INHIBITION OF SODIUM AND POTASSIUM TRANSPORT IN SEPARATED RENAL TUBULE FRAGMENTS INCUBATED IN EXTRACTS OF URINE OBTAINED FROM SALT-LOADED INDIVIDUALS

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SUMMARY

1. Extracts of urine were prepared from salt-depleted and salt-loaded subjects by gel filtration of freeze-dried urine on Sephadex G-50. The extracts were dissolved and adjusted to physiological pH and electrolyte concentration.

2. Separated renal tubule fragments were prepared from rabbit kidney cortex and their intracellular sodium and potassium measured after incubation in the dissolved adjusted extracts. Those concerned with the measurement of intracellular sodium and potassium did not know the identity of the samples.

3. Extracts of urine from salt-loaded subjects inhibited the sodium and potassium transport of the tubules whereas extracts of urine from salt-depleted subjects had no effect.

4. Of ten known hormones that were tested, none had any inhibitory effect on the sodium transport of the tubule fragments.

5. It is concluded that the oral intake of large amounts of salt in normal subjects causes the appearance of an unknown substance in the urine which inhibits sodium transport in separated renal tubule fragments.

Key words: natriuretic hormone, sodium transport, tubule fragments, salt-loaded man.

During the last few years evidence has steadily accumulated to suggest that urinary sodium excretion is controlled in part by hormonal factors other than aldosterone. A rise in sodium excretion has been shown to occur in animals upon expansion of the blood volume with blood with which, in some experiments, the animal was in equilibrium, so that dilutional changes in the blood could be excluded (Bahlmann, McDonald, Ventom & de Wardener, 1967; Tobian, Coffee & McCrea, 1967; Knox, Howards, Wright, Davis & Berliner, 1968; Kaloyanides & Azer, 1971; Lichardus & Nizet, 1971). In some of these experiments the rise in sodium excretion was considered to be associated with a rise in aldosterone level. However, it is now generally accepted that the increase in sodium excretion cannot be explained by changes in aldosterone level alone (Bahlmann, 1970).

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excretion was demonstrated in denervated (Bahlmann et al., 1967) and in isolated kidneys (Tobian et al., 1967; Kaloyanides & Azer, 1971) at a time when there was no change or a fall in arterial pressure. In the experiments of Knox et al. (1968) and Kaloyanides & Azer (1971) the rise in sodium excretion was associated with a decrease in glomerular filtration rate and renal blood flow, which suggested that the rise in sodium excretion is due in part at least to a direct inhibitory effect on tubular transport of sodium. This suggestion has been supported by the results of experiments in vitro. Blood and plasma taken from dogs during blood volume expansion inhibits sodium transport in the frog skin (Nutbourne, Howse, Schrier, Talner, Ventom, Verrout & de Wardener, 1970) and in separated renal tubule fragments (Clarkson, Talner & de Wardener, 1970). Sealey, Kirshman & Laragh (1969) and Sealey & Laragh (1971) found that extracts of plasma or urine obtained from salt-loaded animals or man caused a rise in urinary sodium excretion when injected into rats.

The experiments we describe were performed to determine whether extracts of urine from salt-loaded subjects prepared in the same way as those of Sealey et al. (1969) would have an effect on the sodium and potassium transport of separated renal tubule fragments incubated in vitro. The extracts were prepared from the urine of fourteen normal subjects during salt depletion and salt loading. The intracellular sodium and potassium of tubules incubated in the extracts was measured. Measurements were also made of the effects of some known hormones on sodium and potassium transport in suspensions of renal tubule fragments.

**EXPERIMENTAL PROCEDURE**

Fourteen normal subjects were given a diet containing not more than 25 mmol of Na+/day for 5 days. All the food taken by the subjects during this time was supplied by the metabolic ward kitchen. On the first day of the low salt diet the subjects took 40 mg of frusemide. The 24 h urine collections were made during the last 2 days. On the sixth day of the experiment the subjects returned to a normal diet supplemented with 300 mmol of Na+/day in the form of tablets of wax-based NaCl (Slow Sodium, Ciba Ltd, Basle, Switzerland) which has been shown to be completely absorbed (Clarkson, Curtis, Jewkes, Jones, Luck, de Wardener & Phillips, 1971). All fourteen subjects continued to take the high salt diet for 8 days. The 24 h urine collections were made on the last 2 days. In addition to the sodium supplement of 300 mmol/day, four of the subjects took an extra 150 mmol of Na+/day on the last 2 days of the high salt intake, making their total sodium supplement up to 450 mmol/day. Nine of the subjects began the experiment with the low salt diet and ended with the high salt diet. The order was reversed in five subjects.

