J. Membrane Biol. 13, 165–184 (1973)
© by Springer-Verlag New York Inc. 1973

Sodium Fluxes in Rat Red Blood Cells in Potassium-Free Solutions

Evidences for Facilitated Diffusion

L. A. Beaugé* and Olga Ortiz

División de Biofísica, Instituto de Investigación Médica Mercedes y Martín Ferreyra, Casilla de Correo 389, Córdoba, Argentina

Received 16 March 1973

Summary. Unidirectional as well as net sodium fluxes were studied in rat red blood cells incubated in potassium-free sodium and sodium-substituted solutions. In the absence of ouabain the magnitude of sodium efflux in different solutions followed the sequence Na > choline > tris > Mg; in the presence of 10^{-4} M ouabain the sequence was choline > tris > Na > Mg. In a sodium-magnesium mixture the ouabain-sensitive sodium influx as a function of the external sodium concentration followed more or less an S-shaped curve; at high external sodium there was good agreement with the efflux values, but below 90 mM-Na all efflux points were above the influx ones. Both ouabain-insensitive Na influx and efflux were stimulated by external Na following a linear relationship though with different slopes. In net flux experiments these cells were able to extrude sodium against an electrochemical gradient in K-free ouabain Na–Mg and Na-choline mixture solutions. In K-free-Na-free magnesium media the ouabain-sensitive sodium loss increased proportionally to the square of the internal sodium, whereas the ouabain-insensitive loss went to saturation. In K-free sodium solutions the net Na gain was reduced as internal Na increased and was unaffected by ouabain. These results, plus the changes in the sodium influx/net Na gain ratio and in the rate constant for Na efflux when internal Na was modified, are consistent with the existence of a facilitated diffusion system for sodium movements which contributes, together with leakage, to the net Na gain in K-free sodium solutions.

In previous papers (Beaugé & Ortiz, 1970, 1971*a*) the transport of rubidium and sodium in rat red blood cells, and the relationships between the two ion species and the cardiac glycoside ouabain have been reported. A large Na–Na ouabain-sensitive exchange was described in K-free sodium solutions, which gradually switched to a Na–Rb exchange as the latter cation was added to the external media; the increase in sodium efflux

* Member of the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina. *Present Address:* Department of Biophysics, University of Maryland School of Medicine, 660 W. Redwood Street, Baltimore, Md. 21201.

produced by 5 mM-Rb was only 40% above the K (Rb)-free values. However, in net flux experiments, the loss of internal sodium as a function of time did not differ much in K (Rb)-free choline Ringer's as compared with 5 mM-Rb choline. This finding was not consistent with some of the results described above, and a more detailed study of sodium fluxes in K-free conditions was attempted. The results shown in this paper have been briefly described elsewhere (Beaugé & Ortiz, 1971*b*, 1973).

Materials and Methods

The procedures for blood collection and cell washing have been previously described (Beaugé & Ortiz, 1970).

Solutions

All solutions were prepared with de-ionized water and reagent grade chemicals. Glucose was present at a concentration of 200 mg/100 ml and was added as a solid prior to use. The composition of the solutions was as follows (mM): a) sodium Ringer's: NaCl 150; $MgCl_2$ 1; orthophosphoric acid titrated with tris to give a pH of 7.4 (37 °C) 2; when potassium and rubidium were present, sodium concentration was 150 minus the K or Rb concentration; b) choline Ringer's: the composition was similar to the former, sodium being replaced by equal amounts of choline, which was recrystallized from an ethanol solution before use; c) tris Ringer's: tris-base titrated with HCl to give a pH of 7.4 (37 °C) 174; $MgCl_2$ 1; tris-phosphate 2; d) magnesium Ringer's: $MgCl_2$ 109; tris-phosphate 2. All solutions were Ca-free. ^{22}Na was obtained through the Comisión Nacional de Energía Atómica of Argentina as an aqueous solution of NaCl of high specific activity; before use it was dessicated, redissolved in de-ionized water and the pH adjusted close to 7.0 with tris. Ouabain was supplied by Sigma Chemical Co., USA.

Sodium Efflux

The efflux of sodium was determined from the rate of ^{22}Na lost during 60 to 75 min, taking samples at 15-min intervals. The general procedure has been described previously (Beaugé & Ortiz, 1971*a*). The rate constant for sodium efflux was determined from a semilogarithmic plot of counts remaining in the cells as a function of time.

Sodium Influx

The uptake periods lasted 1 hr. The rate constant for sodium influx was calculated from the equation proposed by Sachs and Conrad (1968) for efflux corrections. A description of the method can be seen elsewhere (Beaugé & Ortiz, 1971*a*). In all cases a simultaneous determination of sodium efflux was performed in order to use the equation mentioned above. In the uptake experiments the cells were preincubated in the same solution and for the same length of time as in efflux experiments.

Counting

Whenever possible, counting time was long enough to reduce errors to 1 to 3%. ^{22}Na activity was assayed in a Nuclear Chicago Automatic gamma counter. All fluxes

were expressed in mmole/liter of cells/hr. The volume of the cells was calculated by comparing the optical density at 541 nm of the unknown hemolysate with a sample of known hematocrit.

Cation Analysis

Sodium and potassium were determined with a Beckman DU spectrophotometer; all readings always fell within 20% of the standards. The cells were washed three times in K-free magnesium Ringer's with 10^{-4} M ouabain in the cold and then lysed in 10 ml of de-ionized water. Since the rate of sodium loss in this washing solution was the lowest found for rat red cells, averaging 0.163 hr^{-1} at 37 °C, no correction factor was introduced for cation loss during this procedure.

