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pH DEPENDENCE OF RUBIDIUM INFLUX IN HUMAN RED BLOOD CELLS

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SUMMARY

An optimum pH range, relatively narrow and close to physiological values, was found for both ouabain-sensitive and -insensitive saturable components of Rb^+ influx in human red blood cells; the deviation of 1.5 units from that optimum resulted in about 75% inhibition in the maximum uptake, both in Na^+-containing and Na^+-free Mg^{2+} Ringer's solution. All changes in the ouabain-sensitive Rb^+ influx were parallel to changes in the ouabain-sensitive Rb^+-activated Na^+ efflux, suggesting that whereas the external H^+ concentration influenced the rate of pumping it did not affect the $Na^+/Rb^+(K^+)$ coupling ratio. The apparent K_m for all these fluxes increased as the pH went from the acid to the alkaline side; however, any speculation on the Rb^+-site affinities and ionization constants is challenged by the complexity of these interactions. All observed effects were independent of the buffer used and all were reversible.

Enzymes are proteins of high molecular weight carrying acid and basic groups whose dissociation is affected by the H^+ concentration in the media [1–3]. In the living cell, proteins are on both the inner and outer surface of the membrane. In addition, carrier-mediated solute movements through that membrane require some structure that seems to be protein in nature [4], and their kinetic analysis shows many similarities to enzyme systems involving the existence of maximum fluxes and affinity constants [5]. It is possible then, that solely on this basis almost any carrier-mediated transport system would be affected by the H^+ concentration in the media. This has been shown for the non-carrier or leak ion movement in red blood cells [6] and has been interpreted on the basis of changes in the dissociation of some fixed charges in the membrane. The aims of the present work are to extend this observation to some carrier-mediated cation translocation in human red blood cells.

Rb^+ influx, and in some experiments Na^+ efflux, were determined as described elsewhere [7] in solutions of the following composition (mM): (a) Na^+ Ringer: $MgCl_2$, 1; Glycine–Tris–maleic acid, 10; NaOH, variable depending on the pH;

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$Na^+ + Rb^+$ concentration was kept constant at 150 mM. As the amount of added base would depend on the desired pH, solutions were prepared in preliminary experiments by trial and error and most of the NaCl (1 M) was added before titration had ended to avoid pH drifts due to changes in the ionic strength. Once the total base needed for a given pH was known, the amount of 1 M NaCl to be added was calculated from the following equation:

$$V_{NaCl} = \left\{ \frac{279 - Rb^+ - \left[0.932 \times 2 \left(\frac{[NaOH]_i \times V_{NaOH}}{V_{sol}} \right) + 30 \right]}{0.932 \times 2} \right\} \cdot \frac{V_{sol}}{M_{NaCl}}$$

where V_{NaCl} is the volume of 1 M NaCl to be added, in ml; Rb^+ is the final RbCl osmolarity; 279 is the total final osmolarity of the solution, in mosmoles/l; 0.932 is the osmotic coefficient for 0.1 M NaCl which was also taken for NaOH; 2 is the number of osmotic-active particles per each molecule of NaCl; (NaOH) is the concentration of the NaOH solution (mM); V_{sol} is the volume of Ringer solution to be prepared, in ml; V_{NaOH} is the volume of NaOH added (variable for every pH), in ml; 30 is the approximate mosmoles/l for a 10-mM solution of glycine–Tris–maleate; M_{NaCl} is the molarity of the NaCl solution, which was set at 1 M. (b) Mg^{2+} Ringer: in this case the titration was done with Tris base of different concentrations from 0.1 up to 3 M. The general composition was (mM): glycine–Tris–maleate, 10; $MgCl_2$, 108 – Rb^+ – Tris (Tris base variable); as Tris is a weak base, the percentage of dissociation had to be taken into account for every pH, and this varied as follows: pH 6.0, 100%; pH 7.4, 65% and pH 8.5, 30%. To calculate the amount of 1 M $MgCl_2$ to be added, the following equation was used:

