Further Characterization of the Natriuretic Factor Derived from Kidney Tissue of Volume-Expanded Rats
Effects on Short-Circuit Current and Sodium-Potassium-Adenosine Triphosphatase Activity

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SUMMARY Boiled homogenates of kidneys from volume-expanded and hydropenic rats were subjected to column chromatography. The fraction eluting within the range of partition coefficients (Kav) 0.76-0.89 (fraction III) was lyophilized and the effects of this semipurified preparation were assessed on short-circuit current (SCC) across isolated frog skin, on rat kidney cortex Na-K-ATPase activity, and on sodium excretion by the rat in vivo. At a dose of 500 μg/ml, fraction III from expanded rat kidney inhibited SCC by 21 ± 5% (P < 0.01), whereas the same fraction from hydropenic rat kidney produced an insignificant change in SCC of 2 ± 8%. In a dose-response study, 50, 150, 500, and 1,500 pg/ml of fraction III from expanded rat kidney inhibited SCC by 4, 8, 19, and 28%, respectively; 500, 1,000, and 1,500 μg/ml inhibited Na-K-ATPase activity by 11, 22, and 49%, respectively. An identical study with fraction III from hydropenic animals showed no significant effect in either assay. Also, fractions from expanded and hydropenic rats, eluted after fraction III (fractions IV and V), had no effect on SCC or Na-K-ATPase activity. Fraction III also produced significant natriuresis in vivo at a dose of 500 μg/ml, confirming our observations that a natriuretic principle may be recovered from the kidneys of volume-expanded rats. We suggest that this natriuretic principle may act by reducing active sodium transport via inhibition of Na-K-ATPase.

SEVERAL STUDIES now have shown that natriuretic factors can be found in both the serum and the urine of volume-expanded or uremic animals and humans. These factors not only produce a natriuresis when injected into test animals but also inhibit transepithelial sodium transport in anuran membrane preparations such as the isolated frog skin or toad bladder. The term "antinatriferic" has been used to describe this latter effect. In a recent study, Kaplan et al. have shown that the natriuretic factor obtained from uremic urine increases intracellular sodium content of and decreases pyruvate oxidation by isolated toad bladder cells. These findings suggest that this natriuretic factor inhibits active sodium transport either through interference with ATP generation via the tricarboxylic acid cycle or with some step in the active transport sequence, possibly the transport enzyme, Na-K-ATPase.

Previous work from this laboratory has demonstrated that a natriuretic factor also can be isolated from kidneys of volume-expanded rats but is not found in kidneys of hydropenic rats. In the present study we have shown in addition that this factor inhibits transepithelial sodium transport by isolated frog skin in a dose-dependent manner. Furthermore, the factor inhibits Na-K-ATPase activity of pooled rat kidney cortex homogenates with a similar dose-response relationship.

Methods

PREPARATION OF MATERIAL

Female Sprague-Dawley rats (250–300 g) were infused with 0.9% saline through a jugular venous cannula. An amount equivalent to 10% of the body weight was infused over a 1-hour period and a sustaining infusion of 0.2 ml/min was maintained for a 2nd hour. Urine flow was monitored during volume expansion through an indwelling urethral catheter; a diuresis of greater than 0.25 ml/min was taken to indicate an adequate response to volume expansion. A second group of rats was made hydropenic by water deprivation on the night before they were killed.

The kidneys from three expanded or three hydropenic rats were removed under ether anesthesia, immediately bisected and placed for 20 minutes in 30 ml of deionized boiling water acidified to pH 3.4 with acetic acid. The kidney tissue was then cooled to 0°C, blotted gently to remove excess water, decapsulated, cut into small pieces, and then weighed and replaced in the original solution, also cooled to 0°C. Sufficient deionized water was added to the mixture to give a 1:10 (wt/vol) dilution of the kidney material, and the mixture was homogenized with a Tri-R tissue homogenizer. The homogenate was centrifuged at 600 g for 15 minutes at 4°C to remove cell debris, and 20 ml of the supernatant fluid were subjected to Sephadex G-25 column chromatography (5 × 45 cm, void volume of gel bed, approximately 360 ml) with 0.1 M acetic acid as eluate.
The ultraviolet absorbance of the eluting fractions was monitored at 280 nm (A280). Fractions having elution volumes of 760-830 ml, 830-900 ml, and 900-970 ml, termed fractions III, IV, and V, respectively, were collected and lyophilized (Fig. 1). Lyophilized material was weighed to the nearest 0.1 mg and assayed immediately or stored desiccated at -20°C.

