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Calcium-Potassium-Stimulated Net Potassium Efflux from Human Erythrocyte Ghosts

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Summary. In the presence of 8 mM external Ca^{++}, the K^+ permeability of human red cell ghosts increases provided K^+ is also present in the medium. This increase does not represent K^+/K^+ exchange but a stimulation of net K^+ efflux. The stimulation is half-maximal at 0.7 ± 0.15 mM ($n=5$). At concentrations above 4.0 mM, external K^+ inhibits net K^+ efflux. Similar stimulatory and inhibitory effects of external K^+ were also observed in intact cells after exposure to Pb^{++} or to Ca^{++} in the presence of fluoride, iodoacetate plus adenosine, or propranolol, suggesting that a common K^+-activated K^+-specific transfer system may be involved under all of these various circumstances. Internal K^+ also stimulates net K^+ efflux from ghosts, but it is uncertain whether internal K^+ is an absolute requirement for the K^+ permeability increase.

In contrast to external Na^+ which slightly stimulates K^+ efflux, internal Na^+ inhibits. The inhibition by internal Na^+ is abolished by sufficiently high concentrations of external K^+, showing that K^+ binding to the outer membrane surface and Na^+ binding to the internal surface are mutually interdependent.

In red cell ghosts the Ca^{++}-K^+-stimulated net K^+ efflux increases with increasing pH until a plateau is reached between pH 7.2 and 8.0. In fluoride-poisoned intact cells, the Ca^{++}-K^+ stimulated flux passes through a maximum around pH 6.8.

Neither internal nor external Mg^{++} interferes with the combined effects of Ca^{++} and K^+. Similarly, external EDTA has no influence at concentrations which are far lower than the Ca^{++} concentration required to produce a maximal response. In contrast, low concentrations of internal EDTA prevent the permeability change.

In human erythrocytes under certain experimental conditions, divalent cations such as Pb^{++}, Ca^{++}, or Mg^{++} are capable of inducing a selective increase in K^+ permeability, which is not accompanied by a corresponding alteration of sodium permeability (for reviews see Passow, 1963, 1964). The present report is primarily concerned with the action of Ca^{++}. In intact red cells, Ca^{++} affects potassium permeability only after treatment of the cells with certain metabolic inhibitors or drugs. In contrast, in ghosts Ca^{++} acts in the absence of added modifiers. Even in the comparatively simple situation existing in experiments with ghosts, the effect of

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Ca^{++} on K^+ permeability depends in a complicated manner on the concentrations of K^+, Na^+, H^+, Ca^{++}, and complexing agents. The complexity of the situation is further augmented by the fact that the same ion may have different effects on the inner and outer surface and that there are transmembrane interactions between the various ion species involved (for review, see Riordan and Passow, 1973). In the experiments described below we have characterized some of these interactions, in particular the effects of K^+ on the Ca^{++}-induced permeability change and the effects of external K^+ on K^+/Na^+ interactions at the inner membrane surface. This is supplemented by additional experiments on the effects of hydrogen ions, Mg^{++}, and complexing agents. Preliminary reports of this work have been presented elsewhere (Riordan & Passow, 1973; Knauf, Riordan, Schuhmann & Passow, 1974).

Materials and Methods

Blood taken from apparently healthy donors was stored for not more than three days in acid-citrate-dextrose buffer at 4 °C. Cells were washed three times with isotonic choline chloride. Ghosts were prepared at 0 °C, pH 6.0 as described by Schwoch and Passow (1973). The desired internal ionic compositions were attained by addition of appropriate concentrated salt solutions at the time of "reversion" to isotonicity.

For measuring K^+ efflux, at the end of the resealing period the ghosts were washed and incubated in a medium whose composition was identical to that of the final incubation medium except that Ca^{++} was omitted. During this incubation period (40 min at 37 °C) and a subsequent wash in Ca^{++}-free medium, the leaky ghosts (type III of Bodemann and Passow, 1972) of the population lost their potassium. Hence during the final incubation in the presence of Ca^{++} only K^+ efflux from successfully resealed ghosts (type II) was measured.

