

The Kinetics of Ouabain Inhibition and the Partition of Rubidium Influx in Human Red Blood Cells

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ABSTRACT In the development of ouabain inhibition of rubidium influx in human red blood cells a time lag can be detected which is a function of at least three variables: the concentrations of external sodium, rubidium, and ouabain. The inhibition is antagonized by rubidium and favored by sodium. Similar considerations could be applied to the binding of ouabain to membrane sites. The total influx of rubidium as a function of external rubidium concentration can be separated into two components: (*a*) a linear uptake not affected by external sodium or ouabain and not requiring an energy supply, and (*b*) a saturable component. The latter component, on the basis of the different effects of the aforementioned factors, can be divided into three fractions. The first is ouabain-sensitive, inhibited by external sodium at low rubidium, and requires an energy supply; this represents about 70–80% of the total uptake and is related to the active sodium extrusion mechanism. The second is ouabain-insensitive, activated by external sodium over the entire range of rubidium concentrations studied, and dependent on internal ATP; this represents about 15% of the total influx; it could be coupled to an active sodium extrusion or belong to a rubidium-potassium exchange. The third, which can be called residual influx, is ouabain-insensitive, unaffected by external sodium, and independent of internal ATP; this represents about 10–20% of the total influx.

INTRODUCTION

The total influx of potassium in human red cells can be separated into two components: one, linear with the external potassium concentration, and assumed to be leak; the other, representing about 99% of the total uptake, which saturates as external potassium rises, and is largely associated with active sodium transport (Glynn, 1957). About 80–90% of the saturable component can be blocked with cardiac glycosides such as ouabain. Solomon et al. (1956) pointed out the possibility of some interaction between the inhibitors and ex-

ternal potassium, and Glynn (1957) gave evidence indicating that this interaction was of a competitive type. On the other hand, Hoffman (1966) suggested an allosteric type of inhibition. In studies on membrane ATPase (Dunham and Glynn, 1961) and potassium influx (Garrahan and Glynn, 1967*b*) in human red cells it was shown that external sodium has an inhibitory action on both systems. Schatzman (1965) suggested that the inhibition by ouabain could be brought about through an enhancement of the sodium effect. Synergy between external sodium and ouabain has been reported on sodium efflux in squid giant axons (Baker and Manil, 1968), where sodium facilitates the kinetics of inhibition. In the experiments described below, the influence of external sodium on rubidium uptake and on its inhibition by ouabain has been studied. A brief report of this work has been made (Beaugé and Adragna, 1969).

METHODS

Rubidium has been used in the present work since it has been shown that it can replace potassium as external activator of the sodium pump (Beaugé and Sjodin, 1968; Maisels, 1968).

Rubidium Influx The technique for influx has been described elsewhere (Beaugé and Ortíz, 1970). Blood samples were taken from healthy donors at the University Hospital, collected in 10% (v/v) isotonic sodium citrate, and stored in a refrigerator for about 5 hr before use. After centrifugation at 1500 *g* for 10 min the plasma and buffy coat were removed by aspiration. The cells were washed three times with cold K-free sodium Ringer and twice with the solution to be used in the uptake experiments in which all the constituents but rubidium and ouabain were present. These media were also used to suspend the cells at about 10 times the final hematocrit. The suspension was then added to the incubation medium in the cold and transferred to a bath at 37°C. At the end of the uptake period it was returned to the ice-cold bath for 5 min, washed three times with ice-cold K-free sodium Ringer, and lysed in 10 ml of deionized water. The volume of the cells was obtained by comparing the hemoglobin content of the final hemolysate with a hemolysate of known hematocrit. Rubidium influx was calculated from the activity of the hemolysate and expressed in millimoles per liter of cells. In general four points 15 min apart were taken; in those cases in which the uptake was shown to be linear with time only one point at 30–60 min was obtained. Unless otherwise stated, all experiments were run in duplicate.

Counting ⁸⁶Rubidium activity was counted in a Beckman liquid scintillation counter without scintillator liquid; counting was continued long enough to obtain a standard error of 1–3%.

Analysis for Sodium and Potassium Sodium and potassium were determined by flame photometry in a DU Beckman spectrophotometer checking against a standard of appropriate concentration in order to obtain readings differing by no more than 20%.

Solutions All solutions were made from analytical grade reagents and de-ionized water. Choline chloride was purified as described elsewhere (Beaugé and Ortíz, 1970). Glucose and ouabain were added just before use, the first as a solid and the second as an aqueous solution prepared the same day. The final glucose concentration was 200 mg/100 ml. The standard K-free sodium Ringer had the following composition (mM): NaCl, 150; CaCl₂, 1; MgCl₂, 1; orthophosphoric acid (titrated with Tris to pH 7.4 [37°C]), 2. When rubidium was present sodium concentration was equal to 150 minus the external rubidium concentration (mM). In the choline Ringer the composition was similar except that sodium was replaced by equal amounts of choline. Different concentrations of sodium were obtained by mixing appropriate quantities of sodium and choline Ringer. Obviously all solutions were potassium-free.