Red Cell Chloride

The cells were washed three times in cold K-free sodium Ringer's and twice in the incubation solution. They were then suspended in the same solution at about 50% hematocrit and incubated for 15 min at 37 °C. Duplicate samples were taken for chloride analysis and microhematocrit determinations. For deproteinization, 1 volume of cell suspension was added to 7 volumes of de-ionized water; when hemolysis was completed, 1 volume of sodium tungstate 10% and 1 volume of 2/3 N sulfuric acid were also added; the tubes were stirred and left for 10 min at room temperature, then centrifuged at $1,500 \times g$ for 10 min. Chloride was determined in the supernatant as well as in a sample of the incubation solution by the Schales and Schales method (1941); the accuracy of the method, using an automatic microburette, was within 1%. Intracellular chloride was estimated on the basis of the chloride in the incubation solution, in the cell suspension and in the suspension hematocrit. Intracellular chloride concentration in mM was calculated assuming a 64% cell water content (Beaugé & Ortiz, 1971*a*).

Results

Sodium Efflux into K-free Solutions

The effect of different sodium substitutes on the efflux of sodium in K-free solutions with and without ouabain was investigated first; these experiments are summarized in Table 1. Taking the mean fluxes in sodium media as a reference, the sodium-free effect was variable, depending on the substance used as a replacement. Thus, whereas in choline there was a slight and nonsignificant reduction, this reduction was greater and significant in tris and even greater in magnesium. On the other hand, in the presence of ouabain in choline and tris media the flux increased, while in magnesium media the flux was reduced by 40%. In this latter medium the control fluxes were about 60% higher than the inhibited ones in sodium. When magnesium replaced external sodium a higher external chloride concentration occurred; as shown below, the intracellular electrical potential became more negative. However, the reduction in efflux also seen in tris, where the external chloride

Table 1. Rate constants, k (hr^{-1}), for ^{22}Na efflux in rat red cells incubated in different K (Rb)-free solutions

	Sodium		Choline		Tris		Magnesium	
	Control	Ouabain	Control	Ouabain	Control	Ouabain	Control	Ouabain
Mean	1.044	0.244	0.922	0.479	0.651	0.537	0.400	0.163
SEM	0.076	0.025	0.059	0.061	0.069	–	0.028	0.038
n	(8)	(5)	(11)	(8)	(4)	(2)	(4)	(3)

After washing three times with 5 mM K-sodium Ringer's the cells were preincubated for 4 hr in the same solution at room temperature with ^{22}Na (5 μC/ml suspension) at about 50% hematocrit. The cells were then washed twice in the same solution and then three times in K-free magnesium Ringer's in the cold and suspended in this latter solution at about 70% hematocrit. The hematocrit of the incubation suspension was 0.5%. The rate constants for ^{22}Na loss were obtained from a semilogarithmic plot of the counts remaining in the cells as a function of time. Ouabain concentration was 10^{-4} M and temperature 37 °C.

was even lower than in sodium media, would indicate that these results are more related to the cation used as a sodium replacement than to a possible change in E_m due to variations in the chloride ratios.

Possible Effect of Potassium Leakage on the Sodium Efflux in K- and Na-free Solutions

From the previous results it is evident that the sodium-free effect on sodium efflux in rat red cells in K-free conditions must be an operational definition always specifying the substance used to replace sodium. From a one-to-one ouabain-sensitive Na/Na exchange as described before (Beaugé & Ortiz, 1971*a*), one should expect the decrease produced by ouabain in sodium media to be equal to the decrease when external sodium was withdrawn in the absence of glycoside. However, these expectations were not met; on the contrary, even in magnesium Ringer's where the greatest decrease was observed, the values of sodium efflux were larger than predicted. An alternative would be introduced in these results if the potassium leakage of the cells could originate a potassium concentration close to the pump sites high enough to stimulate the pump under sodium-free conditions. In Fig. 1 the potassium concentration in a nominally K-free magnesium solution is plotted against the suspension hematocrit after a 1-hr incubation at 37 °C (similar results were obtained in choline). From the graph an average 0.016 mM potassium concentration can be estimated after a half-hour incubation at 0.5% hematocrit. During the same length of time the cell sodium has been reduced to about 2.70 mmole/liter of cells according

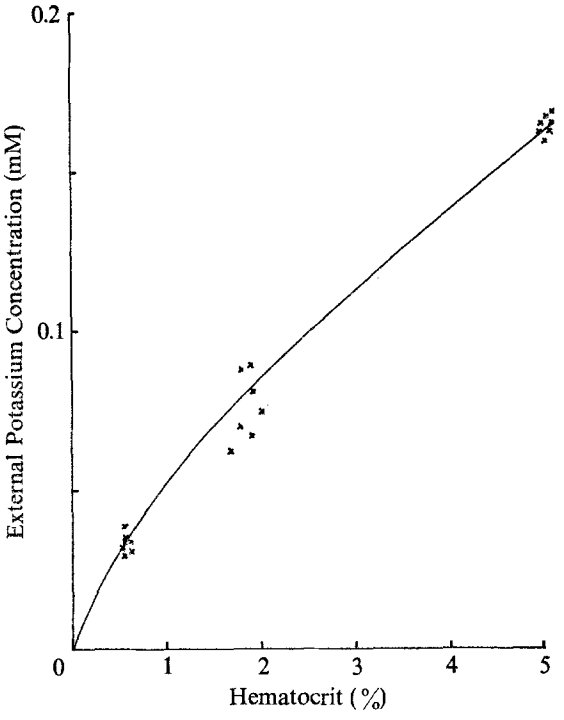

Fig. 1. External potassium concentration as a function of the suspension hematocrit after a 1-hr incubation of rat red cells in a nominally K-free magnesium Ringer's. The cells had been previously washed three times in the same solution. Temperature was 37 °C. Similar results were obtained in choline Ringer's

to the rate constants of Table 1 and the cellular sodium in fresh erythrocytes (Beaugé & Ortiz, 1971*a*). Using the linear relationship between internal sodium and rubidium influx from the above-mentioned paper, and provided that the potassium concentration in the solution is the same as on the pump sites for a sodium-potassium exchange close to one-to-one, an estimate of the potassium-stimulated sodium efflux can be obtained from the activation curve of rubidium influx by external rubidium in Na-free Ringer's (Beaugé & Ortiz, 1970); the values obtained are between 0.4 and 0.7 mmole/liter of cells/hr. In magnesium, the figures approximate 0.64 mmole/liter of cells/hr of ouabain-sensitive sodium loss (attained by multiplying the ouabain-sensitive rate constant for sodium efflux times the average internal sodium concentration during a 1-hr period), while in choline the values are about half of those obtained in magnesium. This point will be treated again in the Discussion.