The washed, resealed ghosts (approximately 80% hematocrit) were equilibrated at 37 °C for about 20 min. The flux experiment was begun by mixing the calcium-containing media with the ghosts. The net K^+ efflux was determined by withdrawing at suitable time intervals accurately measured volumes of ghost suspension, dilution with ice-cold isotonic choline chloride solution, centrifugation, and determination of the amount of K^+ in the pellet by flame photometry. This procedure yields the amount of K^+ in an arbitrarily chosen fixed number of ghosts.

In experiments on the effects of Ca^{++}, the definition of a suitable measure for the rate of net K^+ loss raises problems since the response of the various cells in the population to Ca^{++} may vary and since the rate of loss may be influenced by time-dependent changes of membrane potential and ion concentrations in cells and medium.

Fig. 1 shows that the rate of loss undergoes a marked change after about 2 hr. The internal K^+ concentration at which this change takes place varies with the Ca^{++} concentration in the medium. It reamined unresolved whether this transition from fast to slow rate of loss represents a transition in all cells of the population or if some cells lose K^+ at a low rate and the rest at a much higher rate. In the former case, the effect would represent a graded response and the initial slope would represent a suitable measure of the rate

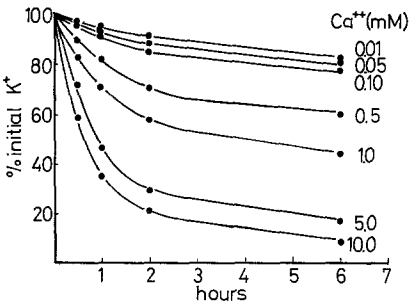

Fig. 1. K^+ loss from red cell ghosts at various Ca^{++} concentrations in the medium. *Ordinate:* K^+ content in percent of initial content. *Abscissa:* time in hours. Ghosts containing 140 mM KCl, 20 mM Tris-HCl, pH 7.4, suspended in media containing 140 mM NaCl, 20 mM Tris-HCl, pH 7.4, and the $CaCl_2$ concentrations indicated in the Figure. Hematocrit 10%, 37 °C. In this and all other experiments during ghost preparation the ratio between cells and hemolysis medium was 1:20

of loss. In the latter case, the effect would represent an all or none response and the half time for the transition from fast to slow rate of K^+ loss would be a measure for the rate of loss in the modified ghosts (*cf.* Riordan & Passow, 1971). In order to avoid ambiguities of the interpretation of the results, all experiments were done at a high Ca^{++} concentration (8 mM) which produces a nearly maximal effect.

As will be shown below, even at a maximally effective Ca^{++} concentration the rate of loss is greatly influenced by the K^+ concentration in the external medium. At high hematocrit values, the K^+ concentration in the medium will continuously change during the time course of K^+ loss. This should in turn continuously increase the permeability coefficient. In all experiments with ghosts we find within the limits of our time resolution that K^+ loss begins immediately after mixing ghosts and medium (Figs. 1 and 2) and that the initital rate of loss represents the maximal rate. The initial portion of the curves relating K^+ loss to time is close to linear. This suggests that the acceleration of net K^+ efflux associated with the progressive accumulation of K^+ in the medium leads to the maintenance of the initial rate for a longer period of time than one would anticipate on the basis of an exponential decay of the rate of efflux. In the present work we chose the initial slope of the curve relating K^+ loss to time divided by the initial K^+ content to obtain a convenient empirical measure of the rate of K^+ loss. This measure will be designated "rate constant" (k). In contrast to the situation with ghosts and intact cells poisoned with lead or Ca^{++} plus propranolol, in intact cells treated with fluoride or iodoacetate plus adenosine, the onset of net K^+ loss is preceded by a lag period. Under these conditions, the maximal rate was used in place of the initial rate for calculating k (*see* Lindemann & Passow, 1960, Fig. 2). It should be emphasized that k is not identical to the permeability coefficient. This coefficient is likely to vary with time under most of our experimental conditions. However, as discussed by other authors (e.g. Blum & Hoffman, 1971; Kregenow & Hoffman, 1972) under such non-steady-state conditions, with time-dependent variations of the permeability coefficient, the determination of an empirical rate constant represents the only feasible approach.

