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High-resolution NMR studies of transmembrane cation transport in uremic patients

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Cation transport in erythrocytes of some uremic patients is impaired. Most studies have focused on the defect of the erythrocyte Na^+/K^+ pump in these diseased states. Herein, this cation transport defect was studied by using nuclear magnetic resonance spectroscopy (NMR) which is a non-invasive method permitting study on living erythrocytes. Firstly, we verified that the Na^+ transport defect in uremic erythrocytes was not due to non-specific causes such as membrane alteration or a modification of the intracellular metabolism. The proton relaxation data, determined using a paramagnetic doping method, are consistent with a lack of erythrocytic membrane damage in uremic patients. Also, ^{31}P-NMR results showed that in our experimental conditions, uremic and normal erythrocytes exhibit similar variations of ATP level over time. Lastly, the use of anionic paramagnetic shift reagent in ^{23}Na-NMR revealed a defect in the Na^+/K^+ pump of erythrocytes from uremic patients with high Na_{in} concentration. This defect seems to be due to a reduced number of pump units and to the presence of an endogenous inhibitor in uremic plasma.

Introduction

The Na^+/K^+ pump is a membrane protein that actively translocates sodium and potassium ions across the cell membrane to maintain the concentration gradient of these cations constant. The normal operation of the pump helps to preserve the integrity of cells and thus to ensure many vital functions in healthy subjects.

Welt and coll. [1] first described an increase in intra-erytrhocytic Na^+ concentration of patients with chronic renal failure (CRF); many subsequent studies focused on the defect of the erythrocyte Na^+/K^+ pump in these diseased states to better understand the physiopathology of these patients [2–12]. All of these studies were carried out using analytical techniques with cell lysis.

Nuclear magnetic resonance (NMR) spectroscopy is a non-destructive and non-invasive method which permits the study of this active membrane transport in living erythrocytes with a three-fold approach. The first is the use of paramagnetic doping method in ^{1}H-NMR [13,14] which allows measurement of water diffusion through erythrocyte membranes and which thus constitutes a membrane status evaluation. The second is the use of anionic paramagnetic shift reagent (SR) [15,16], which makes it possible to separate the intra and extra erythrocyte NMR signals of ^{23}Na and thus to monitor the ionic movements. Lastly, the third is classical ^{31}P-NMR which permits an approach of cellular metabolism with the quantification of metabolite levels and the measurement of intracellular pH [17–19].

Materials and Methods

^{1}H-NMR spectroscopy

Principle. Conlon and coll. [13] first reported on an NMR method for water exchange measurements through erythrocyte membranes. Briefly, in a suspension of erythrocytes, the spin-spin relaxation times, T_2, of water protons are similar in the two compartments: intra- and extra-cellular ($\cong$ 100 ms), and fast exchange through the membrane occurs between them. If Mn^{2+} paramagnetic ions, which do not enter the cell, are added to the suspending solution, the plasma relaxation time becomes much shorter ($\cong$ 1 ms). Because of the

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fast exchange between the two compartments, intra-erythrocyte relaxation time is also modified ($\cong 10$ ms). In these conditions, for CPMG spectra (Carr-Purcell-Meiboom-Gill; NMR pulse sequence which allows T_2 measurements), the expression for the effect of two-site exchange on CPMG decay is the sum of two exponentials [20]:

$$M(t) = P_S \exp(t/T_{2S}) + P_F \exp(t/T_{2F})$$

where S and F represent the slow and fast components, respectively. In this equation, $P_S + P_F = 1$. $1/T_{2S}$ and $1/T_{2F}$ are the relaxation rates of these components.

If concentrations of Mn^{2+} ion are about 20–40 mM then the equation of relaxation rate $1/T_{2S}$ is considerably simplified [14]:

$$1/T_{2S} = (1/T_{2i}) + (P_P/(P_P\tau_i + T_{2P})) \quad (1)$$

where T_{2i} and T_{2P} are the water proton relaxation times of the isolated erythrocytes and of doped plasma, respectively. τ_i is the life time of water proton in the erythrocyte compartment and P_P is the population fraction of water protons in the doped plasma compartment.

The life time τ_i is the variable called the 'water exchange time', T_{we}, by Conlon and Outhred [13]. A second parameter, the activation energy, is measured by an Arrhenius plot of $\ln(1/\tau_i)$ versus reciprocal temperature [21,22]. This parameter reflects the water molecule interaction with the erythrocyte membrane and a modification of its values should reflect a membrane alteration.

Measurement. Freshly drawn heparinized blood samples from healthy controls and patients were divided into two aliquots, one for measurements of T_{2i}, the other for measurement of T_{2S}.

For measurements of T_{2i}, samples were centrifuged (4 °C, 10000 × *g*, 1 h) to obtain tightly packed erythrocytes with a high packed cell volume of about 98%. The plasma and the buffy coat were removed by gentle aspiration. The T_{2i} value determined by this technique is not altered by the trapped plasma. Pirkle and coll. [22] showed an error of 5%, which is within the experimental error.

For measurements of T_{2S}, whole blood samples were prepared prior to the experiment by mixing 2 ml of whole blood with 1 ml of an isotonic doping solution (40 mM manganese chloride/110 mM sodium chloride). The stock solution were always prepared at least one week in advance [22].

T_2 values were measured using the CPMG pulse sequence: D_1–90 °–(D_2–180 °–D_2)$_n$–FID. Relaxation delay D_1 was roughly 5-fold longer than the T_1 of the sample. Delay D_2 was set at 1 ms or 0.6 ms for measurements of T_{2i} and T_{2S}, respectively. Twelve spectra (from 4 ms to 160 ms) were used to compute T_{2i} values in packed erythrocytes. For T_{2S} the number of spectra was eight (from 14.4 ms to 31.2 ms). Moreover, the values of the first spectrum in the sequence was redetermined at the middle and at the end of the sequence to correct, if necessary, any effect of sedimentation [23] on the T_{2S} value. After this sequence, some of these samples were recentrifuged, the erythrocytes were removed and the T_{2P} of doped plasma was measured.