Unidirectional Sodium Fluxes as a Function of the External Sodium Concentration in K-free Conditions

In the following experiments magnesium was used as a sodium replacement in osmotically equivalent amounts; this cation was selected because it gave the lowest sodium efflux in Table 1. Fig. 2 illustrates the changes in ouabain-sensitive sodium fluxes as external sodium was varied; to avoid the assumption of any given relationship the average points were linked by straight lines. However, for sodium influx at least, it would seem that the points could be fitted in a sigmoidal curve. From 90 mM Na up, there were no statistical differences between influx and efflux, but below that concentration all efflux values were above the influx ones. The similarity between the ouabain-sensitive fluxes at high external sodium is in agreement with the one-to-one Na/Na exchange proposed in these cells in K-free sodium Ringer's (Beaugé & Ortiz, 1971*a*). The discrepancy at low external sodium could have more than one explanation: on one hand it could be a consequence of the sodium pump stimulation by the potassium leakage

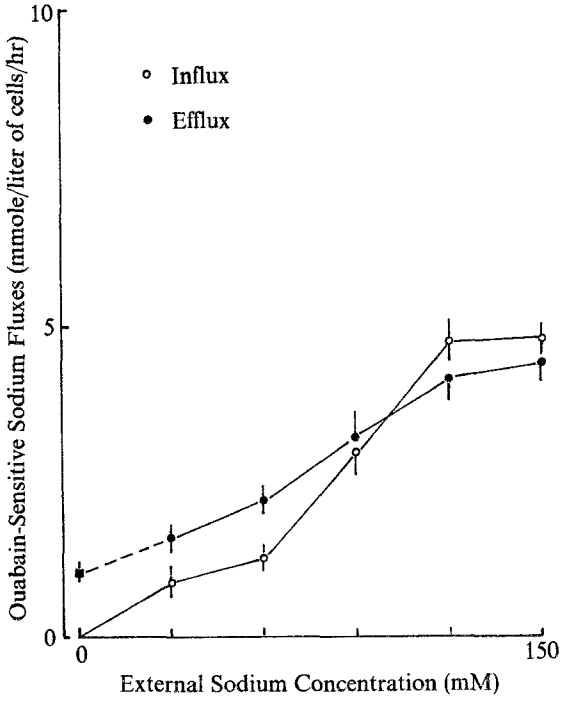

Fig. 2. Ouabain-sensitive initial sodium influx (open circles) and efflux (filled circles) as a function of external sodium concentration in a K-free sodium-magnesium Ringer's. The points are the average of three experiments ± SEM. The filled square is the estimated efflux on the basis of the rate constant from Table 1. Temperature was 37 °C

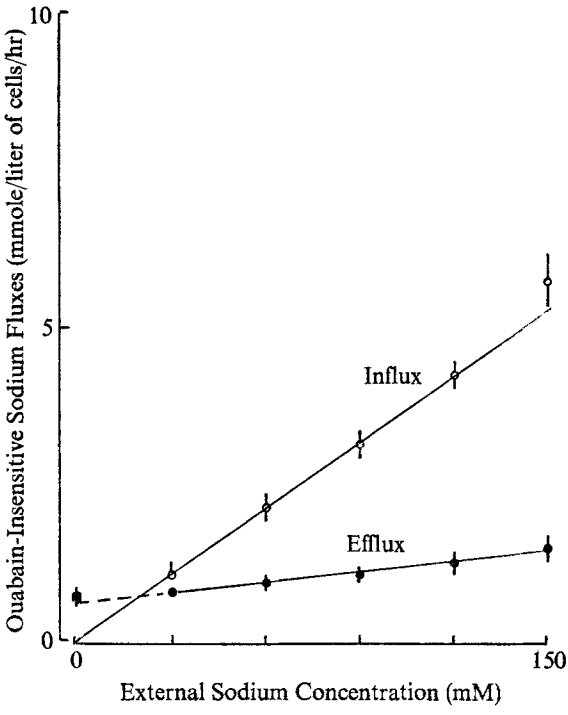

Fig. 3. Ouabain-insensitive initial sodium influx (open circles) and efflux (filled circles) in rat red cells as a function of external sodium concentration in a K-free sodium-magnesium Ringer's. The points are the average of three experiments ± SEM. The filled square is the estimated efflux on the basis of the rate constant from Table 1. Temperature was 37 °C

of the cells; this would be more probable in sodium-free conditions and more or less agrees with the estimations made in the previous section; however, this argument loses strength as external sodium is increased because of its inhibitory effect on the pump. On the other hand, it may be that this ouabain-sensitive Na/Na exchange is not one-to-one over the whole range of external sodium concentration, but shifts to a larger efflux/influx ratio as extracellular sodium is reduced. A ratio larger than one has been reported by Sachs (1971) for human red cells incubated in sodium Ringer's; this ratio was kept constant over a wide range of internal sodium concentrations. As this author has suggested, it may be possible that there is no fixed ratio, but that it varies in differen circumstances. Fig. 3 describes the ouabain-insensitive fluxes as influenced by external sodium concentration. Both influx and efflux followed a linear relationship; the slope of the former was larger and extrapolated to zero, whereas the

slope of the latter extrapolated to a value which is in agreement with the estimated efflux on the basis of the rate constant in Table 1.