In order to measure the unidirectional efflux of potassium, ^{42}K (Harwell, U.K.) was incorporated into ghosts during reversal of hemolysis. The appearance of the isotope in the medium was determined by measuring either the gamma radiation in a Packard Auto Gamma Spectrometer or the Cherenkov radiation in a Packard Liquid Scintillation Spectrometer (Model 3380) without scintillator. The rate constant for unidirectional K^+

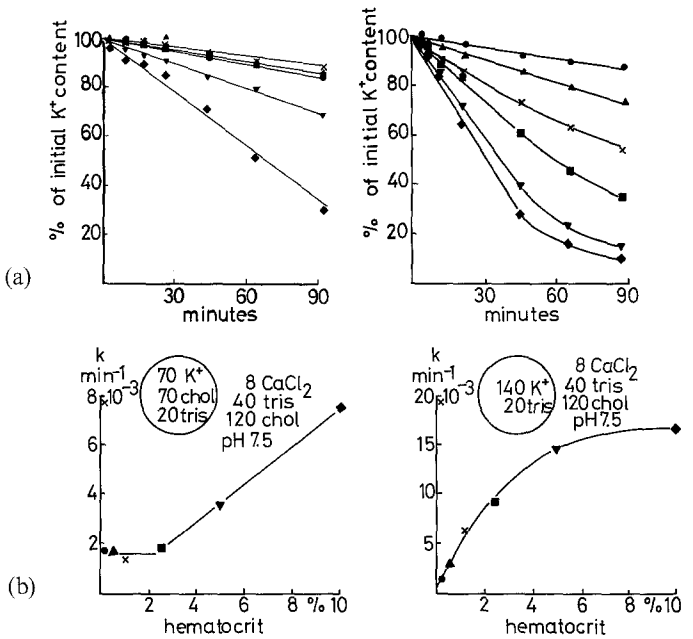

Fig. 2. (*a*) Time course of K^+ loss from human red cell ghosts as measured under the conditions specified in Fig. 2b. From the initial slopes of the curves in Fig. 2*a*, relating K^+ content in percent of the initial value (ordinate) to time (abscissa) rate constants (k) were calculated. (*b*) In this Fig. the calculated values of k are plotted against the corresponding hematocrit values. In Figs. 2*a* and 2*b* corresponding experimental conditions are denoted by the same symbols. Note that the scales of the two ordinates in Fig. 2*b* differ. The temperature was 37 °C

efflux, ${}^0k$, was determined from the initial rate of increase in ^{42}K in a measured volume of supernatant and dividing this by the total ^{42}K in an equal volume of the cell suspension. The latter procedure is permissible since the hematocrit is small (2.5% or less).

In ghosts prepared under identical conditions and suspended in media of the same composition, the standard deviation of eight determinations of k was about 10%. However, the absolute values of k or ${}^0k$ obtained on different days vary to a greater extent (for example, compare Figs. 6 and 12). Nevertheless, the described functional relationships are consistently observed. Represented in the Figures are individual experiments typical of at least 3 and up to 25 replicates.

Since the numerical value of the rate constants depends on the volume of the ghosts, precautions were taken to operate at constant initial ghost volume. In preliminary experiments we found that the ghost volume, as measured at pH 7.5, was independent of the ionic composition of the ghost interior provided the sum of all electrolyte species present in the system (the chlorides of Na^+, K^+, and choline) was maintained at a constant value. For this reason, in all experiments in which the concentrations of K^+ and Na^+ were varied, sufficient choline chloride was added to keep the total electrolyte concentration constant, usually at 160 mM. We adjusted the pH to 7.2–7.6 with Tris titrated with HCl (denoted Tris-HCl). Tris-HCl was present inside the ghosts as well as in the medium. The exact experimental condition are given for each experiment in the corresponding legends to Figures and Tables.