⁸⁶Rubidium was obtained through the Comisión Nacional de Energía Atómica of Argentina as RbCl sterile aqueous solution; it was desiccated, redissolved, and adjusted to pH around 7. Ouabain was supplied by Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Kinetics of Ouabain Inhibition A dose-response curve for ouabain inhibition of rubidium influx is shown in Fig. 1. At low concentrations, the glycoside seems to be more powerful in a sodium medium than in choline, but at high concentrations the situation is reversed. In order to obtain this curve only one point was taken 1 hr after the beginning of the incubation, and there was no preincubation with the glycoside. Two explanations could account for these results: either sodium really diminishes the extent of inhibition or, at low concentrations of ouabain, the absence of sodium reduces the rate of development of inhibition, thus producing an artifact in the curve. In order to test this second possibility the uptake of rubidium as a function of time was measured at 15 min intervals for 1 hr, both in the presence and the absence of sodium and ouabain. Figs. 2 and 3 show two typical experiments. In 10⁻⁴ M ouabain all uptakes were linear with time up to 1 hr. The removal of sodium increased the unpoisoned fluxes but reduced the poisoned ones as reflected by the dose-response curve; therefore, at this concentration, there is no effect of sodium on the rate of development of inhibition, but there is a reduction of the ouabain-resistant uptake. On the other hand, in 10⁻⁶ M ouabain there was a noticeable delay in the inhibition rate when sodium was removed, whereas, with sodium in the bathing solution, it developed instantaneously within the resolution of the method. The final slope of the poisoned flux in choline, though not easy to evaluate, seems to be smaller than in sodium.

When the above results are considered, it should be possible to find ouabain concentrations which inhibit slowly even in the presence of sodium, and one would expect that choline would dramatically slow down the inhibition under these conditions. In fact this was observed starting with 5×10^{-7} M ouabain.

Numerical Estimate of the Inhibition Rate In order to obtain a numerical estimate of the degree to which the removal of sodium affects the rate at which inhibition takes place, other experiments were done in 10^{-7} and 10^{-8} M ouabain. The experimental points were then fitted by an empirical equation of the type

$$Y = y(1 - e^{-kt}) + at$$

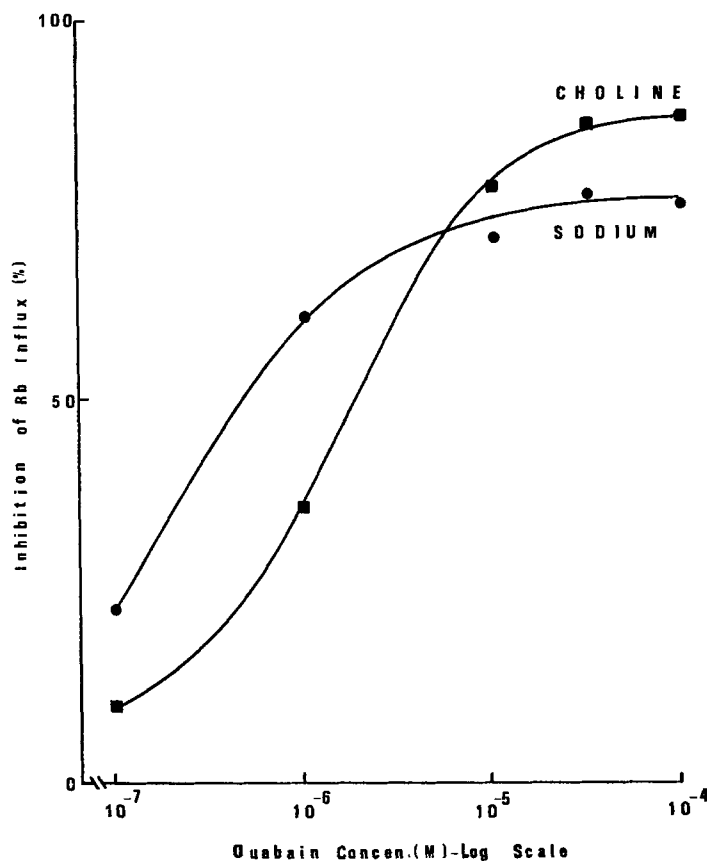


FIGURE 1. Dose-response curve for ouabain inhibition of rubidium influx in human red cells. The points were taken after 1 hr uptake and there was no preincubation with the glycoside. The lines were drawn by eye. External rubidium was 1 mM.

The rationale for this is as follows. In the absence of ouabain the uptake of rubidium as a function of time is linear from the beginning. It is also linear in the presence of a high concentration of glycoside, and this is taken as an indication that inhibition develops at once. In this case the slope of the curve of uptake of rubidium vs. time gives the value of the ouabain-resistant uptake. When there is a delay in the development of inhibition, the uptake as a func-

tion of time can be separated into two components: one is linear and its slope, as in the case of instantaneous inhibition, can be taken as the ouabain-resistant uptake; the other saturates and represents the ouabain-sensitive fraction. These curves can be fitted by equations of the type shown above, where