NMR measurements were carried out at 37, 30, 21, 12, 5 °C (± 0.5 °C) on a Brucker AM 200 spectrometer operating at 200.13 MHz (Service Interuniversitaire de RMN, Faculté de Pharmacie, Marseille). Samples were placed in a 5 mm tube with an inner coaxial capillary containing benzene-d_6 as an external lock. An artificial line broadening of 0.5 Hz was used to improve the spectral signal-to-noise ratio. One sample was used to measure the T_{2S} value at two different temperatures only, in order to prevent a too long contact between sample and manganese ions. Before each T_{2S} measurement, the sample was gently bubbled with humidified air to homogenate the two phases.

^{23}Na-NMR spectroscopy

Principle. If Dysprosium tripolyphosphate (Dy $(PPP_i)_2^{7-}$), a shift reagent which does not enter in the cell, is added in the extra-erythrocyte medium, it is possible to obtain distinct NMR signals for intra- and extra-cellular sodium [15,16,24]. Under these experimental conditions, Ogino and coll. [25] showed that the shift reagent is not toxic for erythrocytes. They asserted that it does not modify the cation transmembrane movements [25], however, note that recently, Sze and coll. [26] showed an inhibition of lymphocyte stimulation by shift reagents. Ogino and coll. found a value of ouabain-sensitive Na^+ net efflux identical with that calculated using flame photometry. Thus, by using a dynamic study, it was possible to evaluate the ouabain effect on intact erythrocytes of uremic patients with regard to this effect on healthy subject erythrocytes.

Measurements

Sample preparation. Freshly drawn heparinized blood was centrifuged (4 °C, 1700 × *g*, 10 min); the plasma and the buffy coat were removed and the cells were washed three times. As concerns erythrocytes of uremic patients before hemodialysis, in order to remain in uremic conditions, the washing solutions were ultrafiltrates obtained from each subject's plasma. For the healthy controls, and uremic patients after dialysis, an artificial solution (NaCl 130 mM, KCl 3 mM, glucose 10 mM, NaH_2PO_4 1 mM, $MgCl_2$ 2 mM, Hepes 10 mM at pH 7.45) was used. To stimulate a hemodialysis session, some pre-dialysis uremic blood samples were washed five times with the artificial solution. The final

wash contained the shift reagent, $Dy(PPP_i)_2^{7-}$. To remain in uremic conditions, we prepared the shift reagent using ultrafiltrate samples from uremic patients before dialysis. The final concentration of this reagent was varied between 2 and 3 mM to separate the resonance for intra- and extra-cellular sodium sufficiently for the two peaks to be accurately integrated. Finally, after the last centrifugation, we discarded the supernatant and stored the packed cells (packed cell volume (PCV) 80–90%) in ice until the first NMR measurement could be made, less than 30 min later. The Na^+ and K^+ concentrations in the solution of final wash were measured by using an ion-selective microelectrode.

Fresh erythrocytes from healthy subjects were incubated for 24 h in normal plasma and in plasma from uremic patients with high Na_{in} concentration. Care was taken to ensure ABO and Rh blood group compatibility. After this incubation, erythrocytes were washed with ultrafiltrate of uremic patients as described above.

Plasma ultrafiltrate. We obtained ultrafiltrates from each patient at the beginning of the dialysis session by reducing the pressure in the dialyzer compartment of a capillary dialyzer (Cordis Labs., Inc., Miami, FL) fitted with a cellulose acetate membrane, before the dialysis fluid was run through the dialyzer.

Ouabain addition and NMR experimental conditions. After obtaining the first NMR spectrum, various volumes of ouabain solution were added (10–30 μl) to the packed cells to reach a final concentration within the following limits ($\cong 5 \cdot 10^{-2}$ mM–$\cong 1$ mM). The samples were homogenized by gentle bubbling with humidified air, then the spectra were acquired every 30 min for 5 h. Before each NMR measurement, the sample was gently bubbled with humidified air to prevent a settling artefact [23]. We verified that the sum of NMR integrations of Na_{in} and Na_{out} signals was constant within experimental error (10%), during the experiments (5 h). This analysis indicates that Na_{in} is 100% NMR visible [16,25,27], under the experimental conditions described. Concomitantly, another sample from the same subject but without ouabain was recorded to verify the integrity of erythrocytes and the stability of SR during the time of experiment.

^{23}Na analysis was conducted at 21° C (±0.5° C) on a Brucker AM 200 spectrometer with a 10 mm probe tuned at 52.94 MHz. Typical experimental conditions were: sample volume 2 ml; pulse width 24 μs ($\cong 60°$); acquisition time 0.254 s without delay between pulses; number of scans 1000. An artificial line broadening of 10 Hz was used to improve the spectral signal-to-noise ratio. All spectra were obtained by use of concentric NMR tube combination: an inner tube inside a 10 mm NMR tube. The inner tube contained an external reference solution in ^2H_2O (to lock the field frequency) in order to compare integration of spectra accurately. Its concentration was monitored by use of an ion-selective microelectrode ($\cong 70$ mM of Na^+ resulting from $\cong 7$ mM of shift reagent).

Calculations of sodium concentrations (expressed in mmol/liter of RBC; experimental error 10%) based on computer integration of the NMR signals were determined according to Gupta and coll. [15] as follows:

$$[Na_{in}] = ((A_{in}/A_{out}) \cdot S_{out}/(1 - S_{out}))[Na_{out}] \quad (2)$$

with

$$S_{out} = (A_{out}/A_{std}) \cdot [Na_{std}]/[Na_{out}]$$

where $[Na_{in}]$ and $[Na_{out}]$ are the sodium concentrations in intra- and extra-cellular media, respectively. A_{in} and A_{out} are the NMR integrals corresponding to Na_{in} and Na_{out} signals, respectively. $[Na_{std}]$ and A_{std} are the sodium concentration and NMR integral of standard solutions for which the NMR spectra were recorded before the packed erythrocyte spectra.