The subjects were students or doctors who arranged to be resident in the hospital during the days on which the urine collections were made. Urine was passed directly into a polythene 24 h collection bottle which was replaced immediately into the laboratory deep-freeze. In this way the urine was quickly cooled and was kept frozen throughout the collection.

**METHODS**

**Preparation of the urine extracts**

Each 24 h urine was freeze-dried in a bulk centrifugal freeze drier (Edwards Model EF6). The freeze-dried urine was extracted with 15–20 ml of 0·1 M-acetic acid and the insoluble
residue was separated in a refrigerated centrifuge (Sorvall). The supernatant solution was applied to a column (2.5 cm x 100 cm) of Sephadex G-50, and eluted with 0.1 M-acetic acid. The fractions appearing before the salt material were combined (Fig. 1), and the remainder were discarded. Crystalline bovine serum albumin (1.2 mg) and 1 drop of 1.0 M-mercaptoacetic acid were added to the combined pre-salt fractions obtained from each 24 h urine. The fractions were then freeze dried. The freeze-dried eluate was kept in an air-tight container in the deep-freeze until assayed.

On the day of the assay the desalted freeze-dried extract of urine obtained from a subject during salt depletion, together with the extract obtained from the same subject during salt loading, was dissolved in 0.01 M-NaCl. The pH of each extract was raised to 7.4 from its initial value of about 4.0 by the addition of 0.5 M-NaOH, and the electrolyte concentration was adjusted to that of the Krebs–Ringer bicarbonate solution in which the tubules were incubated. The Na⁺ concentration was first adjusted to 115 mM by the addition of 1.0 M-NaCl and finally to 140 mM by adding NaHCO₃ to a final concentration of 25 mM. A concentrated solution containing KCl, CaCl₂, MgSO₄ and NaH₂PO₄ was then added so that the final concentration of these ions was 3 mM-K⁺, 1 mM-Ca²⁺, 1.2 mM-Mg²⁺ and 1.2 mM-PO₄³⁻. The Cl⁻ concentration was between 95 and 110 mM according to the amount of NaOH that was added to adjust the pH of the solutions. Experiments in which Cl⁻ and acetate were varied reciprocally between 10 and 100 mM have demonstrated that such differences in Cl⁻ concentration do not affect the intracellular Na⁺ or K⁺ of incubated tubule fragments (E. M. Clarkson, unpublished work). Glucose (200 mg/100 ml) was included. The final volume of the concentrated urine extract was arranged so that 0.17 ml represented 1 h of the original urine.

**Fig. 1.** Elution curves obtained from desalting the dissolved freeze-dried urine on the column of Sephadex G-50. The fractions indicated by the bar were combined. ———, $E_{254}$; ——, Na⁺.
output. At this stage the extracts were relabelled so that those measuring the intracellular sodium and potassium in the tubule fragments did not know which extract was obtained during salt depletion and which was obtained during salt loading.

Preparation and incubation of the tubule fragments

Tubules were prepared from rabbit kidney cortex by the technique of Burg & Orloff (1962) in which the proteolytic enzyme collagenase is used to separate the tubules from the surrounding tissue. Accumulation of Chlorphenol Red by the tubules showed that 90% were proximal tubules. The tubules were incubated at 25° in small flasks that had a side-arm through which 5% CO₂, 95% O₂ was passed. The CO₂ in O₂ maintained the pH of the solution, and oxygenated and mixed the tubules during incubation. Details of the methods used to prepare and to incubate the tubules have been described (Clarkson et al., 1970). A sample (3–4 ml) of each dissolved extract was divided between two of the incubation flasks. Two flasks containing Krebs-Ringer bicarbonate solution were incubated simultaneously. Calf serum was added to all the flasks to a concentration of 5 ml/100 ml of final tubule suspension. When all the solutions were equilibrated with the 5% CO₂, 95% O₂ and the pH was constant, tubule fragments were added to each of the flasks. The final concentration of the urine extracts in the tubule suspensions was such that 5·3 ml of tubule suspension represented a 24 h urine collection. The suspension pH in the fourteen experiments varied between 7·34 and 7·40 but in each individual experiment the pH of the cell suspension containing the urine extracts and that containing the control Ringer solution did not differ by more than 0·03 units. After the tubules had been incubated for 30 min they were separated from the external medium by centrifugation and the tubules were lysed in water. The sodium and potassium in the cell lysate and external medium were measured as described by Clarkson et al. (1970) and the intracellular sodium and potassium concentrations were calculated by using the value of 25·5% for trapped medium.