Y = total uptake in millimoles/liter cells

y = ouabain-sensitive uptake in millimoles/liter cells

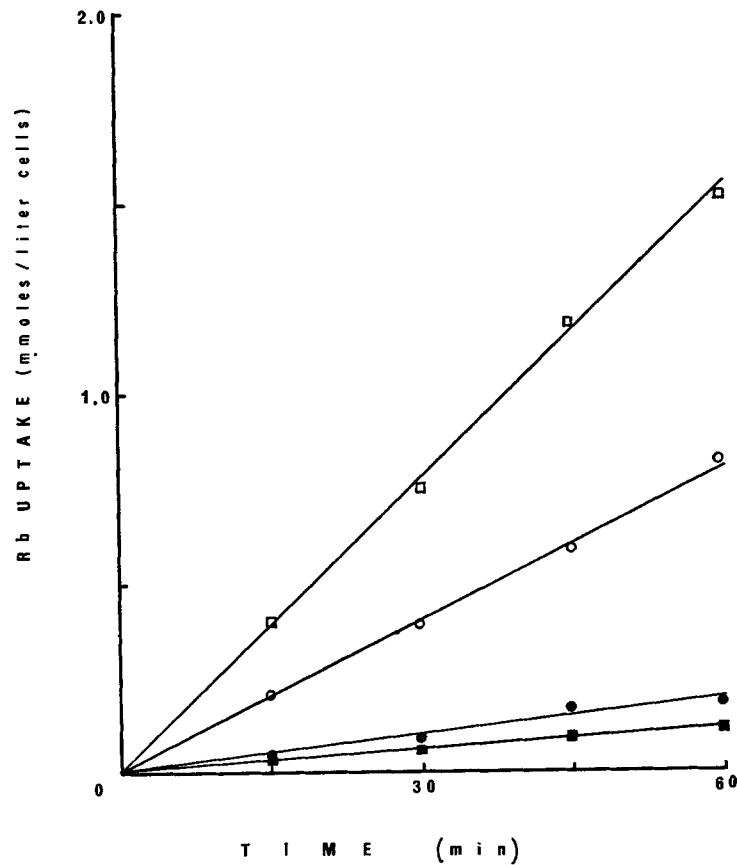


FIGURE 2. The effect of external sodium and ouabain on the influx of rubidium as a function of time in human red cells. Open circles, sodium media; solid circles, sodium media with 10^{-4} M ouabain; open squares, choline media; solid squares, choline media with 10^{-4} M ouabain. External rubidium was 1 mM.

t = time

a = coefficient in millimoles/liter cells \times hour

k = rate constant in time^{-1}

The linear component was determined experimentally for each concentration of ouabain. This was done either by waiting long enough for the uptake to become linear, or by running parallel experiments with and without pre-

incubation of the cells in ouabain. The linear fraction thus obtained was subtracted from the total uptake, thus giving the saturable component. If the latter behaves as an exponential function the semilogarithmic plot of the poisonable uptake which has not been inhibited as a function of time should

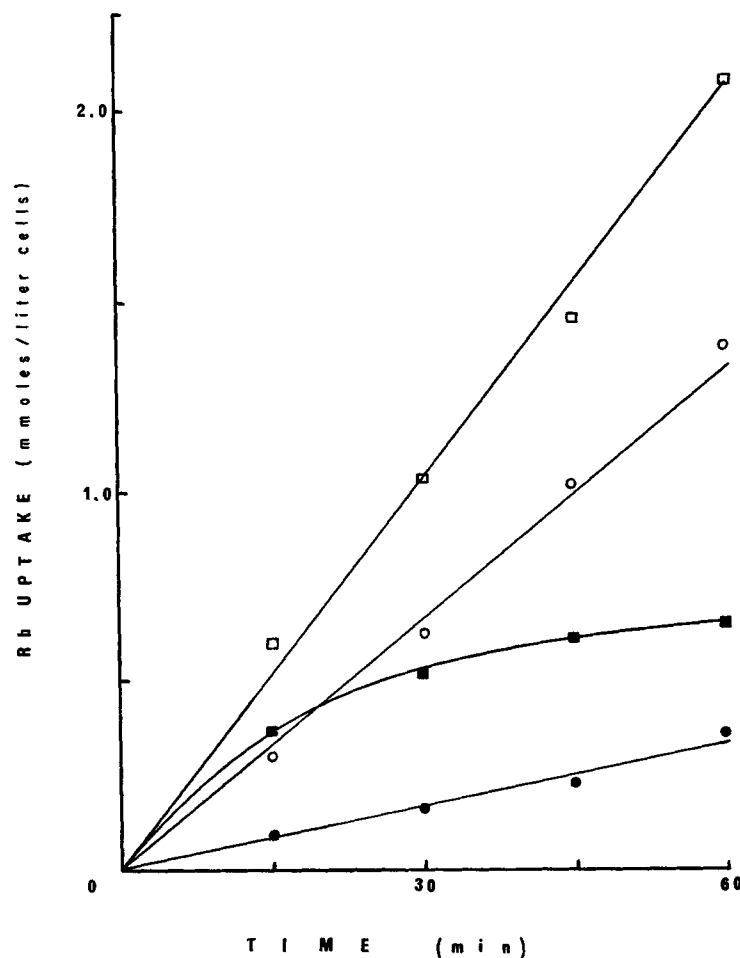


FIGURE 3. The effect of external sodium and ouabain on the influx of rubidium as a function of time in human red cells. Open circles, sodium media; solid circles, sodium media with 10^{-6} M ouabain; open squares, choline media; solid squares, choline media with 10^{-6} M ouabain. External rubidium was 1 mM.

give a straight line; the slope would be k and would serve as a numerical expression for the time taken by the glycoside to exert full inhibition. Fig. 4 shows a semilogarithmic plot of the percentage of the poisonable uptake which has not been inhibited as a function of time in 10^{-7} M ouabain. The time lag for inhibition appears both with and without sodium in the medium, but is

considerably larger in the second case. From the figure the values for k which were calculated are 0.0130 min^{-1} and 0.0835 min^{-1} for choline and sodium, respectively. Thus, the removal of sodium in 10^{-7} M ouabain and 1 mM rubidium increased the time lag for inhibition by 6.5-fold.