$$V_{MgCl_2} = \left\{ \frac{279 - Rb^+ \left[0.932 \times 2 \left(\frac{[\text{Tris base}]_i \times V_{Tris(b)} \times \%\ \text{diss.}}{V_{sol}} \right) + 30 \right]}{3 \times 0.861} \right\} \cdot \frac{V_{sol}}{M_{MgCl_2}}$$

where symbols have the same meaning as for the Na^+ Ringer. All solutions were Ca^{2+}-free. Glucose was added as a solid to give a concentration of 11 mM. When required, ouabain was present at a 10^{-4} M concentration. In some cases both the concentration and composition of the buffer were changed. The determination of pH was made with a Beckman expandomatic pH meter. 0.2 ml of packed cells were added to 9.8 ml of a given non-radioactive solution in order to obtain a final hematocrit similar to that of radioactive samples. After mixing, the tubes corresponding to zero time were centrifuged and the pH was determined in the supernatant. All other tubes were incubated in the same way as the radioactive ones and the pH was determined as before at the end of the incubation period. All readings were made at 37 °C in a specially designed flask. The difference between zero time and postincubation readings was usually within 0.1 pH unit; the average of these two determinations was taken as the corresponding pH of the experiment.

The leakage fluxes are largely influenced by external pH [6] and this would obscure the results on ouabain-insensitive saturable fluxes; therefore, in preliminary experiments the dependence of the linear component of Rb^+ influx on the H^+ concentration (determined as described by Glynn [8]) was tested in cells incubated

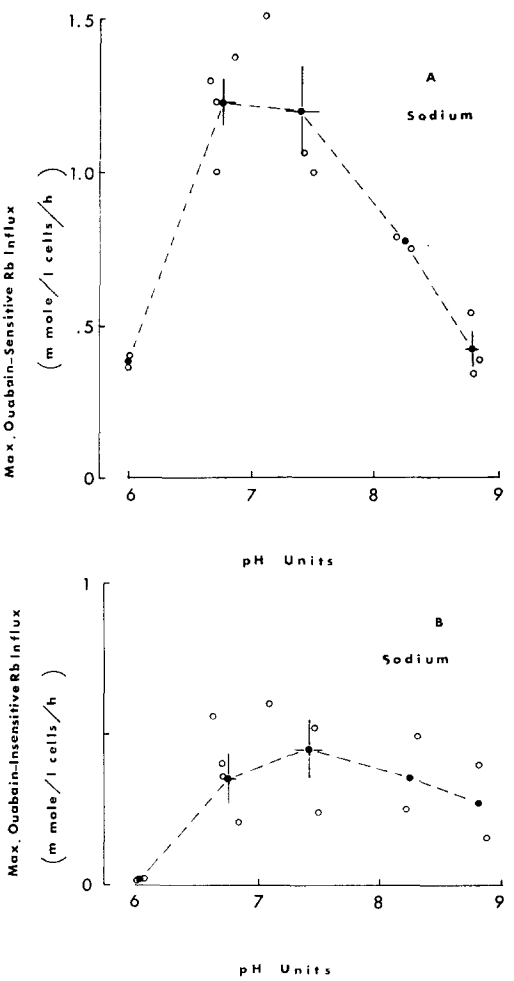
in Na^+-containing and Na^+-free media. From the slope of Rb^+ influx vs external Rb^+ concentration, a constant relationship for a given pH was obtained which had the units of mmole/l cells · h · mM; as this linear component obeys Fisk's first law that factor is directly related to the membrane permeability to Rb^+. Thus, by multiplying it by the external Rb^+ concentration an estimation of the leak component of Rb^+ uptake can be obtained. When pH was changed from 6.0 to 8.7 units this factor increased 6 to 8 times following a parabolic function. In all subsequent experiments the corresponding leakage was subtracted from the total value of Rb^+ influx at any Rb^+ concentration; this left the pH effects on the saturable components only.