Experiments with ten known hormones

Aldosterone, angiotensin, arginine vasopressin, oxytocin, adrenaline, noradrenaline and the prostaglandins A₁, A₂, E₁ and E₂ were added to tubule suspensions in concentrations several times higher than that in which each hormone was likely to have occurred in the urine extracts. The final concentration of the urine extracts in the tubule suspensions described above was such that 5·3 ml of tubule suspension contained the extract prepared from a 24 h urine collection. To test the effects of hormones that may have been present in the urine extracts it was desirable to include in 5·3 ml of tubule suspension the amount of each hormone that might have been excreted in 24 h by the subjects during the conditions of salt depletion and salt loading. As this was not known, approximately five times the amount of each hormone reported to be excreted in 24 h under normal conditions was included in 5·3 ml of tubule suspension. The values for the normal excretions of aldosterone were those given by Minick & Conn (1964) and values for the normal excretion of adrenaline and noradrenaline were obtained from Ritzel & Hunzinger (1963). The excretion of vasopressin was that found by Chaudhury (1960) in dehydrated Indians. Values for the 24 h excretion of angiotensin, oxytocin and the prostaglandins, respectively, were given by Dr A. F. Lever, Professor J. Lee and Professor I. Mills (personal communications), and are shown in Table 1. Table 1 also shows the concentration that would have resulted if this amount of hormone had been included in
<table>
<thead>
<tr>
<th>Hormone</th>
<th>Equivalent concentration in 5-3 ml of tubule suspension</th>
<th>Mean intracellular Na⁺ excretion in 5.3 ml of tubule suspension</th>
<th>Mean intracellular K⁺ excretion in 5.3 ml of tubule suspension</th>
<th>Mean intracellular Na⁺ concentration of cells incubated in:</th>
<th>Mean intracellular K⁺ concentration of cells incubated in:</th>
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<td>Normal 24 h excretion</td>
<td>7.5 µg</td>
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<td>Adrenaline</td>
<td>24 µg</td>
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<td>Noradrenaline</td>
<td>60 µg</td>
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5-3 ml, together with the actual concentration of each hormone that was used in the experiments that were performed.

Each solution of hormone in Krebs–Ringer bicarbonate solution ('hormone-Ringer') was divided between two flasks and incubated simultaneously with two flasks containing Krebs–Ringer bicarbonate solution with no added hormone ('plain Ringer'). Then 5 ml of calf serum/100 ml of tubule suspension was added to all the flasks and the solutions were equilibrated with 5% CO₂, 95% O₂. Tubule fragments were then added and incubation at 25°C was continued for 30 min in the same way as in the experiments with urine extracts. Six experiments were done with each of the ten hormones tested.

**STATISTICAL ANALYSIS OF RESULTS**

The Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) and the Student's *t* test for paired observations were used. The estimates for the probability of the null hypothesis were similar using both tests.

**RESULTS**

In thirteen of the fourteen experiments the intracellular sodium concentration of tubules incubated in extracts of urine obtained from salt-loaded subjects was higher than that of tubules incubated in extracts obtained from salt-depleted subjects (Fig. 2). In the remaining experiment the intracellular sodium was lower. The mean increase in intracellular Na⁺

![Fig. 2. Difference (salt loaded–salt depleted) in intracellular Na⁺ (Naᵢ) and K⁺ (Kᵢ) concentrations between tubule fragments incubated in extracts of urine obtained during salt depletion and salt loading.](image-url)
concentration in the tubules was 5.0 mmol/l of cells, range -0.5 to +10.5 mmol/l of cells (t = 5.0, P<0.0005).

In eleven of the fourteen experiments the intracellular potassium of tubules incubated in extracts obtained from salt-loaded subjects was lower than that of tubules incubated in extracts obtained from salt-depleted subjects, and in the remaining three experiments there was no change. The mean decrease in intracellular K\(^+\) was 5.5 mmol/l of cells, range 0 to -14.5 mmol/l of cells (t = 4.15, 0.0005 < P < 0.0025).