There is evidence in human (Glynn, 1957) as well as in rat red cells (Beaugé and Ortíz, 1970) that potassium and rubidium antagonize the effect

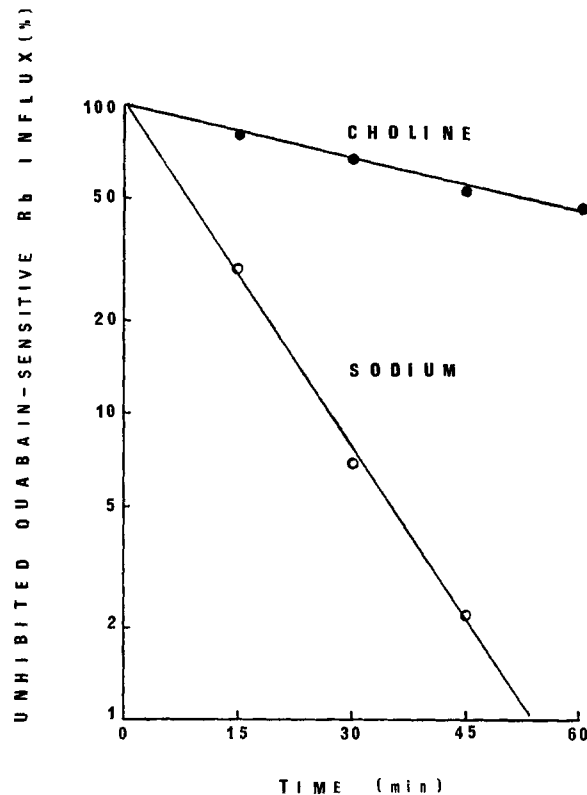


FIGURE 4. Rate of inhibition of the ouabain-sensitive fraction of rubidium influx in human red cells incubated in 1 mM Rb (sodium or choline) Ringer and 10^{-7} M ouabain. The straight lines were drawn by eye according to the equation $Y = y(1 - e^{-kt})$. For details see text.

of cardiac glycosides; in rat cells, this antagonism becomes more apparent with the removal of sodium. Therefore, the delay witnessed above may be due to an increase in the rubidium-ouabain antagonism rather than to the removal of sodium, since the latter is a powerful competitor with rubidium for the external pump sites. In order to test this possibility other experiments were performed at different ouabain and rubidium concentrations, in the presence and absence of external sodium. The rate constants for inhibition were calculated from the equation shown above.

Table I summarizes the results of four experiments. As external rubidium rose, the rate of inhibition was reduced with and without external sodium. For each rubidium concentration the time lag was longer in sodium-free than in sodium medium, and this was more evident at low concentrations of rubidium and ouabain.

Kinetics of Ouabain Inhibition at Different External Sodium Concentrations Fig. 5 describes the sodium dependence of the rate of ouabain inhibition in constant concentrations of rubidium and ouabain. The effect of the removal of sodium began to appear below 90 mM but became most conspicuous between 30 mM and sodium-free media. The experiment was carried out at one concentration of rubidium and ouabain and it is quite possible that the picture would change with varying concentrations.

TABLE I
EFFECT OF EXTERNAL SODIUM, RUBIDIUM,
AND OUABAIN CONCENTRATIONS ON THE RATE
OF INHIBITION OF RUBIDIUM
INFLUX IN HUMAN RED CELLS

Ouabain concentration	Rate constants for inhibition, min^{-1}					
	Sodium			Choline		
	0.2 Rb	1.0 Rb	2.0 Rb	0.2 Rb	1.0 Rb	2.0 Rb
<i>M</i>						
10^{-6}	—	∞	—	—	0.080	—
10^{-7}	0.924	0.085	0.096	0.028	0.013	0.007
10^{-8}	—	0.016	—	—	0.001*	—

* This value is only approximate because of the large error in calculation.

Rubidium Influx As a Function of External Rubidium Concentration Fig. 6 illustrates the activation curve of rubidium influx by external rubidium. In drawing this figure the linear component of the uptake, which was obtained as determined by Glynn (1957), was subtracted from each point. This component was found to be equal to $0.024 (\text{Rb})_e$; i.e., about 2.5% of the total rubidium influx could be considered as simple diffusion. The activation curve was sigmoid with sodium, whereas it had a hyperbolic shape with choline; this has already been described for potassium influx in the same cells (Sachs and Welt, 1967). The maximal influx was 2.27 mM/liter cells per hr in sodium and 2.17 mM/liter cells per hr in choline and the values for K_m were 1.05 and 0.50 mM, respectively. It is interesting to observe that in the presence of 10^{-4} M ouabain the uptake was always lower in the absence of sodium; in other words, the ouabain-resistant influx, which can still be considered part of the saturable component, was lower in sodium-free than in sodium medium. This difference is significant at every point in the curve.