It is not clear to what extent choline affects the experimental results described below. However, it should be borne in mind that the most striking effect, the activation by K^+ can be observed at relatively low concentrations where the variations of the K^+ concentration cover a wide range while the corresponding percentage changes of the choline concentration are more modest. Under these conditions possible effects of varying choline are likely to be small. Under the conditions where there are large variations of both K^+ or Na^+ on the one hand and choline on the other, K^+ and Na^+ have opposite effects. Moreover, the effects of Na^+ and Li^+ which are qualitatively similar show marked quantitative differences. This would suggest that in these experiments the critical variables are the alkali ions although possible superimposed effects of choline cannot be excluded.

Results

I. Effects of External Potassium on Potassium Efflux

(1) Hematocrit and K^+ Efflux. The starting point for the present investigation was the observation (Fig. 2) that the effect of a fixed concentration of external Ca^{++} depends on the hematocrit. The relationship between K^+ efflux and hematocrit is greatly affected by the internal K^+ concentration. This made us suspect that the effect of varying the hematocrit could be related to the presence of traces of external potassium which may be lost from the ghosts by hemolysis or leakage during equilibration prior to the exposure to Ca^{++}. At 140 mM internal K^+ and at high hematocrit the K^+ concentration in the medium could be sufficient to nearly maximally stimulate net K^+ efflux, while at 70 mM K^+ and low hematocrit the concentration could remain too low to induce the permeability change.

(2) External K^+ and K^+ Efflux. The suspicion that traces of external K^+ activate the Ca^{++}-induced net K^+ efflux is confirmed by the experiment in Fig. 3, where the effects of external K^+ on ghosts containing 70 mM K^+ at a hematocrit of 0.25% are presented. The Figure shows that external K^+ increases the rate constant for net K^+ efflux by about one order of magnitude. A maximum is reached between 2–4 mM K^+ in the medium. These data indicate that a stimulatory and an inhibitory effect of external K^+ on net K^+ efflux are superimposed. On the basis of the assumption that the combined effect is equal to the sum of the activating and inhibitory effects of external K^+ (Fig. 3), the K^+ concentration corresponding to half maximal activation was found to be 0.7 ± 0.15 (SEM, $n=5$).

The most interesting question that arises from these observations is whether or not external K^+ is a necessary prerequisite for the increase of K^+ permeability in the presence of Ca^{++}. The results in Table 1 demonstrate that in the absence of external K^+, Ca^{++} causes no significant

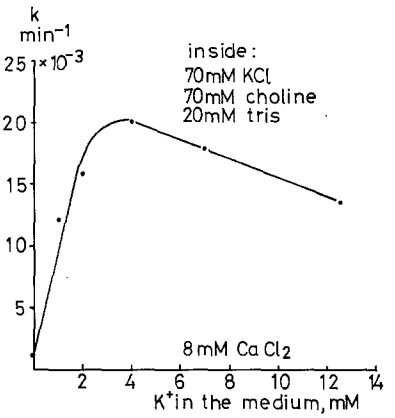

Fig. 3. Rate constant for net K^+ loss from human red blood cell ghosts in the presence of Ca^{++} as a function of external K^+ concentration. The ghosts contained 70 mM KCl, 70 mM choline chloride, and 20 mM Tris-HCl and were suspended in media containing 120 mM choline chloride, 8 mM $CaCl_2$, and 40 mM Tris-HCl, pH 7.2. The KCl concentration was varied by isosmotic replacement of choline chloride. The temperature was 37 °C, hematocrit 0.25%

Table 1. Rate constants for net potassium efflux (k) in the presence or absence of Ca^{++} in the medium[a]

Ca_o^{++} (mM)	0	0	8.0	8.0
K_o^+ (mM)	0	2.5	0	2.5
$k \times 10^3$ (min^{-1})	2.45	3.44	2.00[b]	17.9
SE	±0.84	–	±0.94	±5.15
n	13	3	25	23

[a] Internal composition of the ghosts: 70 mM KCl, 70 mM choline chloride, 20 mM Tris-HCl, pH 7.5. Composition of medium: 120 mM choline chloride, 40 mM Tris-HCl, pH 7.5 and either 0 or 2.5 mM KCl. Ca_o^{++} and K_o^+ represent concentrations of Ca^{++} and K^+ in the external media, respectively. Hematocrit, 0.25%. Temperature, 37 °C.

