$^{23}$Na Nuclear Magnetic Resonance Study of Na$^+$/K$^+$ Pump Inhibition by a Fraction from Uremic Toxins

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An in vitro inhibitor of Na$^+$/K$^+$-transporting ATPase (EC 3.6.1.37) was isolated from uremic plasma and normal urine by liquid chromatography. A $^{23}$Na nuclear magnetic resonance study involving living erythrocytes showed that this inhibitor causes impairment of the Na$^+$/K$^+$ pump of intact erythrocytes. This finding may explain the high intra-erythrocytic sodium concentration in those uremic patients exhibiting a high concentration of this inhibitor. The presence of this same inhibitor in normal urine suggests that it may play a physiological role.

The importance of the Na$^+$/K$^+$ pump in maintaining many vital functions in healthy subjects suggests that study of pump defects in diseased states such as chronic renal failure may yield important insights into their pathophysiology.

Previous studies (1, 2) described decreased Na$^+$/K$^+$-transporting ATPase (EC 3.6.1.37) activity and increased intracellular Na$^+$ in erythrocytes of patients with chronic renal failure. Since these initial studies, other authors (3–5) confirmed that erythrocytic Na$^+$ is increased in some patients with this disease. These reports, and those on decreased Na$^+$/K$^+$-transporting ATPase activity (1, 3, 4), led to the hypothesis that the increase in intracellular Na$^+$ resulted from a diminished Na$^+$/K$^+$ pump activity (3, 6).

Moreover, the findings that dialysis corrects the defect in pump-mediated transport (7–9) and that uremic plasma induces suppression of Na$^+$ pump efflux (4) and Na$^+$/K$^+$-transporting ATPase activity (10) suggest that the defect may be attributed to a factor in the circulation (4, 11–13). These previous results on intracellular Na$^+$ were obtained by classical techniques that require cell lysis. In contrast, $^{23}$Na nuclear magnetic resonance (NMR) with paramagnetic aqueous shift reagent allows the direct determination of intracellular sodium in living erythrocytes, which in turn permits dynamic study of the sodium flux through cell membranes. Therefore, we investigated Na$^+$/K$^+$ pump inhibition in uremic patients by the NMR technique in an attempt to demonstrate the presence of such an inhibitor in their body fluids.

Recently, by $^{23}$Na NMR, we measured the intra-erythrocytic sodium concentration (Na$_{in}$) in living cells of uremic patients (14). These can be distributed into two groups: one with high Na$_{in}$, the other with normal Na$_{in}$. Subsequently, we demonstrated that a fraction isolated from uremic plasma and normal urine inhibited dog kidney Na$^+$/K$^+$-transporting ATPase activity in vitro (15). Measurements of inhibition of the purified ATPase, however, are not sufficiently indicative of the inhibition in intact cells (16). But, as mentioned, $^{23}$Na NMR allows direct measurement of sodium flux through the membrane of living cells. Therefore, we describe here the chromatographic isolation of the Na$^+$/K$^+$-transporting ATPase inhibitory fraction, and we report its effect on the Na$^+$/K$^+$ pump in living erythrocytes, as studied by $^{23}$Na NMR.

**Materials and Methods**

*Isolation of inhibitory fraction*. This fraction was isolated as described elsewhere (17). Briefly, the procedure is as follows. Gel permeation chromatography of uremic plasma ultrafiltrate or normal urine on Sephadex G15 yields peak 2, containing the active fraction. Then anion-exchange chromatography on Sephadex DEAE A25 resolves peak 2 into six components (2-1 through 2-6). Finally, the active fraction (2-3 fraction) is desalted by gel permeation chromatography on Sephadex G15. Then, to improve the purification of the 2-3 fraction, an additional chromatographic step was performed on a 2.5 x 100 cm Sephadex G10 column, with distilled water as eluent. The flow rate was 80 mL/h and absorbance was monitored at 254 nm. Under these conditions, the 2-3 peak was partly resolved into two parts, designated 2-3a and 2-3b in the order of their elution. Finally, these chromatographic fractions were lyophilized. 

Na$^+$/K$^+$-transporting ATPase activity. Chromatographic fractions 2-3a, 2-3b, 2-5, and ouabain were assayed for their ability to inhibit the ATPase, by a previously described method (15). The ATPase from canine kidney (Sigma Chemical Co., St. Louis, MO) was incubated at 37°C for 5 min in a medium containing, per liter, 100 mmol Tris HCl, 1 mmol EGTA, 3 mmol ATP (vanadate free, Sigma), 20 mmol MgCl$_2$, 100 mmol NaCl, and 20 mmol KCl (pH 7.4). Chromatographic fractions were added in an incubating medium volume of 1 mL. Blanks were obtained by parallel incubation performed with and without ouabain, 1 mmol/L, in the absence of chromatographic fractions. The reaction was stopped by adding cold perchloric acid (0.75 mol/L final concentration). The resulting solutions were analyzed for inorganic phosphorus according to Hurst's method (18).