Electrical Membrane Potential in Rat Red Cells from the Chloride Distribution Ratio

The results in Fig. 3 suggest that at about 25 mM external sodium rat red cells are able to maintain a steady internal sodium concentration. Since the cells were incubated at very low hematocrit in a nominally K-free medium with 10^{-4} M ouabain, the absence of the sodium-potassium pump can be assumed; however, in the case of an electrical membrane potential inside negative, this implies a steady internal sodium concentration against an electrochemical potential gradient, which is equivalent to active transport. To be certain of the concentration of external sodium required to keep the internal sodium constant, if passive fluxes are the only mechanism involved, the membrane potential in these cells must be known. So far, the best procedure for its determination seems to be the estimation of the chloride distribution ratio (Cotterell & Whittam, 1971). Table 2 shows these experiments. Normal sodium cells are equivalent to fresh cells; high sodium cells were preincubated for 4 hr in K-free sodium Ringer's at 37 °C. In fresh cells incubated in sodium media, the membrane potential was the same regardless of the presence or absence of external Rb at 5 mM concentration, with a value between −6.30 mV and −6.98 mV (inside negative); besides, when external sodium was replaced by magnesium the membrane hyperpolarized up to −12.64 mV ($P<0.001$). In high sodium cells incubated in sodium Ringer's the membrane potential was somehow lower than in fresh cells, though without statistical significance.

Table 2. Electrical membrane potentials (mV) in normal and high sodium rat red cells incubated in different media estimated on the basis of the chloride distribution ratio

	Normal Na_i cells			High Na_i cells
	K-free Na	5 mM-Rb Na	K-free Mg	K-free Na
Mean	−6.30*,**	−6.98+	−12.64*,+	−3.95**
SEM	1.18	1.31	1.04	0.92
n	(4)	(5)	(5)	(4)

Fresh cells or cells preincubated for 4 hr in K-free sodium Ringer's at 37 °C were washed three times in cold K-free sodium Ringer's and twice in the incubation solution. After being suspended in the same solution for 15 min at 37 °C and about 50% hematocrit, duplicate samples were taken for chloride determination in the suspension. *See text* for details.

*,+ $P<0.001$; ** $P>0.1$.

Net Sodium Fluxes as a Function of the External Sodium Concentration in K-free Sodium-Magnesium Ringer's

The external sodium concentration which would keep internal sodium invariant in fresh cells incubated in K-free sodium-magnesium mixture plus ouabain was obtained so far from unidirectional fluxes. Since sodium influx determinations involve some errors due to the correction factor applied for back diffusion, it was decided to look for the same information by analyzing the changes in intracellular sodium after 1 hr of incubation at different external sodium concentrations. Fig. 4 shows the average of two to five experiments of this type, both in the absence and in the presence of 10^{-4} M ouabain. The cell sodium remained unchanged at around 30 mM external sodium in both instances; from this concentration up there was a net sodium gain which increased linearly with external sodium and which was insensitive to ouabain. Below 30 mM sodium there was a net sodium loss, both with and without ouabain, but the loss was greater in the second case. Fig. 5 is the mean of two experiments where external sodium was varied in 10 mM from Na-free up to 50 mM-Na, also using magnesium to maintain osmolarity. In this case too, at about 30 mM-Na, the red cells kept a constant internal sodium; at a higher concentration there was a net

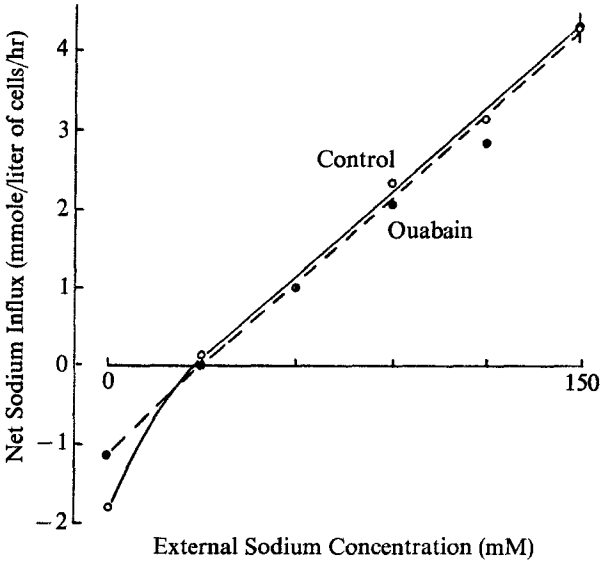

Fig. 4. Net sodium fluxes in rat red cells after a 1-hr incubation at different external sodium concentrations in sodium-magnesium Ringer's in the presence (filled circles) and absence (open circles) of 10^{-4} M ouabain. The points at 150 mM Na are the mean ±SEM of five experiments; all other points are the average of two experiments. The lines were drawn by eye. Temperature was 37 °C

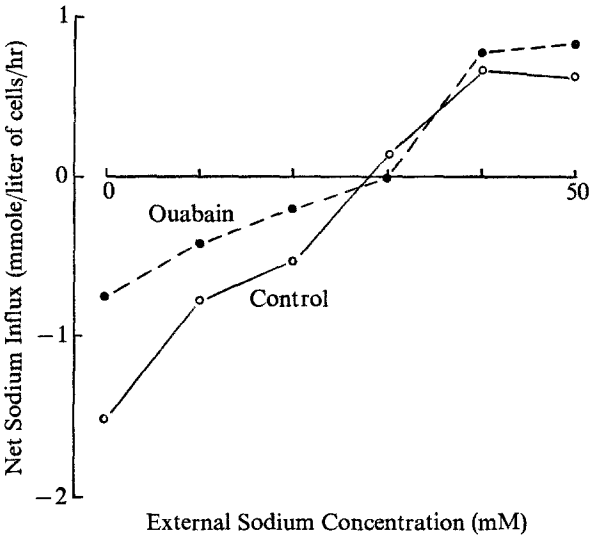

Fig. 5. Same experimental design as in Fig. 3 but in a shorter range of external sodium concentrations. The points are the average of two experiments