![Figure 3](image.png)

**Fig. 3.** Difference (salt depleted - Ringer) in intracellular Na\(^+\) (Na\(_t\)) and K\(^+\) (K\(_t\)) concentrations between tubule fragments incubated in the extracts of urine obtained during salt depletion and in Ringer-calf serum.

The intracellular sodium and potassium concentration of tubules incubated in urine extracts obtained during salt depletion was not significantly different from those incubated in Ringer-calf serum (Fig. 3). The mean intracellular Na\(^+\) and K\(^+\) concentrations of tubules incubated in extracts of urine obtained from salt-depleted subjects was 49.1 and 85.1 mmol/l of cells, and of tubules incubated in Ringer-calf serum was 49.4 and 83.6 mmol/l of cells.

In twelve of the fourteen experiments the intracellular sodium concentration of tubules incubated in extracts of urine obtained from salt-loaded subjects was higher than that of tubules incubated in Ringer-calf serum and in two experiments the intracellular sodium was lower (Fig. 4). The mean increase in intracellular Na\(^+\) concentration was 4.6 mmol/l of cells, range -5.0 to +16.0 mmol/l of cells (t = 3.13, 0.0025 < P < 0.005). In eleven of the fourteen experiments the intracellular K\(^+\) concentration of tubules incubated in extracts obtained from salt-loaded subjects was lower than that of tubules incubated in Ringer-calf serum and in two experiments it was higher. In the remaining experiment no result was obtained for the intracellular K\(^+\) concentration of tubules incubated in Ringer-calf serum. The mean decrease in intracellular K\(^+\) in the thirteen experiments was 4.0 mmol/l of cells, range +5.5 to -15.0 mmol/l of cells (t = 2.42, 0.0125 < P < 0.025).
In the six experiments that were done with each of the hormones aldosterone, angiotensin, arginine vasopressin and oxytocin the intracellular Na\(^+\) and K\(^+\) concentrations of tubules incubated in hormone-Ringer were the same as those of tubules incubated in plain Ringer (Table 1). The intracellular Na\(^+\) concentration of tubules incubated in Ringer solution containing adrenaline or noradrenaline was also similar to that of tubules incubated in plain Ringer but the intracellular K\(^+\) was higher. The tubules incubated in Ringer solution containing the prostaglandins A\(_1\), A\(_2\), E\(_1\) and E\(_2\) had a lower intracellular Na\(^+\) concentration than tubules incubated in plain Ringer solution. This difference was statistically significant with prostaglandin E\(_2\). The effect of the prostaglandins on the intracellular K\(^+\) concentration was variable.

**FIG. 4.** Difference (salt loaded – Ringer) in intracellular Na\(^+\) (Na\(_1\)) and K\(^+\) (K\(_1\)) concentrations between tubule fragments incubated in the extracts obtained during salt loading and in Ringer-calf serum.

**DISCUSSION**

The results show that renal tubule fragments incubated *in vitro* in extracts of urine obtained from salt-depleted subjects are able to maintain the same gradient of Na\(^+\) and K\(^+\) as tubules incubated in Ringer-calf serum. Tubules incubated in extracts obtained from salt-loaded subjects, however, have an impaired ability to maintain a gradient of Na\(^+\) and K\(^+\). The mean intracellular Na\(^+\) concentration of tubules incubated in extracts obtained during salt loading was higher than that of tubules incubated either in the Ringer-calf serum or in the
extracts obtained during salt depletion, and the mean intracellular K\textsuperscript{+} concentration was lower. The mean differences in intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations were small; those concerned with the estimation of intracellular sodium and potassium did not know which extract was obtained during salt depletion and which was obtained during salt loading. Moreover, the final results were the mean of four separate K\textsuperscript{+} and Na\textsuperscript{+} analyses.

The urine from which the extracts were prepared was deep frozen within a few minutes of being passed and was kept deep frozen until it was freeze-dried. This prevented the deterioration of the extract and the growth of organisms. Precautions were taken to avoid differences in electrolyte concentration in the two extracts. Before incubating the tubules in the dissolved extracts the two extracts obtained from each subject were both adjusted to the same pH and electrolyte concentration. The results might also have been caused by differences in the concentration of some of the known hormones in the extracts. However, those hormones that were tested showed no inhibitory effect on the Na\textsuperscript{+} or K\textsuperscript{+} transport of the tubules. Adrenalin and noradrenalin had a slight stimulating effect on K\textsuperscript{+} transport in the tubules, and the prostaglandins had a slight stimulating effect on Na\textsuperscript{+} transport. The mean intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations of tubules incubated in extracts obtained during salt depletion were the same as those of tubules incubated in the Ringer solution. It was therefore unlikely that the increase in intracellular Na\textsuperscript{+} and decrease in intracellular K\textsuperscript{+} that occurred when the tubules were incubated in extracts obtained during salt loading, was caused by the removal of the stimulating effect of these hormones. Moreover the molecular weights of the hormones that stimulated Na\textsuperscript{+} or K\textsuperscript{+} transport in the tubules were all less than 350 and thus during the preparation of the urine extracts were likely to have been eluted from the Sephadex column with the salt and discarded.