^{23}Na- and ^2H-NMR spectroscopies

Principle. Because of the high hemoglobin concentration in erythrocytes, the Na_{in} concentrations obtained by Eqn. 2 were expressed in mmol/liter of erythroctyes. To obtain the Na_{in} values based on the cell water, we determined the fraction of intracellular water volume V_{in}. We chose the method of Jelicks and coll. [28]; briefly, a solution containing both the shift reagent ($Dy(PP_i)_2^{7-}$) and ^2H_2O is added to the cell suspension. Two samples, those of the erythrocyte suspension and of its supernatant were taken for the NMR measurements. Na_{out}, which is complexed by the shift reagent and is membrane impermeable, provides the extracellular volume and ^2H_2O, which is freely diffusable, gives the total water volume, when these two compounds are quantified simultaneously [28,29].

Measurements. Sample preparation for erythrocytes of healthy controls and uremic patients was similar to that described above, except that the final wash containing the shift reagent was prepared in ^2H_2O.

^{23}Na and ^2H_2O analyses were conducted at 21° C (±0.5° C) on a Brucker AM 200 with a 10 mm probe tuned at 52.94 MHz and 30.72 MHz, respectively. For ^{23}Na, experimental conditions were identical with those described above. For ^2H_2O, experimental conditions were: pulse width, 2 μs; acquisition time, 1.819 s; relaxation delay, 6 s; number of scans, 16. An artificial line broadening of 0.5 Hz was used to improve the spectral signal-to-noise ratio.

For the same sample, the comparison of Na_{in} concentration value calculated from Equation 2 and that determined according to Jelicks and coll. [28] (see below) allows calculation of the fraction of intracellular water volume, V_{in}.

$$[Na_{in}] = (A_{in}/A_{out}) \cdot (V_{out}/V_{in}) \cdot [Na_{out}] \quad (3)$$

with

$$V_{in}/V_{out} = [(A_S^D/A_O^D)\cdot(A_O^{Na}/A_{out}) - 1]$$

where $[Na_{in}]$, $[Na_{out}]$, V_{in} and V_{out} are, respectively, the sodium concentrations and the volumes of intra- and extra-cellular media in the erythrocyte suspension, A_{in} and A_{out} are the NMR integrals corresponding to Na_{in} and Na_{out} signals, respectively. A_S^D and A_O^D are the integrals corresponding to ^{2}H-NMR signals of the sample containing the erythrocyte suspension and that containing the supernatant, respectively. A_O^{Na} is the integral corresponding to the ^{23}Na-NMR signal in the supernatant.

^{31}P- and ^{23}Na-NMR spectroscopies

^{31}P-NMR spectroscopy has been widely used to investigate metabolism. In the study of cation transmembrane transport it was important to know the effect of metabolism on this transport in our experimental conditions, when ouabain was not added.

Since it was impossible to record simultaneously the ^{23}Na and ^{31}P spectra with the probe used, we carried out sequential NMR measurements of ^{23}Na- and ^{31}P-NMR. The sample preparation and ^{23}Na-NMR spectra recording (21 ± 0.5 °C) were identical to those described in the section on ^{23}Na-NMR spectroscopy. Immediately after this ($\cong 5$ min) the ^{31}P-NMR spectrum (81.02 MHz) was recorded. The experimental conditions were identical for all erythrocyte samples: pulse width 18 μs ($\cong 60°$); acquisition time 0.655 s; relaxation delay 4 s; number of scans 360; time recording $\cong 30$ min. The theoretical value of relaxation delay was 6 s ($\cong 3 \cdot T_1$), but we observed no difference in NMR integration, within experimental error, between this value of 6 s and the experimental value chosen, 4 s. This latter value was a good compromise because it allowed us to use 350 scans, which is sufficient to improve signal-to-noise ratio, thus permitting ^{23}Na- and ^{31}P-NMR spectra recording during all the 60 min. An artificial line broadening of 10 Hz was used to improve the spectral signal-to-noise ratio. Between each spectrum recording, the sample was gently bubbled with humidified air to prevent settling artefact.

The quantification of intra-erythrocytic phosphorylated compounds was done by using NMR integration. The ATP was monitored through its β-P peak, which is not overlapped by any other peak [17,18]. The results are expressed in arbitrary units of NMR integration, the spectra being obtained in 'absolute intensity'.

The pH_i value was determined by subtracting from the chemical shifts of inorganic phosphate (P_i) and of 2,3-DPG that of [α-P]ATP [30], which served as an internal standard because it is insensitive to pH over the range studied [31]. These pH_i values were also measured using the chemical shift difference between the 3- and the 2-phosphate group resonances of 2,3-DPG [32]. These shifts were compared with those from the simulated intra-erythrocytic environment by titration of hemolysates [18,30,32,33]. The pH_i values given in the manuscript are the means of these various methods.

Hemolysis level measurement

Hemolysis level was measured (Beckman DU 70 Spectrophotometer) in supernatants before and after the NMR measurements. This level was found to be negligible (< 1%).

Mean globular volume (MGV) and hematocrit (Ht)

MGV is the mean cellular volume of the red blood cells measured in the sample, by using a flow-cytometric light scattering measurement [34] with a H1 Technicon apparatus. Ht values were determined using the same technique. MGV were measured before and after NMR experiments.