sodium gain, unaffected by ouabain as before; below 30 mM-Na there was a net sodium loss, already detected at 20 mM, which was appreciably reduced by ouabain. These results clearly show that when normal sodium cells are incubated in K-free sodium-magnesium mixtures, three main conclusions can be drawn: a) a steady internal sodium was maintained at about 30 mM external sodium. Considering the membrane potential values in Table 2, a potential between −6.30 mV (full sodium) and −12.64 mV (sodium-free) might have occurred, quite possibly closer to −12.64 mV. At 6.90 mM internal sodium (Beaugé & Ortiz, 1971*a*), the external sodium concentration needed to maintain equal electrochemical potentials for sodium at both sides of the membrane would be between 4.30 and 5.50 mM; this is one-sixth to one-seventh the concentration at which sodium influx and efflux are balanced. In other words, whereas the E_m values were between −6.3 and −12.6 mV (inside negative), at 30 mM external sodium the estimated E_{Na} was +3.9 mV (inside positive); b) above that "balance" sodium concentration there was a net sodium gain; it was increased linearly with external sodium and it was unaffected by ouabain; c) below that concentration there was a net sodium loss, both against and in favor of (in Na-free media) a gradient, which was reduced by about 50% by ouabain. The fact that even in the presence of the glycoside a net sodium loss was detected at higher external sodium than in the case of electrochemical equilibrium, indicates

that there is some mechanism which takes sodium out of the cells against its electrochemical gradient. The possibility of a cation effect on ouabain binding to explain the results in Figs. 4 and 5 seems unlikely. As it has been shown in these cells (Beaugé & Ortiz, 1970), external sodium favors ouabain inhibition as compared to Na-free media: any reduction on ouabain binding should be expected at low, not at high, external sodium. Thus, the results observed should be the consequence of a completely opposite effect.

Net Sodium Fluxes as a Function of External Sodium Concentration in K-free Sodium-Magnesium and Sodium-Choline Ringers' with 10^{-4} M *Ouabain*

As shown in the first section of this paper, sodium efflux in the absence of both sodium and potassium is a function of the substance used as a

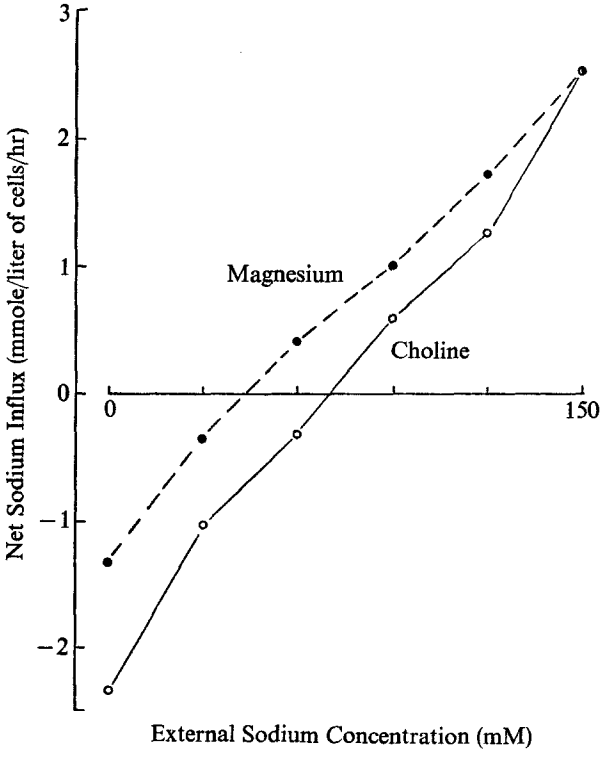

Fig. 6. The effect of the sodium substitute on the net sodium fluxes as a function of the external sodium concentration in the presence of 10^{-4} M ouabain. Filled circles refer to magnesium and open circles to choline. Each point is the average of two experiments. Temperature was 37 °C

sodium replacement; this applies to ouabain-sensitive as well as to ouabain-insensitive fluxes. In those experiments only sodium fluxes in favor of an electrochemical potential gradient were considered. In order to see if the same dependence could be found for movements against a gradient, similar experiments were performed comparing sodium-magnesium with sodium-choline mixtures, both in the presence of ouabain. These results are presented in Fig. 6. In sodium-magnesium, the external sodium concentration needed to maintain a balance of fluxes was somewhat higher than before, around 40 mM; however, in sodium-choline this concentration was even shifted to higher values, reaching 70 mM. Above the balance concentration, sodium gain was lower in choline than in magnesium, and below it the net loss was greater; in other words, choline in some way prevented sodium gain and stimulated sodium loss. That is to say, the ability of rat red cells to extrude sodium against a gradient in K-free ouabain solutions was also a function of the substance used as a partial sodium replacement.

Net Sodium Loss in K-free Magnesium Ringer's as a Function of the Internal Sodium Concentration

From the experiments in Figs. 4 through 6, it can be seen that sodium efflux in K-free-Na-free solutions has one portion which is ouabain-sensitive and another which is ouabain-insensitive. The use of ouabain as a flux partitioner presents difficulties, especially in rat red cells in Na-free media, because it is very hard to know whether the ouabain-sensitive part of a flux has been completely inhibited by the glycoside under a given experimental condition (Beaugé & Ortiz, 1970; *see also* Discussion). Another manner in which fluxes can be characterized is the way they are influenced by changes in the internal or external ionic composition; a similar behavior in both cases would not add any new information. But if the fluxes change, the assumption that they are different processes could receive some support. The experiments performed to test this point were based on the influence of changes in internal sodium concentration. The results were plotted as the ratio of the net sodium change (in its absolute value) over the initial internal sodium concentration as a function of that initial sodium concentration. This ratio has the dimension of time^{-1} and, though it is really a crude approximation of the rate constant for net Na efflux, the datum has proved to be accurate enough for the purposes of the present work (*see* Maizels, 1968); changes in cell volume were checked, and they did not

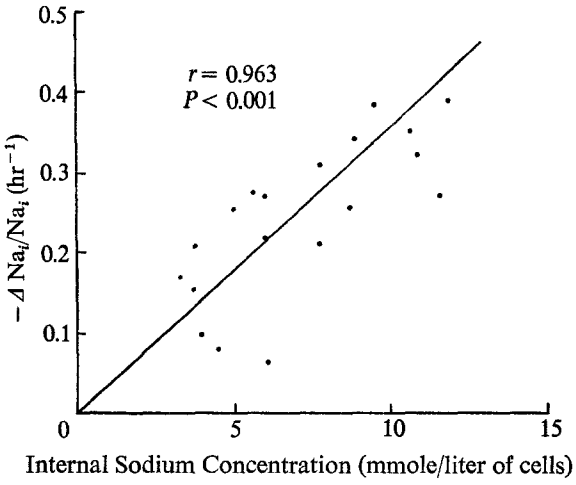

Fig. 7. Ouabain-sensitive fractional net sodium loss ($-\Delta Na_i/Na_i$) as a function of the internal sodium concentration in rat red cells after a 1-hr incubation in K-free magnesium Ringer's. The cells were preincubated for 1 to 4 hr in solutions of different compositions in order to vary the sodium content. The straight line was drawn by the least-squares method. Temperature was 37 °C