[b] Applying Chauvenet's criterion 2 determinations were not included in the average.

increase of K^+ efflux. This absolute requirement for external K^+ is independent of the external Ca^{++} concentration (Fig. 4).

The stimulatory effect of increasing external K^+ is not due to a change of the driving force for net K^+ efflux, since this increase is associated with a reduction of the concentration gradient and a negligible change in potential. On the basis of the Goldman equation, using 0.037 min^{-1} as the value of the chloride permeability, P_{Cl} (Hunter, 1971), changes in the driving force upon raising external K^+ from 0 to 4 mM should cause the rate constant for net K^+ efflux to decrease by 6%, in contrast to the 10-fold increase which is observed (Fig. 3).

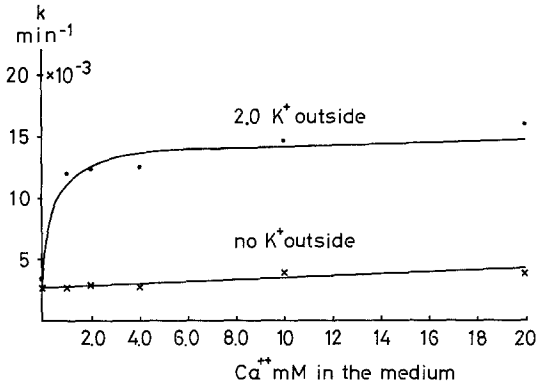

Fig. 4. Rate constant for net K^+ efflux as a function of external Ca^{++} concentration in the presence and absence of external K^+. The internal and external ion concentrations were as described in Fig. 2*b* (left). Ca^{++} was varied by isosmotic replacement of external choline. The pH was 7.5, temperature 37 °C, and hematocrit 0.25%

Similar calculations predict a 13% decrease in the rate constant when external K^+ is increased from 4 mM to 12 mM. Under the conditions of the experiment depicted in Fig. 3, the change in driving force would decrease k to 17.5×10^{-3} min^{-1} at 12 mM K^+. Thus, the inhibitory effect of external K^+ at high concentrations is at least partially related to a decrease of the K^+ concentration gradient, which is partially compensated by a small potential change that tends to facilitate K^+ efflux. Nevertheless, the demonstrated decrease of net K^+ efflux seems to represent some genuine inhibition, since $K^+ - K^+$ exchange shows a dependence on K^+ concentration (*see* section 4 and Figure 6; *cf.* Blum & Hoffman, 1971) which is similar to that of net K^+ efflux.

(3) Internal K^+ and K^+ Efflux. Fig. 5 shows the relationship between the rate constant of K^+ efflux and internal K^+ concentration as measured in the absence or in the presence of 3.0 mM K^+ in the medium. In the absence of added external K^+, there is little if any change of the rate constant for K^+ efflux up to about 80 mM internal K^+. Above this concentration, there is an increase.

As has been shown previously (Fig. 2) with ghosts containing high internal K^+ concentrations, even at the lowest hematocrit used (0.25%), the K^+ concentration in the external medium cannot be maintained below the level at which K^+ efflux is stimulated. Thus, the increase in rate constant at high internal K^+ concentrations is most likely due to the presence of traces of K^+ in the external medium. We suspect therefore that in the complete absence of external K^+ the rate constant for K^+ exit is independent of the internal K^+ concentration. This would resemble