TABLE I

Relaxation rates ($1/T_{2S}$), life time (τ_i), hematocrit (Ht) and mean globular volume (MGV) of erythroctyes from healthy and uremic subjects

	Healthy subjects		Uremic patients			
			normal Na_{in}		high Na_{in}	
	5 °C ($n = 7$)	37 °C ($n = 10$)	5 °C ($n = 5$)	37 °C ($n = 9$)	5 °C ($n = 4$)	37 °C ($n = 6$)
$1/T_{2S}$ (s^{-1}) mean ± S.E.	79.0 ± 1.2	130.2 ± 2.3	71.5 ± 0.7	120.3 ± 2.4	72.2 ± 0.6	119.1 ± 2.0
τ_i (ms) mean ± S.E.	21.1 ± 0.3	8.0 ± 0.1	23.2 ± 0.2	8.5 ± 0.3	23.2 ± 0.4	8.9 ± 0.3
Ht (%) mean ± S.E. ($n = 18$)	45.2 ± 0.5		27.0 ± 0.8			
MGV (fl) mean ± S.E. ($n = 18$)	95.0 ± 1.0		87.1 ± 2.7			

Treatment of data

The mean values of experimental results are expressed with the standard error of the mean (S.E.). Comparative statistical analyses of groups were performed using Student's unpaired *t*-test. Comparison of linear regression slopes was done as described by Dagnelie [35].

Results

Table I shows the $1/T_{2S}$ experimental values and the τ_i values calculated from Eqn. 1, at two different temperatures. The typical T_{2i} and T_{2P} values were 60 ± 3 ms and 1.1 ± 0.2 ms, respectively. The value of P_P parameter (the population fraction of water proton in the doped plasma compartment) was calculated from Eqn. 4 [22]:

$$Ht = (1 - P_P)/(V_i + (1 - V_i)V_P(1 - P_P)) \quad (4)$$

where Ht is the hematocrit level, V_i the fraction of intracellular solvent volume determined by NMR (see below) and V_P the fraction of plasma water volume equal to 0.95 [22]. Ht and MGV parameters are also included in Table I. There was a significant difference between τ_i values in healthy and in uremic erythrocytes (Student's unpaired *t*-test; $P < 0.001$ for 5 °C and $P < 0.01$ for 37 °C).

For uremic patients, we drew the Arrhenius plot of $\ln(1/\tau_i)$ versus reciprocal temperature (data not shown). The points at 12 °C, 21 °C and 30 °C are τ_i mean values from 9, 15 and 10 uremic patients, respectively. Activation energy value was 5.3 ± 0.1 kcal/mol. For healthy subjects, this same value was 5.2 ± 0.1 kcal/mol. The points at 12 °C, 21 °C and 30 °C are τ_i mean values from 7, 8 and 7 healthy subjects, respectively.

The ratio of Na_{in} concentration values calculated from Eqn. 2 and those from Eqn. 3 allows determination of the fraction of intracellular water volume (V_i). For erythrocyte samples of three healthy subjects, $V_i = 0.69 \pm 0.01$, which agrees within 3% of the literature values [22]. For erythrocyte samples of five uremic patients, $V_i = 0.65 \pm 0.01$. There was no significant difference between these values, nor for MGV values (Table I).

Fig. 1 shows an example of ^{31}P and ^{23}Na-NMR spectra acquired sequentially for packed erythrocytes. Each spectrum is marked with the time of the midpoint of acquisition. The assignments of ^{31}P-NMR are as follows: sugar phosphate (SP, 1); 2,3-diphosphoglycerate (DGP, 2, 3); intracellular inorganic phosphate (P_i, 4); γ-, α- and β-P of adenosine triphosphate (γ-ATP, α-ATP, β-ATP, 5, 6, 7). The P_i present in low concentrations in fresh erythrocytes is represented by a small upfield shoulder on the 2-P peak of DPG. But, by