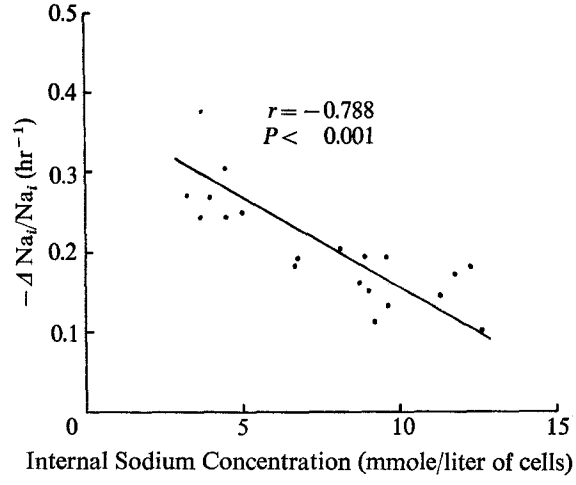

Fig. 8. Ouabain-insensitive fractional net sodium loss ($-\Delta Na_i/Na_i$) as a function of the internal sodium concentration in rat red cells after a 1-hr incubation in K-free magnesium Ringer's. The general procedure was the same as that described for Fig. 7

exceed 1%. Among the possible relationships to be found, and their meaning, are the following: a) a ratio increasing linearly with internal sodium, which indicates the flux increases proportional to the square of

the internal sodium concentration; b) a constant ratio, meaning a linear increase in flux with sodium; c) a ratio with a negative correlation, equivalent to a flux which goes to saturation; d) their combination. Ouabain-sensitive losses are summarized in Fig. 7 and ouabain-insensitive ones in Fig. 8. Clearly, the behavior of both fluxes is opposite over the range of internal sodium concentrations investigated; thus, whereas the ouabain-sensitive ratio increased with internal sodium, the ouabain-insensitive ratio had a negative correlation. It has been shown in human red cells (Maizels, 1968) that the ouabain-sensitive fractional sodium efflux in different Na-free media (K and Rb) first increased and then decreased as internal sodium was elevated; this reflects a type of sigmoidal relationship between pumped sodium and internal Na concentration. Furthermore, in similar experiments in those cells which considered the real rate constant for labeled sodium efflux in magnesium-sucrose Ringer's (Sachs, 1970), the same results were obtained; besides, and in agreement with the experiments in Fig. 8, the ouabain-insensitive rate constant decreased. In view of these results, it is likely that if internal sodium were increased further in Fig. 7, the sign of the slope would change, showing similar characteristics in rat and in human cells. This aspect will be treated in detail in the Discussion.

Sodium Fluxes in K-free Sodium Ringer's as a Function of the Internal Sodium Concentration

In several experiments in the present paper, as well as in a previous paper (Beaugé & Ortiz, 1971*a*), rat red cells have been incubated up to 4 to 5 hr in K-free sodium Ringer's in order to increase their internal sodium. However, there are no experiments performed to see how changes in internal sodium, by themselves or as a consequence of the incubation time, influence sodium fluxes. In Fig. 2 of the above-mentioned paper, the rate of sodium gain seemed to decrease as time progressed up to 1 hr, although no definite conclusions could be drawn. To check this point, fresh cells as well as cells preincubated for 4 hr in different Na-K solutions (*see* the above-mentioned paper) were incubated in K-free sodium media and after an hour the net sodium changes were determined. These results were also plotted as the ratio of the net sodium gain (in mmole/liter of cell water/hr) over the external sodium concentration (mM) against the internal sodium content (mmole/liter of cells) and are illustrated in Fig. 9. Filled circles are controls and crosses refer to 10^{-4} M ouabain. A negative correlation existed between sodium gain and initial internal sodium concentration;

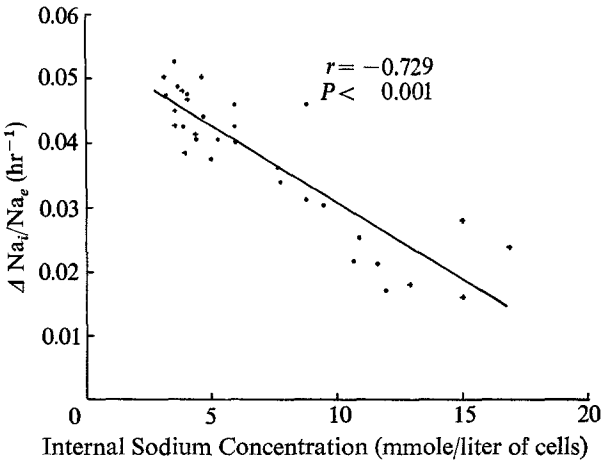

Fig. 9. Fractional net sodium gain ($\Delta Na_i/Na_e$) plotted as a function of the internal sodium concentration in rat red cells after a 1-hr incubation in K-free sodium Ringer's with (crosses) and without (filled circles) 10^{-4} M ouabain. Fresh cells as well as preincubated cells as described in Fig. 7 were used. The straight line was drawn by the least-squares method. Temperature was 37 °C

Table 3. Effect of increasing the internal sodium concentration on sodium influx, net sodium gain and rate constant for ^{22}Na efflux in rat red cells incubated in K-free sodium Ringer's with 10^{-4} M ouabain

	Na_i (mmole/liter of cells)	Na influx (mmole/liter of cells/hr)	Net Na gain	k^e_{Na} (hr^{-1})
Mean	3.76*	5.09+	4.19*	0.316**
SEM	0.11	0.15	0.20	0.012
n	(4)	(4)	(4)	(4)
Mean	14.95*	5.58+	2.09*	0.248**
SEM	0.80	0.45	0.27	0.015
n	(4)	(4)	(4)	(4)