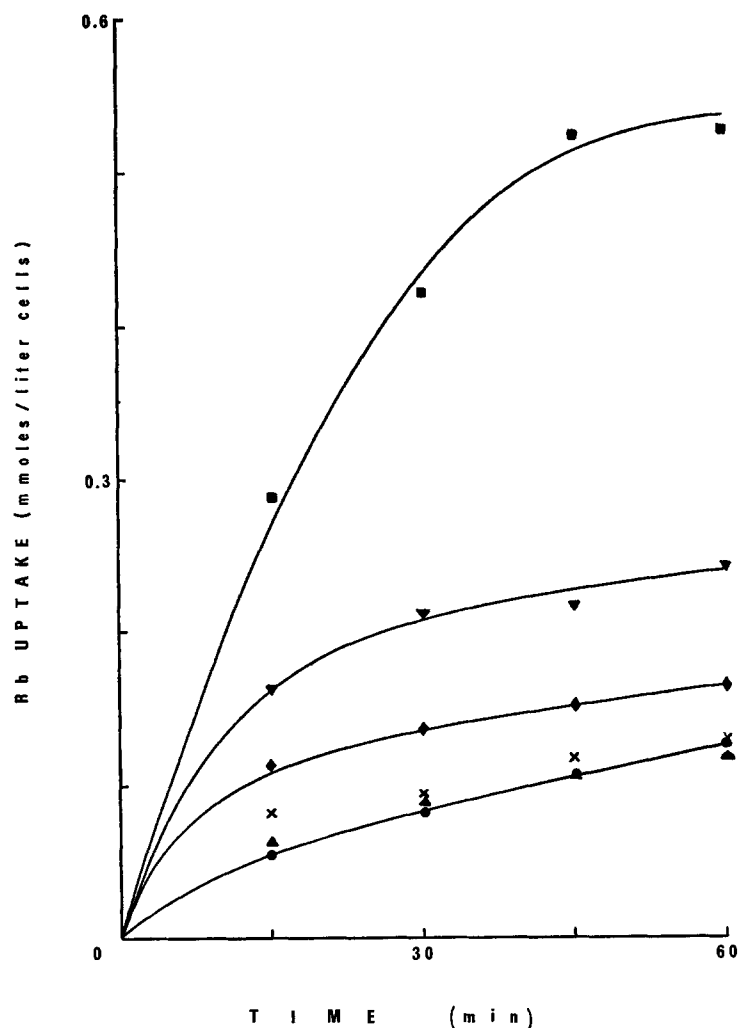


FIGURE 5. Sodium dependence of the rate of ouabain inhibition of rubidium influx in human red cells. The concentration of rubidium was 1 mM and ouabain 5×10^{-7} M. The external sodium was replaced by equal amounts of choline. Solid circles, 150 mM Na; solid triangles, 120 mM Na; X, 90 mM Na; solid diamonds, 60 mM Na; solid reversed triangles, 30 mM Na; solid squares, sodium-free.

Sodium Dependence of the Ouabain-Insensitive Rubidium Influx The experiments of the previous section show that, when sodium is absent, the uptake of rubidium in ouabain is reduced. The values obtained so far are with full sodium and sodium-free media. Experiments were performed to investigate the effect of partial substitution of choline for external sodium. In all cases the uptake was the same in 60 mM sodium and full sodium media. Fig. 7 summarizes the results of two experiments in which the effect of external

sodium was investigated between sodium-free and 60 mM sodium. The figure shows that the uptake increases as sodium rises in a more or less exponential fashion. The K_m of this effect obtained from the figure is about 16 mM.

Extent of Ouabain Inhibition in Sodium and Sodium-Free Solutions So far, the results can be accounted for if outside sodium is necessary for a ouabain-

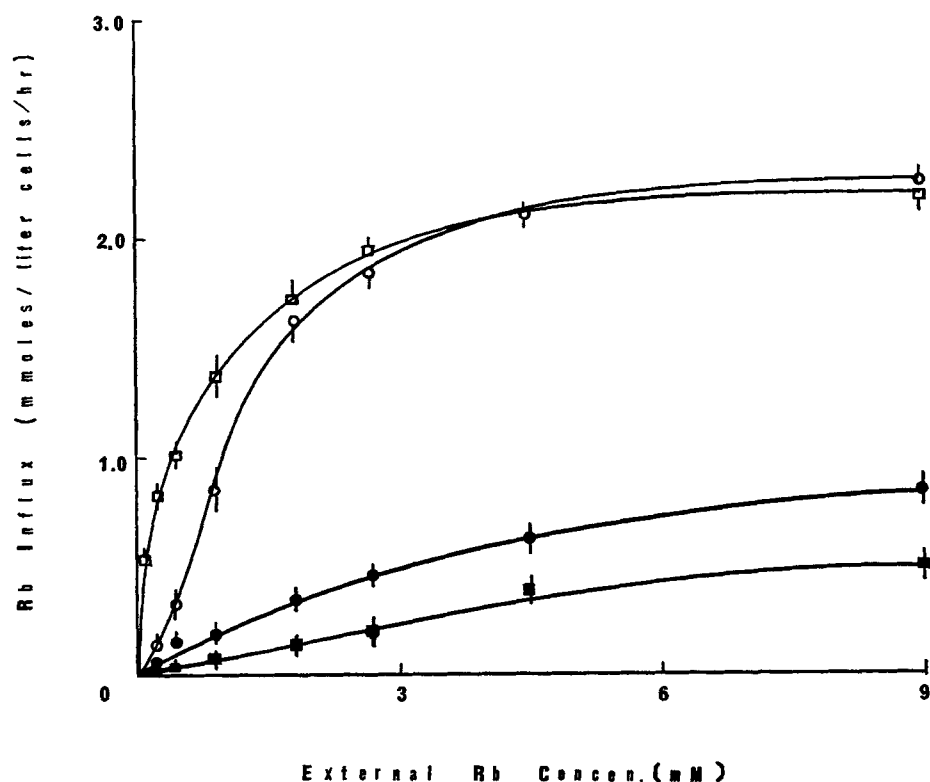


FIGURE 6. The effect of external sodium and 10^{-4} M ouabain on the activation curve of rubidium influx by external rubidium. Open circles, sodium media; solid circles, sodium media with ouabain; open squares, choline media; solid squares, choline media with ouabain. Sodium and choline concentrations were equal to 150 minus rubidium concentration (mM). Each point is the average of two to six experiments plus minus 1 SEM.