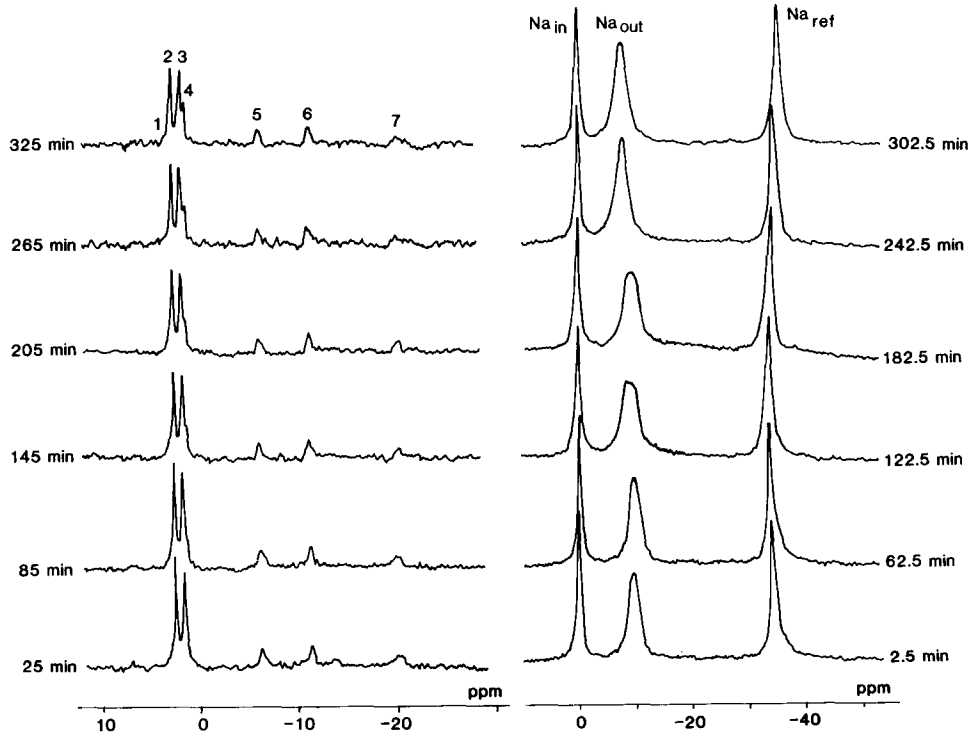


Fig. 1. Example of ^{31}P- and ^{23}Na-NMR spectra acquired sequentially for packed erythrocytes. Each spectrum is marked with the time of the midpoint of acquisition. The assignments of ^{31}P-NMR are as follows: sugar phosphate (SP, 1); 2,3-diphosphoglycerate (DPG, 2, 3); intracellular inorganic phosphate (P_i, 4); γ-, α- and β-P of adenosine triphosphate (γ-ATP, α-ATP and β-ATP, 5, 6, 7). The assignments of ^{23}Na-NMR are as follows: intra-erythrocytic sodium (Na_{in}); extracellular sodium (Na_{out}); capillary sodium (Na_{ref}).

using an artificial line broadening of 1–2 Hz we could pinpoint this resonance accurately. Moreover the integrations of P_i resonance were obtained using a Lorentzian deconvolution. For ^{23}Na-NMR spectra, the assignments are as follows: intra-erythrocytic sodium (Na_{in}); extracellular sodium (Na_{out}); capillary sodium (Na_{ref}).

Time dependence of spectral data, i.e., mean quantity values of intra-erythrocytic phosphorylated compounds, intra-erythrocytic pH (pH_i) and mean concentration of Na_{in} for four healthy controls and six uremic patients before dialysis (three with normal Na_{in} concentration and three with high Na_{in} concentration) are shown in Fig. 2. The presence of paramagnetic shift reagent with different concentrations in the external medium modifies the susceptibility gradient from one sample to another. The use of α-ATP as internal reference allows correction of these variations, leading to correct pH_i values. The DPG quantities are classically higher in uremic than in healthy erythrocytes [1,36]. With time they both decrease whereas the P_i quantities increase for erythrocytes of healthy controls and for those of uremic patients. Acidification of intra-erythrocytic medium was observed in both cases. Lastly, the Na_{in} concentration was stable within experimental error during the experiment time. In our experimental conditions, we observed no significant difference between the variation of phosphorus compound levels of healthy erythrocytes and that of uremic erythrocytes (with high or normal Na_{in} concentrations).

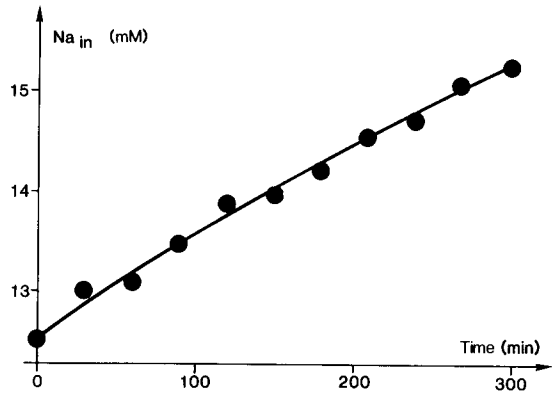


Fig. 3. Example of the time course of the Na^+ entry induced by the ouabain ($1.5 \cdot 10^{-4}$ M) in packed erythrocytes (PCV ≅ 80%).

Fig. 3 shows an example of the time course of the Na^+ entry induced by the ouabain on packed erythrocytes (PCV ≅ 80%). The Na^+ and K^+ concentrations of extracellular media varied within the following limits: $152 < Na^+ < 171$ mM and $2.5 < K^+ < 2.9$ mM. In contrast with classical techniques of cell lysis, NMR measurements of the Na_{in} entry into intact erythrocytes due to ouabain effect require a non-modification of intracellular volume or at least the knowledge of this modification to correct the Na_{in} concentration, if necessary.

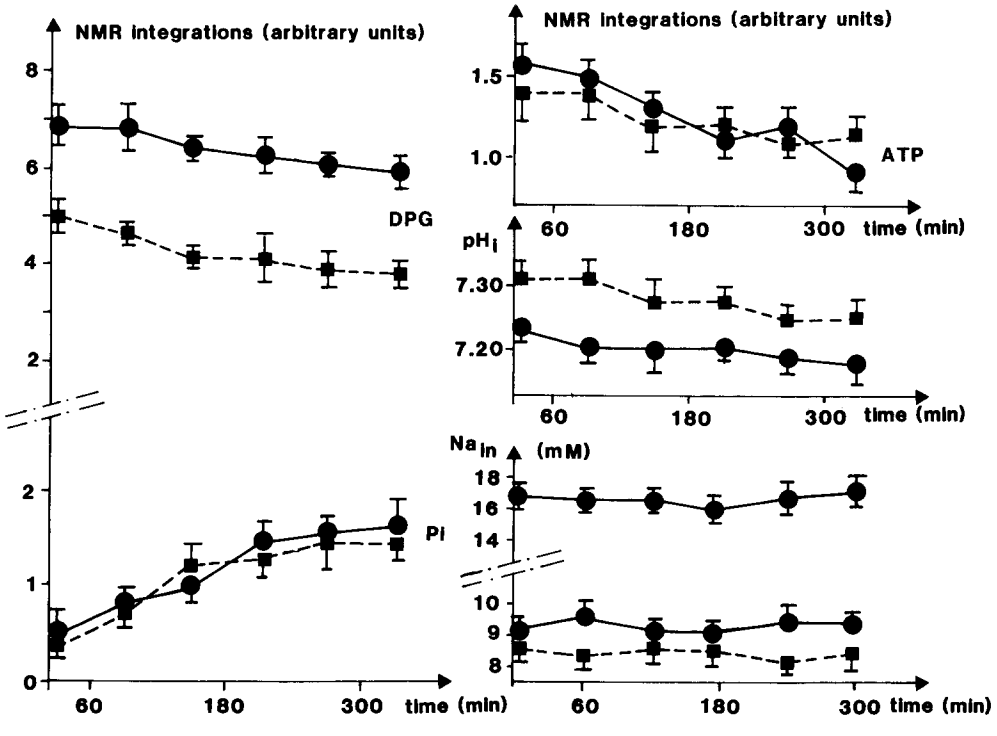
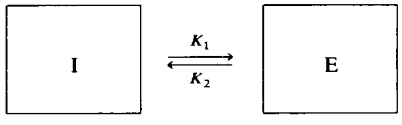