The difference between results obtained in the absence and in the presence of ouabain was considered as the Na$^+$/K$^+$-transporting ATPase activity. The enzymic activities obtained with chromatographic fractions were compared with those obtained with ouabain and expressed as a percentage of its inhibitory effect.

$^{23}$Na NMR study. The intracellular sodium concentration was measured in living erythrocytes as previously described (14, 19). Briefly, $^{23}$Na NMR with aqueous paramagnetic shift reagent (dysprosium tripolyphosphate, not toxic to erythrocytes) allows resolution of distinct resonances of intracellular and extracellular sodium in intact erythrocytes, because the shift reagent does not cross the biological membrane. By remaining in the extracellular medium, shift reagent causes only the extracellular $^{23}$Na NMR signal to shift, whereas the signal for intracellular $^{23}$Na is unshifted. Accurate integration of the signals allows determination of sodium concentrations in the two compartments.

In a first step, the intracellular sodium concentration...
(Na\textsubscript{in}) in normal erythrocytes was determined. In a second step, various doses of 2-3a or 2-3b fractions were added and the variations in Na\textsubscript{in} were measured over time (5, 120, and 900 min). As controls, 2-5 fraction, NaCl, and ouabain were also tested under the same experimental conditions. The results were expressed as a percentage of the Na\textsubscript{in} increase.

Sample preparation. Freshly drawn heparinized blood from healthy volunteers was centrifuged, the plasma was removed, and the cells were washed three times with a washing solution (NaCl 140 mmol/L, KCl 5 mmol/L, glucose 5 mmol/L, pH 7.4). For the final wash, the washing solution was modified by adding the shift reagent. A final concentration of this reagent ranging between 4 and 6 mmol/L was used to sufficiently separate intracellular and extracellular sodium signals, in order to integrate them accurately. After the last centrifugation, the supernatant fluid was removed and the packed cells (packed cells volume 90–95%) were stored in ice (−4 °C) until NMR measurements were performed.

NMR spectroscopy. Analyses were conducted at the Service Interuniversitaire de RMN, Marseille, with a Bruker AM 200 spectrometer equipped with a 10-mm probe tuned at 22.94 MHz. Typical experimental conditions were: pulse 20 μs; acquisition time 254 ms, without delay between pulses; number of scans 500; and temperature 37 °C. Spectra were obtained by use of a concentric NMR tube combination: an inner tube inside a 10-mm (o.d.) NMR tube. The inner tube contained an external reference solution: 50–70 mmol/L Na\textsuperscript{+} and 7–8 mmol/L shift reagent in \[^2\text{H}_2\text{O}\] (to lock field frequency) for integration (the technique is fully described elsewhere (14)).

Results

Gel permeation chromatography (Sephadex G15) allows isolation of compounds in a molecular mass range of 200–2000 Da. Their purification by ion-exchange chromatography on Sephadex DEAE A25 indicates the anionic character of these compounds. Finally, the last gel permeation chromatography (Sephadex G10) separates the fraction of interest (2-3 fraction) into two parts: 2-3a and 2-3b. However, the poor resolution of this chromatographic step leads to contamination of the 2-3b fraction with material in peak 2-3a.

Table 1 shows that these fractions, obtained from uremic plasma and normal urine, inhibited purified dog kidney Na\textsuperscript{+}/K\textsuperscript{+}-transporting ATPase. Results are expressed as the mean ± SD of three experiments. Whatever its origin (normal urine or uremic plasma), the 2-3a fraction decreased the activity of this ATPase by about half. The 2-3b fraction exerted a slight inhibitory effect; and the 2-5 fraction, tested as control, showed no effect.

Figure 1 presents typical \(^{23}\text{Na}\) NMR spectra obtained with ouabain (1 mmol/L) or 2-3a fraction (2 g/L) at the beginning of experiments (upper spectra) and 900 min later (lower spectra). In this figure, Na\textsubscript{in}, Na\textsubscript{out}, and Na\textsubscript{ref} resonances represent intracellular sodium, extracellular sodium, and external reference sodium, respectively. At the beginning of the experiments, the 2-3a fraction assay exhibited a higher Na\textsubscript{out} signal and a Na\textsubscript{out} downfield shift as compared with ouabain. This double effect was due to a sodium load resulting from the addition of the 2-3a fraction, isolated as the sodium salt, as proved by the modification of the starting ratio of Na\textsubscript{out}/Na\textsubscript{in}: 3.5 and 9.0 in the ouabain assay and the 2-3a fraction assay, respectively. This sodium
load increases the free sodium in the medium, thus modifying the ratio between free Na and Na bound with the shift reagent. Consequently the Na* _obs* resonance will be downfield-shifted with regard to ouabain assay. In addition, in the cases of ouabain and 2-3a fraction assays, there was a slight Na* _out* and Na* _ref* downfield shift, probably attributable to a weak hydrolysis of shift reagent after 900 min.