FROG SKIN ASSAY

Active sodium transport was measured as the short-circuit current (SCC) across the isolated ventral skin of Rana pipiens (Los Angeles Biologicals). The frogs were maintained in tap water at room temperature. The Ussing-type cell15 which we employed had three separate chambers bored into the Lucite chamber halves. Each chamber had a surface area of 1 cm² and a volume of 4 ml. This allowed concurrent measurement of potential difference (PD) and SCC across three sections from the same piece of skin. PD was monitored continuously (open circuit) and SCC applied every 10 minutes by the method described by Sullivan et al.16 PD was measured via agar-Ringer bridges and paired calomel reference electrodes connected to a digital voltmeter (Orion Research, model 801). SCC was passed through Ag/AgCl electrodes connected to the chambers of agar-Ringer bridges. The Ringer’s solution used in all experiments contained 111 mM NaCl, 2.0 mM KCl, 2.4 mM NaHCO3, 1.0 mM CaCl2, and 50.0 mM glucose. The pH was 7.6 and the osmotic pressure 220 mOsm. The frogs were doubly pithed and the ventral skin was mounted between the two chamber halves. After a 60-minute equilibration period, measurements of PD and SCC were started. When the SCC values for each chamber varied by < 10% for 30 minutes, the experimental treatment was begun. If the control SCC of any of the three chambers was <20 μA/cm², or if the interchamber variation in SCC was greater than 20%, the preparation was discarded.

To determine initially which fraction contained antinatriergic activity the lyophilized material was dissolved in frog Ringer’s solution to give a concentration of 500 μg/ml. Samples (4 ml) of two of the fractions were added to the inner (serosal) chamber halves bathing two of the three sections of the skin and fresh Ringer’s solution was added to the serosal half of the control chamber. The pH and osmolality of the Ringer’s solution were checked before and after addition of each of the lyophilized fractions and were found to be unchanged. The outer half of all three chambers was refilled with fresh Ringer’s solution. PD and SCC were recorded for the following 60 minutes. At this time the Ringer’s solution in inner and outer chambers bathing all three sections of the skin was replaced with fresh solution. After 30 minutes, PD and SCC again were monitored continuously. When SCC varied by <10% for an additional 30 minutes, the remaining fraction was assayed in the manner described above. The order in which the three fractions were assayed was random.

After we found that the antinatriergic substance was located in fraction III, a dose-response relationship was established for the inhibition of SCC by this fraction. Doses of 30, 150, 500, and 1,500 μg of fraction III per ml of frog Ringer’s solution were prepared and assayed as above except that two doses of fraction III were assayed relative to the control chamber during each experimental period. Changes in SCC and PD from control were evaluated after incubation for 60 minutes with each fraction.

Na-K-ATPase ASSAY

Rat kidneys were dissected free of the adipose and connective tissue which constituted the capsule. We removed slices of cortex, leaving a wide corticomедullary junction to ensure that only cortical enzyme would be assayed. Homogenates of the cortex were prepared in a 1:10 (wt/vol) dilution with Tri-R tissue homogenizer at 0°C with 0.015 mM ethylenediaminetetraacetic acid (EDTA) and 2.4 mM sodium deoxycholate (DOC). Samples (1 ml) of each homogenate preparation were stored at -20°C prior to use. This constituted the enzyme preparation. Enzyme activity was assayed no earlier than 3 days and no later than 3 weeks after preparation. During this time interval we found total ATPase activity and the ratio of Na-K-ATPase activity to Mg-ATPase activity to be constant.

The frozen homogenate was thawed at 4°C and diluted to 1:10 with cold distilled water prior to the assay of ATPase, which was carried out by a procedure described previously from this laboratory17 and modified as follows: For determination of total ATPase activity, incubation tubes contained 0.5 ml of substrate solution and provided final concentrations of 1 mM ATP, 1 mM Mg²⁺, 10 mM imidazole-HCl buffer, pH 7.2, 100 mM Na⁺, and 20 mM K⁺; 0.1 ml of 5 mM ethylene glycol bist(β-dimethyl ether)-N,N'-tetraacetic acid (EGTA); 0.3 ml of material from each fraction dissolved in water was added to each assay tube, and the tube was placed in the water bath at 37°C. A control was prepared in the same manner, but 0.3 ml of distilled water was used as eluate. The range of partition coefficients (Kav) is given for fractions III, IV, and V, which were lyophilized for bioassay. Note the relatively even distribution of Co¹⁺ in the three fractions.