Fig. 2. Time dependence of spectral data (mean quantity values of intra-erythrocytic phosphorylated compounds, intra-erythrocytic pH (pH_i) and mean concentration of Na_{in} for erythrocytes of four healthy controls (■------■) and six uremic patients (●———●) (three with normal Na_{in} concentration and three with high Na_{in} concentration).

We measured MGV variations after ouabain addition and at the end of NMR experiments (5 h). These were $2.0 \pm 2.5\%$ and $2.2 \pm 1.6\%$ for four healthy subjects and six uremic patients, respectively. Thus errors obtained for the Na_{in} concentration values are negligible.

The crude results shown in Fig. 3 cannot be used as such. Indeed the passive influx of sodium ions and the pump activity are dependent on concentration gradients [37], as is ouabain inhibition of Na^+/K^+-ATPase [38]. To the best of our ability, we tried to keep the K^+ extracellular concentration very close from one sample to another. But for Na^+ extracellular concentrations, the $Dy(PPP_i)_2^{7-}$ added as a sodium salt in the last washing modified these concentrations. Moreover, although the whole packed cell volume studied was 2 ml for all samples, the volumes of intra and extracellular compartments were different (PCV $\cong$ 80–90%) because it was difficult to discard exactly the same volume of supernatant from one sample to another.

To evaluate quantitatively the ouabain effect on Na^+ active efflux, we modeled the time course of Na^+ entry. In the closed system studied, a two compartment model was the simplest kinetic model explaining the changes in sodium concentrations (Fig. 3).



Erythrocyte-V_I Supernatant-V_E

In this model I and E represent erythrocytes and supernatants, respectively; K_1 and K_2 are the apparent exchange constants between the two compartments. These constants are only the mathematical interpretation of the transmembrane ion transport; V_E and V_I are the volumes of the two compartments. The differential equations developed according to this model are:

$$V_I \, dC_I/dt = -K_1 V_I C_I + K_2 V_E (C_E - C_I)$$

$$V_E \, dC_E/dt = -K_2 V_E (C_E - C_I) + K_1 V_I C_I$$

Differential equations were solved using the Laplace transform method. Values of the equation parameters were obtained by using a non-linear regression with an iterative procedure [39].

For Na_{in} concentration we obtained: $Na_{in} = A + B \exp(-\alpha t)$ with $A = K_2(C_I(0) + C_E(0)V_E/V_I)/(K_1 + K_2(1 + V_E/V_I))$, $B = C_I(0) - A$ and $\alpha = K_1 + K_2(1 + V_E/V_I)$.

$C_I(0)$ and $C_E(0)$ are the initial sodium concentrations in the two compartments. For $C_E(0)$, the dilutions due to ouabain addition were taken into account. The volumes are calculated after NMR determination of the Na_{in} concentration. Indeed, NMR integration is proportional to the volume observed and to the concentration, and thus $V_I = A_{in}[Na_{out}]V_{whole}/(A_{out}[Na_{in}] + A_{in}[Na_{out}])$. Finally the V_E value took into account the ouabain addition.

Furthermore, the same sample but without ouabain was used as a reference. Indeed, because of the stability

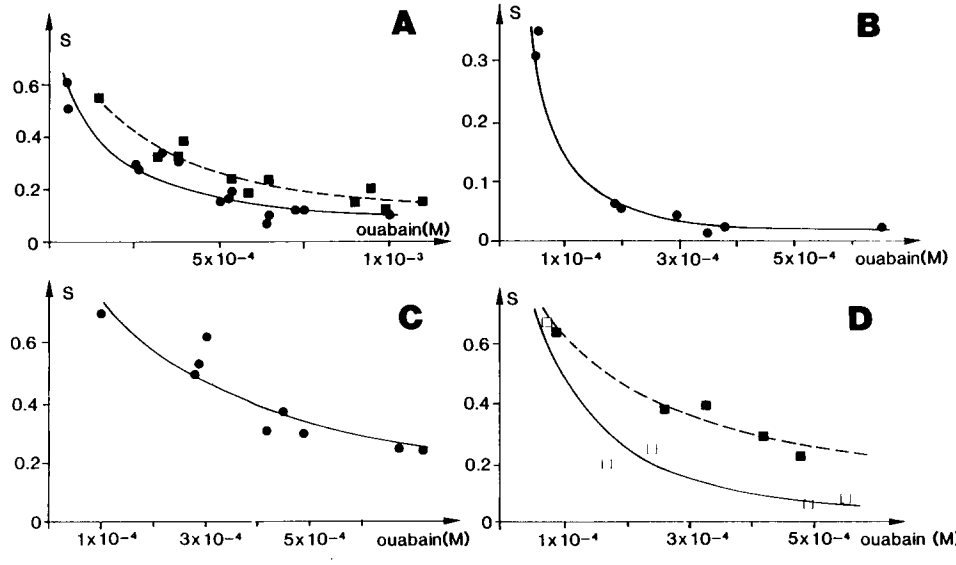


Fig. 4. Ratio S, representing the ouabain effect, versus ouabain concentration: (A) six healthy samples ($Na_{in} = 8.7 \pm 0.3$ mM, ■------■) and eight uremic samples washed with their own ultrafiltrates, before hemodialysis, with normal Na_{in} ($Na_{in} = 9.2 \pm 0.3$ mM, ●———●); (B) three uremic samples washed with their own ultrafiltrates, before hemodialysis, with high Na_{in} (16.9 ± 0.6 mM); (C) the same three uremic samples (with high Na_{in}) washed with artificial solution (see text); (D) two healthy samples ($Na_{in} = 7.9 \pm 0.3$ mM) incubated in normal plasma (■------■) and in uremic plasma (□———□).

of Na^+ NMR integrations of samples during the time of study (see above results), $V_I\ dC_I/dt = 0$ and thus $K_2/K_1 = C_I(0) \cdot V_I/V_E \cdot [C_E(0) - C_I(0)]$. This ratio ($R_{ref}$) represents the equilibrium state of the sample when ouabain was not added.