insensitive uptake. Nevertheless, it might be possible that sodium antagonizes the action of ouabain despite the fact that it favors the rate of inhibition. In order to investigate this possibility, rubidium uptake was studied in sodium and sodium-free solutions at concentrations of the glycoside at which the dose-response curve showed maximal inhibition. If sodium has some type of competitive effect, it could be detected as the concentration of the inhibitor is reduced. From Fig. 7 it seems that a difference might be detected between

sodium-free and 10 mM sodium solution. Based on these findings, three different experiments were performed comparing 10, 50, and 150 mM sodium with sodium-free media at four concentrations of ouabain (5×10^{-5} to 10^{-3} M). Fig. 8 shows the result with 10 mM sodium. As can be seen, a 20-fold increase in the concentration of ouabain does not influence the differential uptake between sodium and sodium-free media. Similar results were obtained with 50 and 150 mM sodium. This indicates that inhibition by ouabain is not affected in a competitive way by external sodium.

Table II illustrates the influence of two extreme concentrations of rubidium, sodium, and ouabain on the influx of rubidium. In 140 mM sodium there is

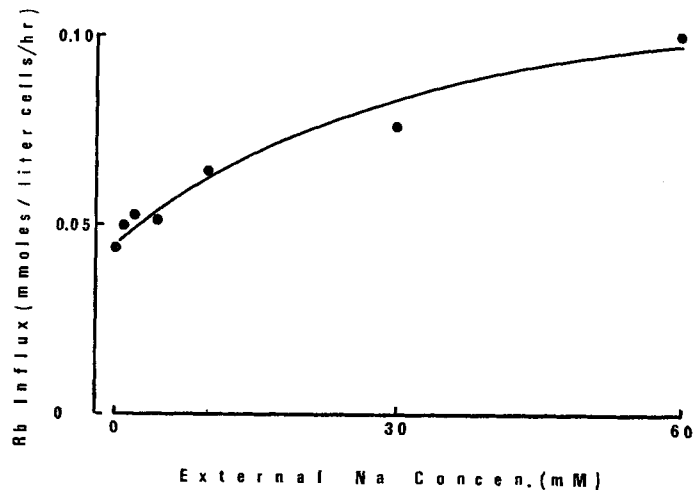


FIGURE 7. Sodium dependence of the ouabain-resistant rubidium influx in human red cells incubated in 1 mM Rb (sodium-choline mixture) Ringer with 10^{-4} M ouabain. Each point is the average of two experiments.

no difference in the inhibition produced by both concentrations of the glycoside either at 1 or 10 mM rubidium; in 0.1 mM sodium a statistically significant difference is found at 1 mM rubidium, whereas the uptake is the same at 10 mM rubidium. The sodium-dependent fraction is present in all cases. The latter observation can be interpreted as a consequence of the rubidium-ouabain antagonism.

Effect of ATP Depletion on the Ouabain-Insensitive Rubidium Influx So far, the experimental evidence shows that a fraction of the saturable component of rubidium influx, quite possibly carrier-mediated, is not affected by ouabain but depends on the external sodium. This entry of rubidium could be balanced by the exit of another monovalent cation, potassium or sodium. Alternatively, some type of ouabain-insensitive active transport could develop. Contrary to the case of the classical ouabain-sensitive sodium-potassium exchange, this

would not be revealed by net change experiments because the differences between poisoned cells in sodium and choline would obscure the results. However, if an energy requirement could be demonstrated, this mechanism, though not proved, would have some support.

This point was checked by incubating cells for 16 hr in 5 K-Ringer with and without iodoacetic acid (5 mM); after this period rubidium influx was determined as described above in sodium and sodium-free media, in the presence and absence of ouabain. These experiments are summarized in Table III. After incubation the control cells keep their sodium-dependent influx, whereas it disappears in those exposed to IAA.¹ Though no ATP analysis was performed, the comparison of influx in IAA cells with and without ouabain

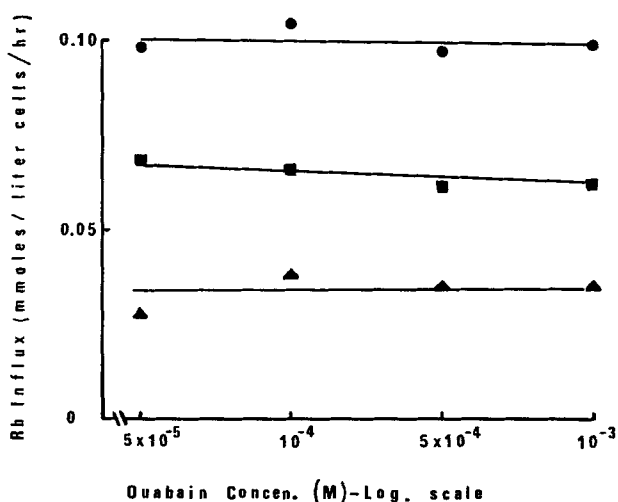


FIGURE 8. Rubidium influx in human red cells in the presence and absence of sodium at different ouabain concentrations. Solid circles, 10 Na-135 choline; solid squares, sodium-free; solid triangles, difference between both media. External rubidium was 5 mM. All points correspond to one experiment done in triplicate.

