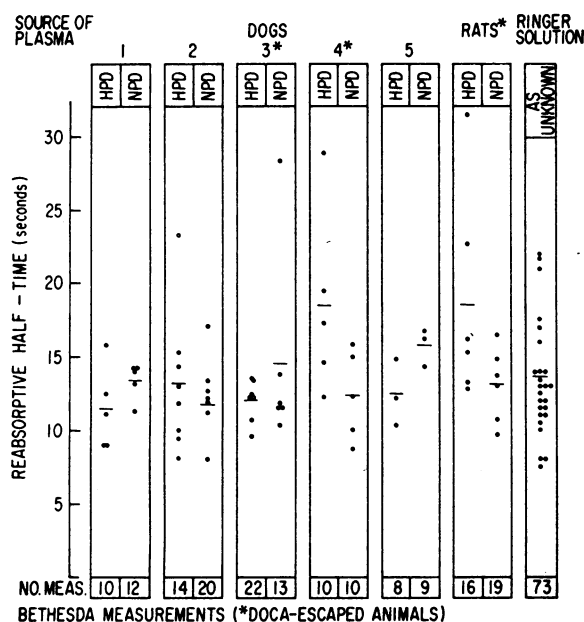


FIGURE 3 Reabsorptive half-times of shrinking drops measured in 10 antidiuretic rats. The nature of the fluid in the shrinking drops was not known to the experimenter and was either a dialysate of hydropenic plasma (HPD), a dialysate of natriuretic plasma (NPD), or (in 8 of the 10 rats) Ringer's solution. Closed and open circles indicate different assay rats; each symbol shows the average of repeated measurements in a single tubule. The total number of measurements is shown at the bottom of each column. Horizontal bar indicates the mean values for all tubules.

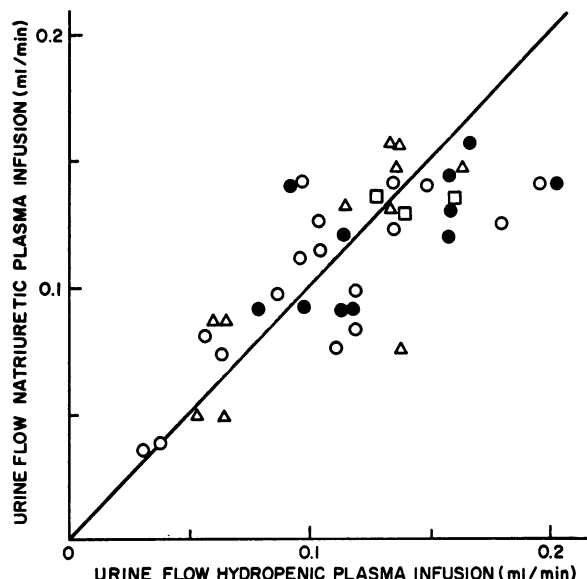


FIGURE 4 (London) Urine flow rates of rats undergoing water diuresis measured during infusion of hydropenic and natriuretic plasma. Values are means of two to four 10-min collection periods during which hydropenic plasma and natriuretic plasma were infused alternately. Plasma from a peripheral vein was infused into Wistar rats that had been pretreated with DOCA (open circles) or rats that were not given DOCA (closed circles). Plasma from a jugular vein was infused into Wistar rats that had been pretreated with DOCA (squares) or rats with hereditary diabetes insipidus (triangles). Values found during infusion of natriuretic plasma are plotted against means of values found during infusion of plasma from hydropenic dogs, the hydropenic plasma being infused both before and after the natriuretic plasma.

can be seen in Fig. 5, dialysates of plasma from saline-loaded dogs did not systematically prolong reabsorptive half-time to values greater than those seen with hydropenic plasma.

DISCUSSION

The factors that operate within the mammalian nephron to control sodium excretion have been the subject of considerable interest in a number of laboratories, including those of the authors. The report describing methods sufficiently sensitive to allow detection of a humoral inhibitor of proximal sodium reabsorption in the plasma of saline-loaded animals (4) led us to apply these assay procedures in our continuing studies of the regulation of sodium excretion. For these assays to be useful they of necessity should be both sensitive and relatively free of false negative and false positive results. In our experiments none of the four reported methods met these requirements.

With regard to the sensitivity of the methods, it is

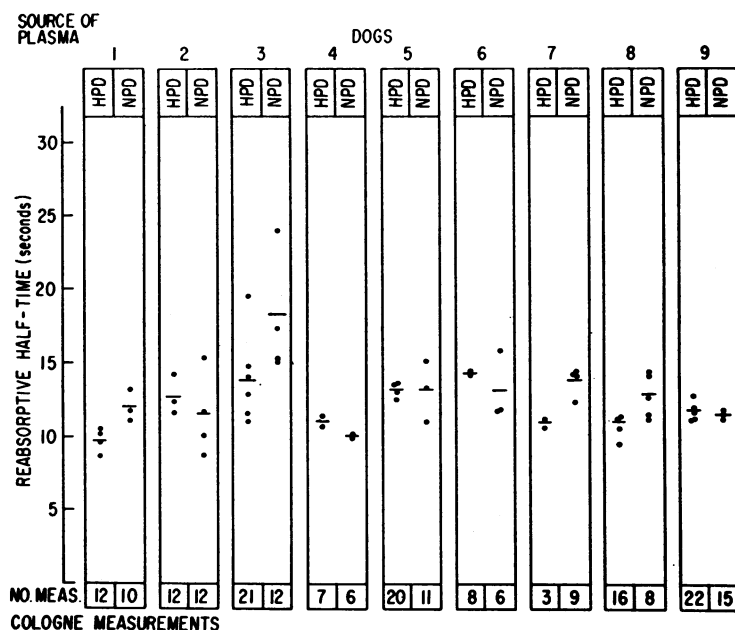


FIGURE 5 Reabsorptive half-times of shrinking drops measured in 10 antidiuretic rats. The nature of the fluid in the shrinking drop was not known to the experimenter and was either a dialysate of hydropenic plasma (HPD) or of natriuretic plasma (NPD). Closed and open circles (in experiment No. 8) indicate different assay rats; each symbol shows the average of repeated measurements in a single tubule. The total number of measurements is shown at the bottom of each column. Horizontal bar indicates the mean value for all tubules.