It was possible to calculate another value of this ratio (R_{ouab}) from the K_1 and K_2 values determined from the model, when ouabain was added to the sample. The ratio $S = R_{ref}/R_{ouab}$ represents the ouabain effect for the sample. It is equal to 1 if the ouabain concentration is equal to 0 and it decreases when the ouabain concentration increases.

This model takes into account the gradient concentrations and the volumes, thus permitting comparison of the various samples. Fig. 4 shows the results:

Fig. 4A. Six healthy samples ($Na_{in} = 8.7 \pm 0.3$ mM and eight uremic samples, washed with their own ultrafiltrates, with normal Na_{in} ($Na_{in} = 9.2 \pm 0.3$ mM);

Fig. 4B. Three uremic samples, washed with their own ultrafiltrates, with high Na_{in} ($Na_{in} = 16.9 \pm 0.6$ mM);

Fig. 4C. The three uremic samples (with high Na_{in}), washed with artificial solution (described in Materials and Methods);

Fig. 4D. Two healthy samples ($Na_{in} = 7.9 \pm 0.3$ mM) incubated in normal plasma and in uremic plasma.

In a first approximation, the best linear regressions were $\ln[S/(1-S)] = a\ \ln[\text{ouabain}] + b$; slope values and regression coefficients are summarized in Table II. A significant difference between samples from uremic patients with high Na_{in}, and those of healthy controls and uremic patients with normal Na_{in} was observed ($P < 0.05$). After washing with artificial solution, the slope decreased ($P < 0.2$). A significant difference between samples incubated in normal plasma and in uremic plasma was observed ($P < 0.1$).

Lastly, Table III shows the Na_{in} concentration values for eight uremic patients with high Na_{in} and three uremic patients with normal Na_{in} before and after a hemodialysis session. We observed no corrective effect by hemodialysis on Na_{in} concentrations for patients.

TABLE II

Slope (a) and regression coefficient (r) of linear regressions ($\ln[S/(1-S)] = a\ \ln[\text{ouabain}] + b$) from data obtained with healthy and uremic erythrocytes

Erythrocytes	a	r
Healthy samples	−1.01	0.95
Uremic patients, normal Na_{in} samples	−0.94	0.92
Uremic patients, high Na_{in} samples	−1.55	0.94
Uremic patients, after washing with artificial solution	−1.12	0.92
Healthy samples, incubated in normal plasma	−1.05	0.96
Healthy samples, incubated in uremic plasma	−1.68	0.96

TABLE III

Na_{in} concentrations of erythrocytes from healthy subjects and from uremic patients before and after an hemodialysis session

n.s., not significant.

Erythrocytes	Na_{in} concentrations (mM; mean ± S.E.)		Significance
	before dialysis	after dialysis	
Healthy samples ($n = 6$)	8.7 ± 0.3		
Uremic patients normal Na_{in} samples ($n = 3$)	9.0 ± 0.4	9.3 ± 0.4	n.s.
Uremic patients high Na_{in} samples ($n = 5$)	11.2 ± 0.3	11.3 ± 0.2	n.s.
Uremic patients very high Na_{in} samples studied in this work ($n = 3$)	16.9 ± 0.6	17.4 ± 0.5	n.s.

Discussion

Our results allow the study of three causes which may be involved in the occurrence of high Na_{in} concentrations in some uremic patients [3–5,8,40]. These three causes may be summarized as: a defect of erythrocyte membrane which would lead to an increase in the passive Na^+ influx, and/or a metabolism defect in the intracellular medium due, for example, to the lower pH_i in uremic erythrocytes [33,41], and/or the inhibition of Na^+/K^+ pump, which would lead to a decrease in the active Na^+ efflux.

Failure in erythrocyte membrane

To calculate the τ_i values, we chose the equation of Morariu and coll. [14] rather than the simpler equation of Conlon and coll. [13] because the former takes into account the population fraction of plasma water proton, P_P, which is different in uremic patients as compared with healthy controls (see Table I, Ht data). Moreover, this method gives τ_i values which are in agreement with the water exchange time through erythrocyte membrane, as determined by tracer methods [42].

We observed higher τ_i values in uremic patients as compared with healthy controls (Table I). τ_i is pH dependent [14], and thus the lower intraerythrocytic pH value observed in uremic patients might explain their higher τ_i values [33,41]. But these pH variations are so weak ($\cong 0.1$ pH unit) that they fail to explain the higher τ_i values observed [14]. The difference in τ_i values are

due to variations in $1/T_{2S}$ data (Table I). It should be underlined that $1/T_{2S}$ results are in agreement with observations of longer relaxation times for cellular water protons in pathologic tissue and, moreover, that they are not specific to any particular disease [43].

The similar values of activation energy obtained for uremic patients and healthy subjects are consistent with the membrane integrity of eyrthrocytes because this parameter reflects the water molecule interaction with the erythrocyte membrane [21]. Such an integrity of the membrane structures [44] involved in water transport leads to the hypothesis that the passive influx of sodium behaves similarly. This hypothesis is in good agreement with previous studies, which showed a normal Na^+ passive influx in uremic patients [8,11].