suggests that almost all, if not all, the ATP has disappeared. The fact that the same results were obtained when the IAA preincubation was done in 150 mM potassium proves that the rise in internal sodium is not responsible for these results. When the preincubation in IAA lasted only 4 hr the ouabain-sensitive part of the total influx did not disappear completely and the same difference between the resistant influx in sodium and choline was detected in the control and IAA cells. This does not support the idea that the results are the consequence of the pretreatment with IAA by itself.

¹ Abbreviations used in the text are as follows: ATP, adenosine-5'-triphosphate; IAA, iodoacetic acid.

TABLE II
EFFECT OF EXTERNAL SODIUM, RUBIDIUM,
AND OUABAIN CONCENTRATIONS ON THE
OUABAIN-RESISTANT RUBIDIUM INFLUX
IN HUMAN RED CELLS

	140 mM sodium				0.1 mM sodium			
	1 mM Rb		10 mM Rb		1 mM Rb		10 mM Rb	
	5×10^{-5}	10^{-3}	5×10^{-5}	10^{-3}	5×10^{-5}	10^{-3}	5×10^{-5}	10^{-3}
	0.145	0.165	0.61	0.55	0.062	0.051	0.26	0.25
	0.150	0.145	0.38	0.56	0.065	0.049	0.25	0.24
	0.159	0.157	0.57	0.60	0.062	0.048	0.26	0.23
	0.113	0.116	0.67	0.53	0.058	0.046	0.25	0.23
	0.095	0.111	0.69	0.54	0.064	0.045	0.25	0.26
	0.094	0.097	0.61	0.55	0.059	0.041	0.23	0.25
Mean	0.126	0.132	0.588	0.558	0.062*	0.046*	0.25	0.24
± SE	0.012	0.011	0.045	0.034	0.0011	0.0015	0.004	0.005

* $P < 0.001$.

TABLE III
THE EFFECT OF 16 HR PREINCUBATION IN
IAA ON THE OUABAIN-RESISTANT SODIUM-DEPENDENT
RUBIDIUM INFLUX IN HUMAN RED CELLS

	Sodium			Choline			Difference sodium-choline	
	Control	IAA	Difference	Control	IAA	Difference	Control	IAA
	<i>mmoles/liter cells/hr</i>							
	0.42	0.11	0.31	0.13	0.15	-0.02	0.29	-0.04
	0.42	0.12	0.30	0.18	0.15	0.03	0.24	-0.03
	0.33	0.10	0.23	0.12	0.12	0.00	0.21	-0.02
	0.33*	0.13	0.20	0.09	0.14	-0.05	0.24	-0.01
	0.33*	0.11	0.22	0.07	0.12	-0.05	0.26	-0.01
	0.30	0.14	0.16	0.11	0.13	-0.02	0.19	0.01
Mean	0.355	0.118	0.237	0.117	0.135	-0.018	0.238‡	-0.018‡
± SE	—	—	—	—	—	—	0.0105	0.0063

One group of cells was preincubated for 16 hr in 5 mM K sodium Ringer (or 150 mM KCl buffered with Tris) with the addition of 5 mM iodoacetic acid to deplete the internal ATP. The control group was preincubated in the same solution but without iodoacetic acid with the addition of glucose (200 mg/100 ml). Both solutions were calcium-free and had 0.1% streptomycin. Temperature was 37°C.

* The preincubation was done in 150 mM K.

‡ $P < 0.001$.

DISCUSSION

The influx of rubidium in unpoisoned cells and the characteristics of the activation curve with external rubidium are in close agreement with results pre-

viously reported for potassium in the same cells (Sachs and Welt, 1967; Garahan and Glynn, 1967*b*), both from a qualitative and quantitative point of view. Consequently, rubidium can be used as well as potassium to study the coupled sodium-potassium transport mechanism.

Kinetics of Inhibition by Ouabain

The present results show that a time lag for ouabain inhibition can be detected in a sodium medium provided that the concentration of the glycoside is below 10^{-6} M. This result is in agreement with those reported by Glynn (1957) for scillaren at 2×10^{-8} g/ml. Using a method for rapid solution changes, Baker and Manil (1968) found the same lag in squid giant axons; indeed they were able to detect changes with a resolution of 1 sec, by which delay was observed even in 10^{-3} M ouabain. With the methodology employed in the present work, this cannot be done; as a comparison, in squid axon at 10^{-6} M ouabain the time lag observed was 130 sec and in this work inhibition developed instantaneously. However, both works concur in that as the concentration of the drug was reduced the lag period increased.

In order to develop its action, a drug must attach itself to a specific site or receptor; as concentration decreases, the probability of occupying these sites becomes less and consequently will take longer to exert full action.