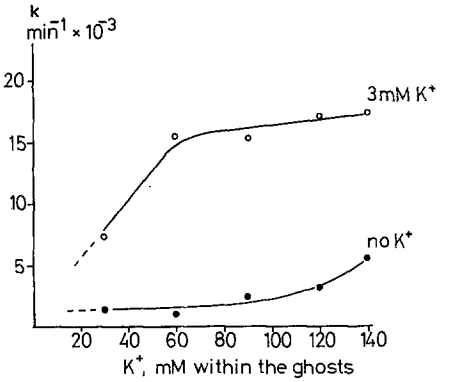

Fig. 5. Rate constant for net K^+ efflux (k) in the presence of Ca^{++} as a function of internal K^+ concentration. The ion concentrations inside and outside the ghosts were as described in Fig. 3, except that the internal K^+ concentration was varied by isosmotic replacement of choline chloride. The pH was 7.5; temperature 37 °C; hematocrit 0.25%

the behavior of K^+ efflux through the leak pathway in the intact cell (Shaw, 1955). In the presence of external K^+, the rate constant increases with increasing internal K^+, and seems to approach a plateau above 60 mM. It would seem plausible to relate this change of the rate constant to variations of the membrane potential (Glynn&Warner, 1972; Hoffman&Knauf, 1973). However, an analysis in terms of the Goldman equation, again using the value of P_{Cl} determined by Hunter (1971), shows that the potential changes associated with the alterations of internal K^+ concentration are insufficient to explain the variations of the net rate constant as defined in the present work. For example, when the internal K^+ concentration is increased from 30 to 60 mM, the rate constant should decrease by about 2% whereas approximately a twofold increase is observed. Hence we conclude that internal K^+, like external K^+, facilitates the increase of K^+ permeability in the presence of Ca^{++}.

(4) K^+ Equilibrium Exchange at Varying K^+ Concentrations. Since there is no unequivocal way of calculating the membrane potential, it seemed useful to measure K^+/K^+ exchange at various K^+ concentrations in the absence of variations in the electrical potential difference. For this purpose ^{42}K efflux was measured with identical K^+ concentrations inside the ghosts and in the media. The result (Fig. 6) demonstrates that there are changes of the rate constant with the K^+ concentration which cannot be related to concomitant changes of the potential. In experiments of this type the various stimulatory and inhibitory effects of internal and external K^+ are superimposed and cannot be separated. However, it is obvious by comparison of Fig. 6 with Figs. 3 and 5 that the results

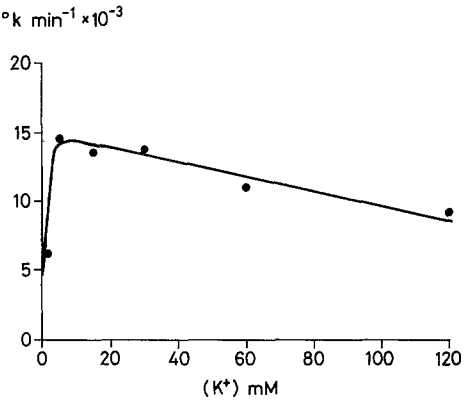

Fig. 6. Rate constant for ^{42}K exchange (^0k) in the presence of Ca^{++} as a function of internal and external K^+ concentration. K^+ concentrations inside and outside the ghosts were always identical, and were varied by replacement with choline chloride, such that the total concentration of choline plus K^+ was 140 mM. Both ghosts and media contained 20 mM Tris-HCl, pH 7.6; the media also contained 8 mM $CaCl_2$. The temperature was 37 °C and the hematocrit 2.5%. The rate constant for ^{42}K efflux, ^0k, was determined as described in Materials and Methods

obtained in the absence of variations in membrane potential and K^+ concentration gradient are compatible with those obtained under net flow conditions.

(5) Effect of External K^+ on Metal-Induced Net K^+ Efflux from Intact Red Cells. Under a variety of special experimental conditions, divalent metal ions bring about a specific increase of K^+ permeability in intact cells, similar to that which we have observed in ghosts (*see* Discussion). The question may be asked whether or not this increase is also dependent on external K^+ concentration. Fig. 7 shows the effects of external K^+ on net K^+ loss induced by Pb^{++} or by Ca^{++} in the presence of iodoacetate plus adenosine, fluoride, or propranolol. Although the effect of external K^+ on net K^+ efflux varies with the experimental conditions, stimulation and inhibition are always clearly apparent.