![Figure 1](http://circres.ahajournals.org/Downloaded from)
was substituted for the kidney fraction. To start the reaction we added 0.1 ml of the enzyme preparation. We stopped the incubation after 10 minutes by adding 1 ml of ice-cold 10% trichloroacetic acid (TCA). After centrifugation at 1,700 g for 5 minutes, we assayed 1 ml of the supernatant fluid for inorganic phosphate by the method of Fiske and Subbarow (18). Mg\(^{2+}\) ATPase activity was assayed with 1 mM ouabain in the incubation medium, and Na-K-ATPase was calculated as the difference between total ATPase and Mg\(^{2+}\) ATPase activities. Protein determinations were obtained for each enzyme preparation by the method of Lowry et al. The protein content of 0.1 ml of enzyme preparation varied from 0.160 to 0.180 mg. ATPase activity was then expressed as micromoles of inorganic phosphate per milligram of protein per hour.

**RAT ASSAY IN VIVO**

In order to verify the fact that fraction III possessed natriuretic activity, the natriuretic response in vivo was assayed according to the procedure described by Sealey and Laragh (type IV assay) as modified in this laboratory. After a 90-minute equilibration period and after three stable 15-minute control periods had been obtained, 500 \(\mu\)g of fraction III from kidneys of volume-expanded animals were dissolved in 0.3 ml of 0.9% saline and injected through the femoral cannula.

Glomerular filtration rate (GFR), urine flow (V), absolute sodium excretion (U\(_{Na}\)V), and fractional excretion of sodium (F\(E_{Na}\)) were calculated from the average of the three 15-minute control periods and from the average of the three 15-minute periods following injection of the test fraction. Fraction III from hydropenic rats was not tested because our earlier experiments had demonstrated clearly that the fraction from expanded, but not from hydropenic, rat kidneys contained natriuretic activity. Sodium, potassium, and calcium ions were spread throughout fractions III, IV, and V. The distribution of Ca\(^{2+}\) is shown in Figure 1. In no case, however, were the concentrations of these ions sufficiently high to affect any of the assays. The final calcium concentration in the Na-K-ATPase assay never exceeded 0.1 mM at the highest concentration of fraction III (1,500 \(\mu\)g/ml).

**FROG SKIN**

The changes in PD and SCC produced by the experimental treatments were evaluated in relation to the values of PD and SCC for the control section of each skin by the equation

\[ \frac{E_t - C_t}{E_c} \times 100 \]

where \(E\) and \(C\) are the experimental and control sections of the skin, respectively, and \(e\) and \(c\) refer to the control and experimental periods, respectively. The percent changes (mean ± 1 SEM) in SCC for six experiments are shown in Figure 2.

When fractions III, IV, and V, at a concentration of 500 \(\mu\)g/ml, were tested immediately after lyophilization, antinatriuretic activity was found only in fraction III (Fig. 2b) from expanded rats. The mean changes in SCC and PD produced by fraction III were \(-22.7 ± 2.2\%\) and \(-22.2 ± 5.4\%\), respectively (\(P < 0.001\)). The mean change in resistance, calculated as the ratio of PD/SCC, was \(-6.4 ± 9.1\%\) (\(P > 0.5\)). Data from a typical experiment are shown in Figure 3. Fractions IV and V from expanded rat kidneys and fraction III from hydropenic rat kidneys produced no statistically significant changes in PD or SCC. The change in short circuit current (SCC) produced by adding the lyophilized fractions, dissolved in frog Ringer's solution, to the inner surface of the skin at a concentration of 500 \(\mu\)g/ml. Changes in short-circuit current are expressed as percent change in a section of a frog skin treated with one of the fractions relative to a section of the same skin bathed with fresh Ringer's solution at the inner surface. a = fraction III from hydropenic rats; b = fraction III from volume-expanded rats; c = fraction III from expanded rats assayed after storage for 1 week, as lyophilized powder, in a desiccator at \(-20\°C\); d = fraction IV from volume-expanded rats; e = fraction V from volume-expanded rats. All fractions except (c) were assayed immediately after lyophilization. The histogram is the mean; the vertical bar, ±SEM (n = 6;* = P < 0.001).
significant inhibition of SCC (Figs. 2a, d, and e). Fraction III from expanded rat kidneys was shown to lose inhibitory activity after storage in the lyophilized form for 1 week at -20°C (Fig. 2c).

In the dose-response study with expanded fraction III (mean of five experiments) inhibition of SCC increased linearly with the log of the dose. On the other hand, hydropenic fraction III failed to produce a significant inhibition of SCC even at the highest concentration tested (Fig. 4).