Table 2 reports the variation in Na* _in* over time, obtained with NaCl, ouabain, and 2-5 fraction as controls, and with 2-3a fractions isolated from both uremic plasma and normal urine and 2-3b from uremic plasma. Results, expressed as the percentage increase in Na* _in*, are the mean ± SD of three experiments. Data showed that NaCl and the 2-5 fraction had no effect. As expected, the ouabain test provoked an increase in Na* _in*. With the 2-3a fraction from uremic plasma, an increase in Na* _in* (26% and 71% for 1 and 2 g/L, respectively) was observed. A similar result was obtained with the 2-3a fraction from normal urine (20% for 1 g/L). For the 2-3b fraction there was no effect at a concentration of 2 g/L (cf. that of the 2-3a fraction); but an increase in Na* _in* was observed at higher concentrations, beginning at 3 g/L.

### Discussion

The inhibition of this ATPase by the 2-3a fraction (Table 1) agrees with previous studies reporting the presence of such inhibitors in human pathological body fluids (20–24). The effect of the 2-3a fraction, observed both on Na*+/K*+-transporting ATPase and on Na* _in* concentration in living erythrocytes, is specific. The slight effect observed for the 2-3b fraction (Table 1) results from poor chromatographic resolution of these two fractions (see Results). So a contamination of fraction 2-3b with the material in peak 2-3a is to be considered. Moreover, all controls performed with the 2-5 fraction were negative (Table 1). Thus the effect of the 2-3a fraction is not ascribable to an artifact of the chromatographic procedure. Similarly, an artifact resulting from a drug being taken by the patients may be discounted, because the 2-3a fraction from normal urine exhibits the same activity as the uremic one.

This in vitro effect on Na*+/K*+-transporting ATPase leads to an inhibition of Na* pump in living erythrocytes, as proved by the results with 22Na NMR. A possible shift-reagent toxic effect on ion transport and cellular integrity may be discarded, as reported by Ogino et al. (25).

The possibility of an NMR technical artifact may be excluded because the 22Na NMR study showed that the total sodium (Na* _in* + Na* _out*) remained unchanged during the experiment. Indeed, the sum of Na* _in* and Na* _out* integrations is the same at the beginning and the end of the experiment, within the experimental error (Figure 1).

The amounts of chromatographic fractions used (1 or 2 g/L) resulted in ~50 or 100 mmol of sodium per liter in the medium, respectively. Thus we performed NMR experiments with such sodium concentrations to check for a possible effect of ionic strength. As proved by the results (Table 2), these sodium loads have no effects. Also, controls performed with the 2-5 fraction were negative, thus confirming the results obtained on this ATPase in vitro (Tables 1 and 2). Moreover, these data exclude the possibility of a change in passive sodium transport through the erythrocyte membrane. Indeed, NMR experiments performed with NaCl and the 2-5 fraction (Table 2) show no variation over time of Na* _in* under our experimental conditions.

Finally, data show an increase in Na* _in* and, simultaneously, a decrease in Na* _out* in normal erythrocytes in the presence of the active 2-3a fraction, as proved by the variation in the integration values of these signals (Figure 1). Similar phenomena were observed with ouabain. Thus our findings reflect a disturbance in active transport of Na and may be interpreted as an impairment of the Na* pump in living erythrocytes by the 2-3a fraction of uremic toxins.

We thus isolated an inhibitor of the Na*+/K*+ pump from uremic plasma. This finding confirms the hypothesis formulated by other authors postulating the existence of such a factor in uremic patients (4, 11, 13). Moreover, our study directly demonstrates the effect of this Na*+/K*+ pump inhibitor on the intracellular sodium concentration. Thus the fraction we isolated may be, at least partly, responsible for the intracellular sodium overload observed in some uremic patients.

Because this inhibitory factor is found in normal urine as well as in uremic plasma, these results suggest that it may play a physiological role. Consequently, in patients with chronic renal failure, the toxicity of this factor might be ascribed to its accumulation in uremic body fluids. Further purification and identification of this 2-3a fraction is currently in progress.

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### References


### Table 2. Effect of Samples on Intracellular Sodium Uptake

<table>
<thead>
<tr>
<th>Samples</th>
<th>Final concn</th>
<th>Incubation time, min</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
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<tr>
<td>NaCl</td>
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<tr>
<td></td>
<td>100 mmol/L</td>
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</tr>
<tr>
<td>Oubain</td>
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<td>2-5 fraction</td>
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<tr>
<td>2-3a fraction</td>
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</tr>
<tr>
<td>(from uremic plasma)</td>
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<td>0</td>
</tr>
<tr>
<td>2-3a fraction</td>
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<td>0</td>
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<tr>
<td>(from normal urine)</td>
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<td></td>
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<tr>
<td>2-3b fraction</td>
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<tr>
<td>(from uremic plasma)</td>
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</tr>
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</table>

*Results, expressed as a percentage of Na* _in*, increase, are mean ± SD of three experiments.

*NS, not significant.