The effect of extracts of urine obtained from salt-loaded subjects on Na\textsuperscript{+} transport in tubule fragments is similar to the effect of plasma obtained from dogs during blood volume expansion (Clarkson et al., 1970). In the experiments with plasma, the inhibitory effect of blood volume expansion on the Na\textsuperscript{+} and K\textsuperscript{+} transport of the tubules was observed against a background that was also inhibitory. This was due to an inherent property of plasma and was unrelated to the superimposed effect of blood volume expansion. In the experiments with urine extracts described here, there was no background of inhibition. The tubules incubated in extracts obtained during salt depletion had the same mean intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentration as tubules incubated in the Ringer solution.

The results also support those of Sealey et al. (1969), Sealey & Laragh (1971) and P. R. Brown, K. G. Koutsaimanis & H. E. de Wardener (unpublished work). Sealey et al. (1969) found that extracts of urine similarly prepared from salt-loaded subjects caused a rise in urinary Na\textsuperscript{+} excretion when injected into anaesthetized Brattleborough rats. P. R. Brown, K. G. Koutsaimanis & H. E. de Wardener (unpublished work) have confirmed these results by using conscious Wistar rats; some of the extracts that were tested were prepared from the same urine samples used in the present work. The evidence suggests that expansion of the body fluids by the oral intake of large amounts of salt causes the appearance of some substance in the urine which inhibits Na\textsuperscript{+} and K\textsuperscript{+} transport in separated renal tubule fragments incubated in vitro and also causes a rise in urinary Na\textsuperscript{+} excretion in the rat. It is unlikely that this substance is one of the known hormones that were tested, for none of them inhibited the Na\textsuperscript{+} transport of suspended renal tubules.

The experiments do not distinguish whether the rise in intracellular Na\textsuperscript{+} in the tubules
was due to an inhibitory effect on the \( \text{Na}^+ \) pump or to a change in the permeability of the tubule cell membrane. Nutbourne et al. (1970) measured the short-circuit current across a frog skin bathed with the blood of a dog which had had its blood volume expanded. They found that the fall in short-circuit current that occurred was not associated with a rise in electrical resistance. They concluded therefore that the fall in short-circuit current was due to an inhibition of the \( \text{Na}^+ \) pump produced by a change in the circulating concentration of some unknown substance. This is in agreement with a deduction that can be made from the results described here, when combined with those of P. G. Brown, K. G. Koutsaimanis & H. E. de Wardener (unpublished work) if it is assumed that the change in intracellular \( \text{Na}^+ \) concentration of the separated tubule fragments and the change in urinary \( \text{Na}^+ \) excretion of the intact kidney have been produced by the same substance. In the experiments of P. G. Brown, K. G. Koutsaimanis & H. E. de Wardener (unpublished work) the injection of extracts obtained from salt-loaded subjects into rats caused a rise in urinary sodium excretion. If this effect was due to the substance changing the permeability of the cell membrane of the tubules in the intact kidneys, the rise in urinary \( \text{Na}^+ \) excretion would have to be due to a decrease in permeability. However, if the rise in intracellular \( \text{Na}^+ \) concentration described here was due to the substance changing the permeability of the tubule cell membrane, the permeability would have had to be increased, that is, the change in permeability would have to be in the opposite direction to that postulated for the intact kidney. It is unlikely therefore that if the effects on the intact kidney and the separated tubule fragments are due to the same substance, the substance acts on the permeability of the cell membrane. It is tentatively concluded that the unknown substance present in the urine of salt-loaded subjects which impairs the ability of suspended renal tubules to maintain a gradient of \( \text{Na}^+ \) and \( \text{K}^+ \) produces this effect by inhibiting the \( \text{Na}^+ \) pump.

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Urine extracts and transport in tubules