Level of phosphorus compounds

Na_{in} efflux is an active transport which is Na^+/K^+-ATPase enzyme system dependent. Thus it was of a great interest to monitor the erythrocyte ATP level variations in uremic patients and healthy subjects to check for a possible influence of this parameter on the ouabain-dependent Na^+ entry.

The results in Fig. 2 were obtained with normal and uremic erythrocytes without ouabain addition. They classically showed that there is a weak decrease in ATP and DPG concentrations and an increase in P_i concentration during the NMR experiment time [17,45]. These data are similar in uremic (with normal or high Na_{in} concentration) and healthy erythrocytes; they show identical variations in levels of phosphorus compounds in the two types of erythrocytes in our experimental conditions. Moreover, note that the erythrocyte acidification during the NMR experiment is certainly due to the lactate formation [45].

In conclusion, neither the passive Na^+ influence, which is correlated to membrane integrity, nor the active Na^+ transport, which is correlated to Na^+/K^+-ATPase activity, are modified by our experimental procedures, as indicated by the stability of Na_{in} concentration during the NMR experiments without ouabain addition (Fig. 2). Thus the variations observed between uremic and normal erythrocytes when ouabain is added are specific effects (Fig. 4).

Inhibition of Na^+/K^+ pump

We found no difference in uremic patients, whatever their Na_{in} concentration, as concerns membrane integrity and metabolism. But the existence of these two distinct groups (normal Na_{in} and high Na_{in}) [40] suggests that these two populations should exhibit different sensitivity to ouabain. We therefore selected some patients with very high Na_{in} ($Na_{in} > 15$ mM) to be sure to emphasize the studied effect, because of the weak sensitivity of the NMR technique. But there are few patients with very high Na_{in}, which explains the limited number in this study. In the same way, the other Na^+-transport pathway, such as furosemide-sensitive Na^+/K^+ cotransport was not studied because it represents only $\cong 2\%$ of whole Na^+ transport for Na^+-loaded cells, a level which is not detectable by NMR.

The results showed in Table II are in agreement with the two distinct groups of patients because the high Na_{in} group exhibited a greater sensitivity to ouabain than did the patients with normal Na_{in} or the healthy subjects. The similar behavior of the latter two groups is in agreement with two studies [9,11] which found no decrease in Na^+/K^+ pump activity in uremic patients because the patients selected had a mean Na_{in} concentration similar to that of normal subjects.

Thus, for the same ouabain concentration, inhibition is higher in the high Na_{in} patients. These data may reflect a decrease in the number of Na^+/K^+ pump active units in erythrocyte membrane for these patients as compared with that of healthy controls or of normal Na_{in} patients.

This hypothesis is in perfect agreement with the study of Cheng and coll. [8]. These authors showed that the [^{3}H]ouabain-binding ability of erythrocytes with high Na_{in} was markedly reduced for uremic patients as compared with that of erythrocytes from normal subjects. They also showed that for these uremic patients, ouabain-binding ability and the Na_{in} concentration are correlated.

As shown in Table II, the restoration of the slope value near to normal value after erythrocyte washing with artificial solution may be explained by the existence of an endogenous inhibitor which is removed by this washing. Its presence has been suggested by different authors [2,3,5]. Moreover, in a recent paper we demonstrated the existence of such a factor in uremic plasma [46]. Such results agree with those of previous reports showing on the one hand, a decrease in erythrocyte Na^+/K^+-ATPase activity after incubation with uremic plasma [2,5] and, on the other hand, a significant decrease in this activity in uremic patients [6]. Furthermore, Quarello and coll. [10] demonstrated an increase in this activity in erythrocytes after hemodialysis session.

Also, it seems that this endogenous factor is tightly bound to the erythrocyte membrane since the washing of uremic erythrocytes does not restore the normal slope value perfectly (Table II). Thus, filtration of this substance during the hemodialysis session will be difficult, which explains the weak restoration (16%) of ouabain-sensitive Na^+ efflux [10] and the non-modification of Na_{in} concentration observed in this work (Table III) and in other studies [10,47].

It was appealing to think that the decrease in the number of Na^+/K^+ pump active units, as suggested above, was due to the presence of this endogenous inhibitor. But Cheng and coll. [8] found no difference in

the ouabain-binding ability after incubation of normal erythrocytes in uremic plasma. They concluded to a decreased synthesis of Na^+/K^+ pump units. This apparent discrepancy may be explained if we consider that there is no competition between ouabain and the endogenous inhibitor. Indeed, if such a competition exists, the endogenous inhibitor should be removed from its bindings with Na^+/K^+-ATPase enzyme at the ouabain concentrations used in our experiments, even if the affinity of an endogenous substrate is generally greater than that of an exogenous substance like ouabain.

This hypothesis is in agreement with our experiments where normal erythrocytes were suspended in uremic plasma. The results show that uremic plasma induces significant modification of the slope value after incubation (Table II). Normal erythrocytes become 'similar' to uremic erythrocytes.

Thus, in uremic high Na_{in} erythrocytes, there is a decrease in the number of Na^+/K^+ pump units, a part of which are blocked by an endogenous inhibitor. After washing, these erythrocytes recovered a quasi normal sensitivity to ouabain despite their less numerous pump units. This finding confirms the hypothesis that, in high Na_{in} erythrocytes, there is a kinetic activation of the Na^+/K^+ pump due to the high intracellular level of Na^+ [8,12].

In conclusion, the increase in the intra-erythrocytic sodium concentration in some uremic patients seems to originate from two factors among others. Firstly, a reduced number of pump units due to a decreased synthesis, secondly, the presence of an endogenous inhibitor in uremic plasma.

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