Low sodium cells were preincubated for 5 hr at 37 °C in 5 mM K-sodium Ringer's at about 50% hematocrit with (efflux cells) or without (influx and net flux cells) ^{22}Na (10 μC/ml suspension). High sodium cells were first incubated for 3 hr at 6% hematocrit (in 50-ml centrifuge tubes) in K-free sodium Ringer's at 37 °C; the cells to be used in influx as well as net flux experiments were maintained in the same conditions for 2 more hours; those cells to be used in efflux experiments were transferred into 10-ml centrifuge tubes in the same conditions as the others with the addition of ^{22}Na. At the end of the preincubation period all cells were washed 5 times in the cold as follows: a) low sodium cells in K-free magnesium Ringer's; b) high sodium cells twice in K-free sodium and 3 times in K-free magnesium Ringer's. In all cases the solutions contained 10^{-4} M ouabain. The cells were then suspended at about 100 times the final hematocrit in K-free magnesium Ringer's and samples were taken for cold as well as for ^{22}Na assays at zero time. The incubation period lasted 1 hr at 37 °C and about 0.5% hematocrit. In all experiments low and high sodium cells were taken from the same rat.

* $P<0.001$; ** $P<0.02$; + $P>0.2$.

this was a consequence of the internal sodium and not of the incubation time. Moreover it was insensitive to ouabain.

A net flux is always the consequence of a difference between unidirectional fluxes. The observed reduction in the net sodium gain could be a consequence of two opposite effects (isolated or in combination): a) a reduction in sodium uptake, and b) an increase in sodium efflux. To decide between these possibilities, simultaneous determinations of ^{22}Na influx and efflux and net sodium gain were performed in cells preincubated for 4 hr in 5 mM K or K-free sodium Ringer's. The incubation took place in K-free sodium for 1 hr with the addition of 10^{-4} M ouabain. The results are presented in Table 3. A fourfold increase in internal sodium produced the following effects: a) a slight and nonsignificant ($P>0.2$) increase in sodium influx; b) a slight but statistically significant ($P<0.02$) reduction in the rate constant for ^{22}Na efflux; c) despite the former reduction in the rate constant when the latter was multiplied by the internal sodium concentration, there was a marked increase in the sodium efflux ($P<0.001$); d) a twofold reduction in the net sodium gain ($P<0.001$). As a consequence, the ratio sodium influx/net sodium gain increased from 1.21 in low sodium cells to 2.67 in high sodium cells. The implications of these events are extremely important for the mechanism of sodium movements through rat red cell membrane and will be extensively treated in the Discussion.

Discussion

Considering the experiments on sodium fluxes into K-free sodium Ringer's in the present paper and in a previous paper (Beaugé & Ortiz, 1971*a*), more than half of sodium efflux is ouabain-sensitive and exchanges with external sodium, quite possibly through the sodium pump. In Fig. 2, the exchange seems to be one-to-one at high external sodium; at low sodium, efflux is larger than influx, and under these conditions a ouabain-sensitive net sodium loss would occur. In human red cells incubated in Na-K-free conditions there is evidence that sodium efflux is mediated through the mechanism which is responsible for the Na-K exchange (Sachs, 1970), although not all efflux is stimulated by external potassium; the same interpretation could be applied to rat cells and extended to sodium solutions up to 90 mM. In cells treated with ouabain a net sodium extrusion against an electrochemical gradient was found; this agrees with reports in human red cells (Sachs, 1971) and frog skeletal muscle (Sjodin & Beaugé, 1973). In rat cells the net sodium loss was greater in choline than in magnesium,

whereas in human cells it was the same; however, the lack of response in human cells to potassium Ringer's indicates that in this preparation too, there is an effect of the external media on the flux. The results in human red cells and muscle have been interpreted as an indication that active transport must be performed to achieve these fluxes. The finding that the operation of the system is also a function of the sodium replacement could suggest the possibility that another mechanism might provide the free energy necessary for this work; this would be the free energy derived from the magnesium and choline gradients going into the cells, which in fact are much greater than the opposing sodium gradient going out, resulting in a counter transport process. Evidences are given below for a "carrier mediated" net sodium movement independent of the sodium pump; since such systems are potentially capable of producing counter transport, the proposed mechanism is theoretically possible. Obviously, net influxes of magnesium and choline are required. Unpublished and preliminary data indicate that choline influx is about 5.0 mmoles/liter of cells/hr in choline Ringer's; this value is high enough to account for a sodium-choline exchange. However, it must be stressed that to prove this hypothesis it is necessary to gather experimental evidence showing that the net sodium efflux disappears when the choline or magnesium gradient is in balance with the sodium gradient.

The experiments in Figs. 7 and 8 show that ouabain-sensitive and insensitive sodium effluxes in K-free Mg Ringer's are affected differently by internal sodium concentrations over the range investigated. For the ouabain-sensitive flux in human red cells (Sachs, 1971) it has been proposed that two or three ions must interact with the system before translocation develops; this interpretation could also fit with the present data, provided that the fractional sodium efflux should be reduced if the internal sodium were further increased. The ouabain-resistant flux, as suggested also by the same author, could follow a Michaelian kinetic. Accordingly, it is possible that in the presence of ouabain the properties of the system are altered, and this modified system interacts in a different way with internal sodium. In connection with this hypothesis, there is experimental evidence that the binding of ouabain changes the reactivity of the pump sites to external potassium (Baker & Connelly, 1966; Beaugé & Ortiz, 1970); also, it modifies the ATPase preparation, where it not only changes the affinity for the activating cations but makes possible the phosphorylation on the enzyme by inorganic phosphate, in contrast with the native enzyme which requires the presence of ATP (Albers, Koval & Siegel, 1968; Sen, Tobin & Post,

1969). In squid axons, at normal internal ATP, strophanthidin inhibited sodium outflux (Brinley & Mullins, 1968), whereas when ATP was reduced to the micromolar range the same doses of glycoside increased the efflux of sodium. It could be conceived then that the removal of sodium, by itself or depending on the substitute, and the presence of ouabain (or other substances), might change the properties of the translocation system, producing the appearance of fluxes which are not present under normal or physiological conditions. The implications of this notion would be fundamental to the understanding of transport processes because different mechanisms proposed for sodium transport in skeletal muscle (Beaugé & Sjodin, 1968; Keynes & Steinhardt, 1968), squid axons (Brinley & Mullins, 1968; Baker *et al.*, 1969) and red cells (Hoffman & Kregenow, 1966; Garrahan & Glynn, 1967) are merely operational definitions, which evidently would depend on the experimental conditions in each case.