The increase of the delay in sodium-free media suggests that this ion in some way facilitates the attachment of the drug to the membrane; eventually ouabain molecules will get into the sites anyway, and this is evidenced by the fact that inhibition develops also in choline after some time, and that, when present at a high enough concentration, the kinetics are similar both in sodium and sodium-free solutions. Thus, though sodium ions speed the rate of inhibition, their removal does not prevent it. A rubidium-sodium antagonism could explain these findings; this would be supported by the increment in delay even in sodium media when external rubidium is raised. As was shown earlier, sodium removal increases the affinity of the external sites for rubidium; this might in turn slow the rate of ouabain binding, either in the case of competition for the same site or of conformational changes. However, any specific effect of sodium as a cofactor for binding cannot be completely ruled out. In studies with tritiated ouabain, Hoffman (1969) found about 20% reduction in the ouabain bound when sodium was replaced by choline in a K-free medium; though this might not be significant, it should be stressed that those experiments were not performed to study the rate of binding but total binding, and only one point at 30 min was considered. This way, some early differences could have been unobserved. On the other hand, Hoffman's studies were done with broken ghosts and the effect might well have been exerted on the inside of the membrane.

It is also interesting to note that, whereas in red cells there is a marked

antagonism between potassium or rubidium and ouabain, squid axons seem peculiarly different; thus, the rate of inhibition in nerve was faster in potassium than in sucrose when these were used as a replacement for sodium, and took an intermediate value between full sodium and sucrose.

In summary, the development of ouabain inhibition in red cells is a complicated function of at least three variables: the concentration of glycoside, external sodium, and external rubidium. In addition, whichever mechanisms are involved on the extracellular side of the sodium pump, ouabain and sodium cooperate with each other in favoring inhibition, whereas rubidium antagonizes it.

TABLE IV
COMPONENTS OF RUBIDIUM INFLUX
IN HUMAN RED BLOOD CELLS

I. Nonsaturable
(Na) e -insensitive
(Na) e -independent
Energy independent
Ouabain-insensitive
II. Saturable
a. (Na) e -sensitive at low (Rb) e
ATP-dependent
Ouabain-sensitive
b. (Na) e -dependent
ATP-dependent
Ouabain-insensitive
c. (Na) e -insensitive
(Na) e -independent
ATP-independent
Ouabain-insensitive

e means external.

As to the advantages of the present methodology for evaluating the binding rate, it should be emphasized that ouabain not only attaches to the pump sites, but also there seems to be a nonspecific binding (Hoffman, 1969). By using labeled ouabain both processes would be detected together, whereas, in studying fluxes, the nonspecific binding would be automatically disregarded.

The Partition of Rubidium Influx

The classical division put forward by Glynn (1957) is followed with some modifications and summarized in Table IV. The nonsaturable component, which has been shown to be unaffected by external sodium or ouabain, does not require energy input and follows a linear relationship with the external rubidium concentration; this strongly suggests that it obeys simple electrodiffusion laws. It represents about 2.5% of the total influx, indicating that the

passive membrane permeability to rubidium could be a little larger than to potassium.

On the basis of the effect of external sodium, ouabain, and internal ATP, the saturable component of rubidium influx can be divided into three fractions: (a) the largest one, representing 70–80% of the total influx, is inhibited by external sodium at low external rubidium, is also fully inhibited by ouabain, and requires internal ATP. In net flux experiments it is associated with active sodium extrusion (Maizels, 1968; unpublished personal observations). It then fulfills all the requirements in order to be considered part of the active sodium-potassium (rubidium)-coupled transport.

The remaining influx has the characteristics of ouabain insensitivity, which by no means signifies passive transport, and behaves differently regarding the effect of external sodium and internal ATP. Thus, part of it (b) requires simultaneously external sodium and internal ATP, though there is no proof of ATP consumption. It seems to be equivalent to the sodium-dependent fraction of potassium influx found by Lubowitz and Whittam (1969) which was inhibited by ethacrinic acid. Their data show no difference in lactate production associated with this influx, thus favoring the idea of no energy requirement; but, as they pointed out, due to the large errors in lactate determination, there was no way to exclude completely some ouabain-insensitive active ion movements. Our results on ATP requirements suggest that these movements in fact exist. In order to prove it, ATP consumption would have to be demonstrated. However, this flux (about 15% of the total uptake) represents at most 0.33 mM/liter cells per hr; assuming an ATP/ion stoichiometry of one-third (Garrahan and Glynn, 1967 *c*), this would imply an ATP consumption of no more than 0.1 mM/liter cells per hour, which would be very difficult to detect.

Furthermore, there is no way to determine net fluxes by comparing media with sodium and choline in ouabain; with sodium, the gain due to the inhibition of the pump in sodium would obscure the results and the changes in internal potassium would be too small to be detected. On the other hand, if this influx is part of a rubidium-potassium exchange, there should be a sodium-dependent, ouabain-insensitive fraction of potassium efflux. With the use of ^{86}Rb as a tracer, the rate constant for ^{86}Rb efflux in ouabain was found to be between 0.020 and 0.028 hr^{-1} ; an influx of 0.33 mM/hr of rubidium with an internal potassium concentration around 110 mM/liter cells would result in a 0.003 hr^{-1} change in that rate constant, which would not be easy to detect. Thus, this fraction of rubidium influx which depends on external sodium and internal ATP could be a part of ouabain-insensitive active sodium transport or an exchange with internal potassium. The latter case would be different from the ATP-dependent Na-Na exchange described by Garrahan and Glynn in that it is insensitive to ouabain.

The residual influx (c), unaffected by external sodium and ouabain or internal ATP, also remains obscure, though it could possibly be part of a rubidium-potassium exchange.

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