The changes in the maximal ouabain-sensitive and ouabain-insensitive Rb^+ uptake in the presence and absence of external Na^+ were studied as a function of external pH. In Na^+-containing media V was taken as the value of Rb^+ influx at the external Rb^+ concentration at which the activation curve saturated. In Na^+-free media, it was estimated either as before, or from an Eadie plot [9] of v (Rb^+ influx) as a function of v/s (Rb^+ influx/Rb^+ concentration). The difference between the two methods usually did not exceed 5 %. The results are illustrated in Figs 1A through 1D. Both ouabain-sensitive and -resistant influx showed similar changes with an optimum pH close to the physiological range. A departure of 1.5 units from that optimum either into the acid or alkaline side brought about a large reduction in the uptake, which in some instances reached 90 %. This behavior was the same regardless of the presence or absence of external Na^+. The apparent K_m were also obtained from the same activation curves, either as the necessary Rb^+ concentration to get one-half the maximal uptake (Na^+ media) or from the Eadie plots (Na^+-free media). In all cases the apparent affinity of the translocation system for Rb^+ diminished (apparent K_m increased) as the external pH was increased from 6.0 to 9.0 units. In another group of experiments glycine–Tris–maleate was replaced with equal concentration of Tris–phosphate, Tris–HCl, phosphate or acetylglycine–Tris–maleate. In all cases the observed effect was independent of the buffer used and related only to the pH in the media. When checked they also showed to be reversible; therefore, any irreversible denaturation of the system can be disregarded.

The fact that when the pH is varied to the acid or alkaline side of the optimum a reduction in Rb^+ influx occurs does not necessarily mean that the same mechanism is responsible for that effect. Thus, it could be possible that in the alkaline range there is an actual inhibition of the translocation machinery, whereas in the acid range the large increase in H^+ concentration (about 100 times) could induce competition between them and external Rb^+ for the external site of the transport mechanism [10]. If this were the case for the coupled $Rb^+(K^+)$-Na^+ transport, and H^+ could be transported in exchange for cellular Na^+, a reduction of the ouabain-sensitive Rb^+ influx would not be followed by a similar reduction in the ouabain-sensitive Rb^+-activated Na^+ efflux. To test this point the efflux of Na^+ and influx of Rb^+ were simultaneously determined in red cells incubated in 10 mM Rb–Na^+ Ringer with and without the addition of 10^{-4} ouabain, at two extremes and an intermediate pH. Fig. 2 shows the ouabain-sensitive Rb^+ influx and Na^+ efflux which were normalized, giving a value of 1.0 to the maximal flux. Both were altered almost identically upon changing external pH. This would indicate that either the pH effect is in the dissociable group of the transport system or that if H^+ compete with external $Rb^+(K^+)$ they

are not translocated through the pump; in view of the findings on the alkaline range the first explanation seems more likely. In addition the results suggest that pH has no effect on the $Na^+/Rb^+(K^+)$ coupling ratio. Furthermore, they allow the consideration of the alterations of the ouabain-sensitive Rb^+ influx or actual change in the Na^+–Rb^+ (K^+) active transport.

The present results indicate that the H^+ concentration on the external side of the red-cell membrane is of paramount importance for all mechanism of Rb^+ (K^+) translocation. A deviation from the physiological range reversibly influenced the passive (leak or saturable) component as well as the active Rb^+ influx. The increase in cation leak as the external pH is raised has already been described [6]; as other changes in the external media, such as reduction in the ionic strength, also reversibly modified the loss of K^+ [11–13] and Na^+ [13], it is possible that all these effects are consequences of changes in membrane matrix [6–14]. As regards the saturable component of Rb^+ influx, by using ouabain it is possible to separate two compounds, although the response of these fluxes is remarkably similar upon changing the H^+