Another experimental evidence worth discussing, indicating that a simple pump leak cannot account for net sodium movements in rat red cells, comes from the fact that net as well as unidirectional fluxes are a function of internal sodium concentration in K-free sodium solutions in the presence of 10^{-4} M ouabain. In low sodium cells the sodium influx/net sodium gain ratio predicted from electrodiffusion would be 1.025, whereas the observed ratio was 1.21; in high sodium cells, the ratio which is in accordance with electrochemistry is 1.16 and the observed one was 2.67. In other words, as internal sodium increased the difference between the expected and observed ratios also increased, with the consequent reduction in the net sodium gain; this discrepancy is unlikely to be produced by any activation of the sodium pump by internal sodium because the experiments were performed in media containing ouabain. While the sodium pump is inhibited, these results agree with one of the most important criteria for the identification of facilitated diffusion systems (Stein, 1967). Among other criteria for such a mechanism, which were also found in the present experiments, are the reduction on the rate constant for sodium efflux as internal sodium is increased (indicating that Fick's first law is not obeyed) and the stimulation of sodium efflux by external sodium. Thus, in rat red cells, and maybe in many others, the net gain of sodium does not occur by simple leakage but there is a facilitated diffusion mechanism which, together with the former, moves sodium in favor of its electrochemical gradient. It must be stressed that this facilitated diffusion is not an exchange diffusion as proposed by Levi and Ussing (1948) and postulated by Lubowitz and Whittam (1969) to explain part of the ouabain-insensitive sodium fluxes in human red cells; in the latter mechanism the exchange is one-to-one without

net movement of the substrate, whereas net sodium fluxes have been clearly demonstrated in the present experiments.

This work was supported by Grants No. 3667a/70 and 3118b/71 from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina to L. A. Beaugé.

References

Albers, R. W., Koval, G. J., Siegel, S. 1968. Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. *Mol. Pharmacol.* **4**:324

Baker, P. F., Blaustein, M. P., Keynes, R. D., Manil, J., Shaw, T. I., Steinhardt, R. 1969. The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* **200**:459

Baker, P. F., Connelly, C. M. 1966. Some properties of the external activation site of the sodium pump in crab nerve. *J. Physiol.* **185**:270

Beaugé, L. A., Ortiz, O. 1970. Rubidium, sodium and ouabain interactions on the influx of rubidium in rat red blood cells. *J. Physiol.* **210**:519

Beaugé, L. A., Ortiz, O. 1971*a*. Sodium and rubidium fluxes in rat red blood cells. *J. Physiol.* **218**:533

Beaugé, L. A., Ortiz, O. 1971*b*. Sodium fluxes in rat red blood cells. *Proc. XXV Int. Congr. Physiol. Sci.*, p. 126

Beaugé, L. A., Ortiz, O. 1973. Net sodium fluxes in rat red cells by a facilitated diffusion system. Biophysical Society Meeting

Beaugé, L. A., Sjodin, R. A. 1968. The dual effect of lithium ions on sodium efflux in skeletal muscle. *J. Gen. Physiol.* **52**:408

Brinley, F. J., Mullins, L. J. 1968. Sodium fluxes in internally dyalized squid axons. *J. Gen. Physiol.* **52**:181

Cotterell, D., Whittam, R. 1971. The influence of the chloride gradient across red cell membranes on sodium and potassium movements. *J. Physiol.* **214**:509

Garrahan, P. J., Glynn, I. M. 1967. The behaviour of the sodium pump in red cell in the absence of external potassium. *J. Physiol.* **192**:159

Hoffman, J. F., Kregenow, F. M. 1966. The characterization of new energy dependent cation transport processes in red blood cells. *Ann. N.Y. Acad. Sci.* **137**:566

Keynes, R. D., Steinhardt, R. A. 1968. The components of the sodium efflux in frog muscle. *J. Physiol.* **198**:581

Levi, H., Ussing, H. H. 1948. The exchange of sodium and chloride ions across the fiber membrane of the isolated frog sartorius. *Acta Physiol. Scand.* **16**:232

Lubowitz, H., Whittam, R. 1969. Ion movements in human red cells independent of the sodium pump. *J. Physiol.* **202**:111

Maizels, M. 1968. Effect of sodium content on sodium efflux from human red cells suspended in sodium-free media containing potassium, rubidium, caesium or lithium chloride. *J. Physiol.* **195**:657

Sachs, J. R. 1970. Sodium movements in the human red blood cell. *J. Gen. Physiol.* **56**:322

Sachs, J. R. 1971. Ouabain-insensitive sodium movements in the human red blood cell. *J. Gen. Physiol.* **57**:259

Sachs, J. R., Conrad, M. 1968. Effect of tetraethylammonium on the active cation transport system of the red blood cell. *Amer. J. Physiol.* **215**:795

Schales, O., Schales, S. 1941. A simple and accurate method for the determination of chloride in biological fluids. *J. Biol. Chem.* **140**:879

Sen, A. K., Tobin, T., Post, R. L. 1969. A cycle for ouabain inhibition of sodium- and potassium-dependent adenosine triphosphatase. *J. Biol. Chem.* **244**:6596

Sjodin, R. A., Beaugé, L. A. 1973. An analysis of the leakages of sodium ions into and potassium ions out of striated muscle cells. *J. Gen. Physiol.* **61**:222

Stein, W. D. 1967. The Movement of Molecules Across Cell Membranes. Academic Press Inc., New York