agreed that the changes in rates of sodium reabsorption by the proximal tubule can be measured by these methods. Both $(TF/P)_{Na}$ and shrinking drop measurements have demonstrated decreased sodium reabsorption after infusions both of isotonic saline and of hyperoncotic albumin. Decreases in fractional sodium reabsorption measured after volume expansion with both saline (12-15) and albumin (16, 17) have been in the range of 32-42%. Decreases in absolute rates of sodium reabsorption determined from shrinking drop measurements after infusions of saline (14, 15, 18) and albumin (17) have ranged from 30-39%. A recent investigation of volume expansion with varying amounts of isotonic saline reports that decreases in fractional reabsorption as small as 12% can be detected (19). These repeatedly observed changes in reabsorption define an upper limit to the magnitude of change that could have been produced by the plasma transfers in the present experiments and still have gone undetected. No information is available that permits a speculation about a lower limit for changes detectable by these methods. If there is a natriuretic hormone, it remains possible that the chief limitations of these assay methods are factors related not to the micropuncture techniques but to the plasma transfer procedures such as volumes transferred, concentration

of an inhibitor at the transport site, and, possibly, species differences.

Efforts were made to use experimental protocols that were similar to those used by others. Methods for collection and storage of blood samples, preparation of plasma dialysates, and techniques of micropuncture were the same as those reported previously (4). Nevertheless our results for the four types of experiments differ from the findings already reported. Although we are unable to explain the differences in results, we consider it worthwhile to describe our negative findings because of the importance of this question to our over-all understanding of the renal regulation of salt and water homeostasis. Furthermore, because an attempt to catalog the many possible sources of such differences would be entirely speculative, we consider it worthwhile to comment only on the results of the present experiments.

In experiments in which plasma samples were infused intravenously into the assay rats (Fig. 1) it may be noted that the $(TF/P)_{Na}$ ratios were actually lower after the infusion of hydropenic plasma than during the earlier period when natriuretic plasma was infused. This change, therefore, could not be attributed to a specific inhibitory factor present in plasma, but is more likely to have resulted from continued infusion and progressive

expansion of extracellular fluid volume. It has been shown that expansion of blood volume is associated with a depression of fractional sodium reabsorption by the proximal tubule (16, 19). Such a change in the assay animals could be responsible for some false positive results.

In initial experiments using the Gertz method with shrinking drops of isotonic saline solution, our results were sufficiently variable that we chose to perform the assay experiments using coded samples. The identity of the samples remained unknown to the investigator until the micropuncture manipulations and the analyses of the serial photographs had been completed. Using this approach no difference was seen between the effects of natriuretic and hydropenic plasma. Occasionally, and unpredictably, long reabsorptive half-times were observed with shrinking drops made of Ringer's solution, as well as with either type of dialysate. We could not attribute this variability to such technical details as viscosity of the oil, length of the oil columns, length of the aqueous drop, or variations of intratubular hydrostatic pressure.

In experiments in which the urine flow rate during water diuresis was used as an index of proximal sodium reabsorption, infusion of natriuretic plasma was both preceded and followed by an infusion of hydropenic plasma. The urine flow rate during the infusion of natriuretic plasma was, therefore, compared separately to the flow rate during each period of hydropenic plasma infusion. These comparisons, as shown in Fig. 4, reveal no differences in the effects produced by hydropenic and natriuretic plasmas. Continued infusion of plasma occasionally did increase the rate of urine flow, but this appeared to be related to the duration of infusion rather than to the nature of the plasma source. It is unlikely that the water load administered to the assay rats before each experiment could have obscured differences between the effects of hydropenic and natriuretic plasma. Davis, Knox, and Berliner have shown, at least in the dog, that a large water load has no demonstrable effect on proximal sodium and water reabsorption (20).

Of the possible mechanisms involved in the control of sodium reabsorption, a humoral inhibitor of sodium reabsorption has been invoked on both theoretical and experimental grounds (1, 3). Experiments in which blood was cross-circulated from saline-loaded dogs to hydropenic dogs yielded results which suggest that a transferable factor present in the blood of the donor dog is capable of promoting sodium excretion in the recipient (21-23). However, the small magnitude of the natriuresis and changes in the composition of blood make interpretation of these experiments difficult. Other experiments in which efforts were made to control sev-

eral such compositional changes have, by exclusion, offered further indirect evidence consistent with the presence of a humoral natriuretic factor (24).

The studies recently reported (4) offered the first direct evidence for the existence of an inhibitory factor in plasma. Although infusion of plasma from saline-loaded animals did not increase sodium excretion (4), a dialyzable constituent was thought to inhibit proximal sodium reabsorption. Using the same methods we have been unable to detect an inhibitor of proximal sodium reabsorption in the plasma, or dialysates of plasma, from saline-loaded animals. Nevertheless, the results of these experiments do not eliminate the possible existence of a hormone that augments sodium excretion. Rather, we find that if such a hormone is present these four assay methods are not sufficiently sensitive to permit its detection.

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