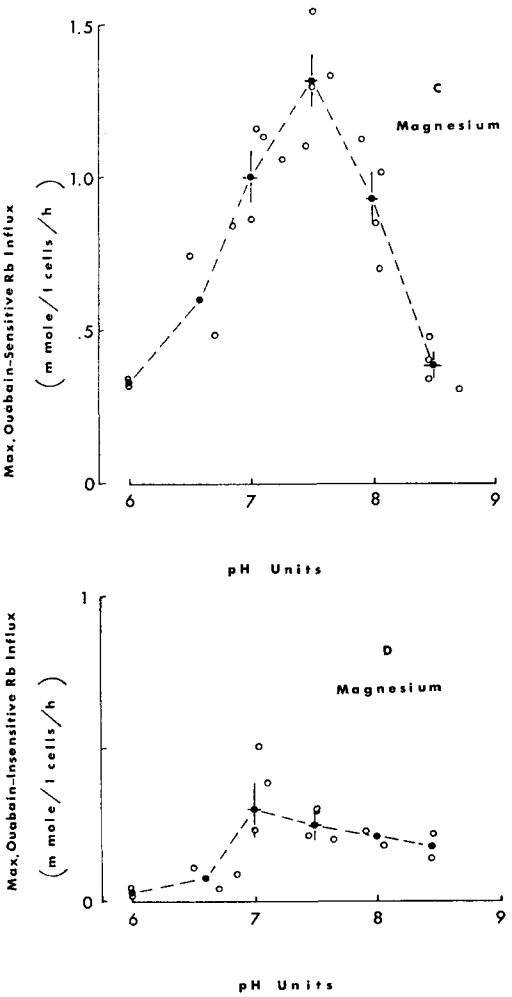


Fig. 1. Effect of external pH on the maximal ouabain-sensitive (A and C) and ouabain-insensitive (B and D) Rb^+ influx in human red blood cells incubated in Na^+-containing (A and B) and Na^+-free Mg^{2+} (C and D) Ringer solutions. Open circles are individual determinations taken from activation curves of Rb^+ influx by external Rb^+ concentration; filled circles are the average value $\pm$S.E. from flux (vertical bars) and pH (horizontal bars). When used, ouabain was present at 10^{-4} M concentration. See text for details.

concentration. This again raises the question if there really are two separable entities, with two different responses to ouabain, or whether the same mechanism, modified by the presence of the glycoside, is responsible for Rb^+ translocation in both instances. If both fluxes are not carried out by the same mechanism, it seems at least that they have in common functional groups with similar ionization properties. The physical interpretation of the changes in the kinetic parameters is extremely difficult, especially in Na^+-containing solutions, because of the characteristics of the site–substrate interactions [16–17]. It would seem useless to try any quantitative approach to obtain the apparent pK of the functional groups on the basis of Dixon's model

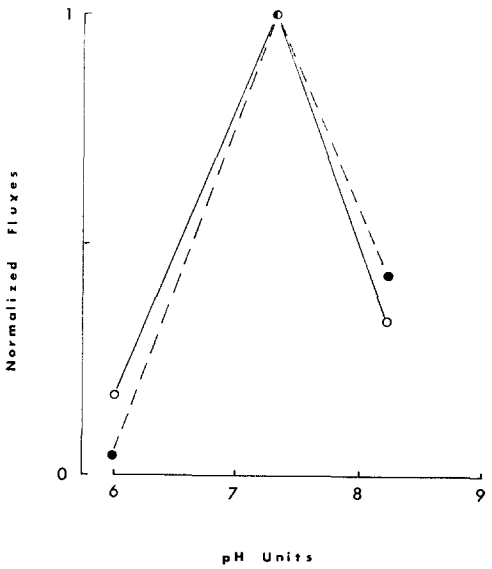


Fig. 2. Effect of external pH on the ouabain-sensitive Rb^+ influx (open circles) and ouabain-sensitive Rb^+-activated Na^+ efflux (filled circles) in human red blood cells incubated in 10 mM Rb^+–Na^+ Ringer. Both fluxes have been normalized by giving a value of 1.0 to the maximum flux of each cation. Each point is the mean of two experiments carried out simultaneously. When used, ouabain was at 10^{-4} M concentration.

[18]. Finally, it is interesting to note that although the union between ions and proteins is usually affected by the different types of buffer used by competitive interactions [19], the union between Rb^+ and the external site of the translocation mechanism did not seem to be influenced by the buffers used in the present work.

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