**Na-K-ATPase**

The changes in total, Mg-activated, and Na-K-activated ATPase activity produced by hydropenic and expanded fractions III and IV (1,500 μg/ml) and fraction V (500 μg/ml) are shown in Table 1. (The smaller concentration of fraction V was necessitated by a very low yield of this fraction.) Assays were performed in duplicate (fractions IV and V) or triplicate (fraction III). Only fraction III from expanded rats produced significant inhibition of Na-K-ATPase. Mg ATPase activity was unaffected by any of the fractions. In Figure 5 is a dose-response curve for fraction III from expanded and hydropenic rats. As with the frog skin SCC assay, Na-K-ATPase inhibition increased progressively with increases in the log of the dose of expanded fraction III but not with hydropenic fraction III.

**RAT ASSAY IN VIVO**

Table 2 shows that 500 μg of fraction III from expanded rat kidneys exert significant natriuretic and diuretic activity. Urine flow increased by a mean of 90% and fractional excretion of sodium by a mean of 123% after injection of the test fraction. The mean increase in fractional excretion of sodium is almost identical to that demonstrated in our previous study, in which we utilized a comparable quantity of natriuretic substance.14

**Discussion**

The results of our present study indicate the kidneys of volume-expanded rats yield a fraction (fraction III) which in vitro is both antinatriferic and an inhibitor of Na-K-
Effect of Fraction III from Expanded Rat Kidneys on Glomerular Filtration Rate (GFR), Urine Flow (V), Sodium Excretion ($U_{Na}V$) and Fractional Excretion of Sodium (FE$_{Na}$) in Assay Animals

| Experiment no. | GFR (ml/min) | V ($\mu$l/min) | $U_{Na}V$ (µEq/min) | FE$_{Na}$ (%) |
|---------------|--------------|----------------|---------------------|--------------|
|               | Control      | Experimental   | $\Delta$            | Control      | Experimental   | $\Delta$  | Control      | Experimental   | $\Delta$  |
| 1             | 1.04         | 1.08           | +4%                 | 13.0         | 16.4           | +26%      | 2.33         | 3.91           | +68%      |
| 2             | 1.04         | 0.92           | -13%                | 12.5         | 17.6           | +40%      | 1.53         | 1.88           | +23%      |
| 3             | 1.02         | 1.04           | +2%                 | 7.5          | 19.4           | +159%     | 1.70         | 5.52           | +255%     |
| 4             | 1.04         | 1.04           | 0%                  | 6.0          | 15.0           | +150%     | 0.92         | 2.90           | +215%     |
| 5             | 0.98         | 0.95           | -3%                 | 6.7          | 11.7           | +75%      | 0.43         | 0.78           | +81%      |
| Mean          |              |                |                     |              |                |           |              |                |           |
|               | 1.01         | 1.04           | +3%                 | 13.0         | 16.4           | +26%      | 2.33         | 3.91           | +68%      |

Compared are the mean results of three 15-minute periods prior to injection of the test fraction (control) with the mean results of the three 15-minute periods following injection (experimental). $\Delta$ = percent change between control and experimental values.
ATPase in vitro. Further purification of the fraction will be necessary to establish whether these properties arise from a single molecular species.

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Effect of Thoracic Blood Volume Changes on Steady State Cardiac Output

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SUMMARY We have investigated the extent to which shifts of blood volume out of or into the thoracic region influence the steady state cardiac output. The systemic circulation of anesthetized dogs was replaced with an artificial circuit which simulated the pertinent mechanical characteristics of an intact circulation. As in the normal animal, the steady state venous return was proportional to the pressure gradient for venous return (i.e., mean systemic minus right atrial pressure). Cardiac function was altered either by administration of epinephrine or by changes in left ventricular afterload. At a constant mean aortic pressure of 100 mm Hg, epinephrine administration increased the steady state cardiac output by 55%. Half of this increase resulted from the lowered mean right atrial pressure (caused by improved cardiac function); the remainder resulted from an increased mean systemic pressure (caused by the volume shift to the systemic circulation). Increases in afterload transferred sufficient volume to the heart-lung compartment to reduce significantly the mean systemic pressure and, hence, the steady state venous return. Our results indicate that the heart-lung compartment contains a significant volume which is under cardiac control. In addition to being able to alter the right atrial pressure, the heart can modulate the steady state cardiac output by adjusting the mean systemic pressure. To this degree the heart can adjust its own venous return.

SHIFTS OF volume into or out of the thoracic region have been thought to be too small to cause any significant changes in the systemic blood volume and mean systemic pressure (Pms). In the steady state, the cardiac output must equal the venous return; and since the steady state venous return is a function of the pressure gradient for venous return [i.e., Pms minus right atrial pressure (Pra)], it follows that such shifts of thoracic blood volume by...
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