(6) Effect of pH on K^+ Stimulation of Net K^+ Efflux. The previous experiments with ghosts were done at pH values between 7.2 and 7.5. Fig. 8 shows that in this pH range the K^+-stimulated K^+ transfer system is nearly maximally activated. With decreasing pH there is a continuous decrease of the rate constant. At the hematocrit used in these experiments, without added external K^+ the K^+ concentration in the medium is too low to activate the Ca^{++}-K^+-stimulated K^+ transfer (*cf.* Figs. 2, 4 and Table 1). Since the control flux contains no Ca^{++}-K^+-stimulated com-

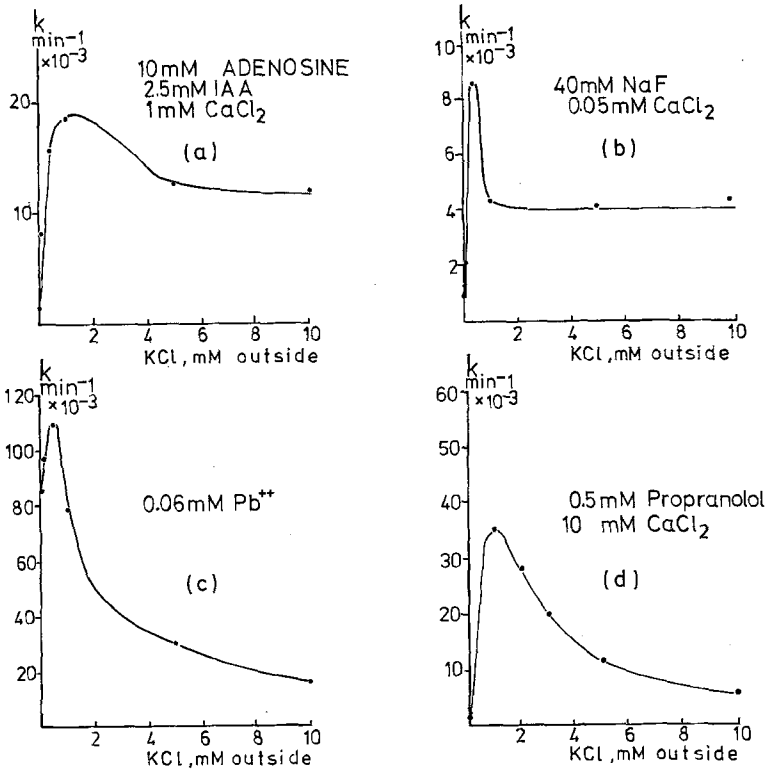

Fig. 7. Dependence of the rate constant for net K^+ efflux from intact cells on external K^+ concentration under various experimental conditions. A specific increase in K^+ permeability was induced by various means as indicated in each panel. The rate constant for net K^+ efflux, k, was calculated from maximal slopes as described in Materials and Methods. In *c* and *d*, the maximal slopes are identical to the initial slopes, but not in *a* and *b*. Isotonicity was maintained with sodium chloride. The other conditions were as follows: (*a*) pH 6.9, 25 °C, 0.25% hematocrit; (*b*) pH 7.05, 37 °C, 0.25% hematocrit; (*c*) pH 7.10, 37 °C, 0.25% hematocrit; (*d*) pH 7.5, 37 °C, 1% hematocrit. IAA = iodoacetate

ponent one may, therefore, conclude that the pH dependence of the control in the absence of external K^+ and that of the fully activated system are qualitatively similar.

Fig. 9 shows that the Ca^{++}-K^+-stimulated K^+ transfer in fluoride-treated intact cells is also markedly pH dependent, but the exact relationship to pH differs from that observed in ghosts in the absence of fluoride and with 8 instead of 0.05 mM Ca^{++} in the medium (Fig. 8). In the fluoride-poisoned intact cells, the Ca^{++}-K^+-stimulated K^+ transfer passes through a maximum around pH 6.8, while in the ghosts a maximal value is approached around pH 7.2. Moreover, in the fluoride-poisoned cells, at high pH the effect of external K^+ is very much smaller than in the ghosts.

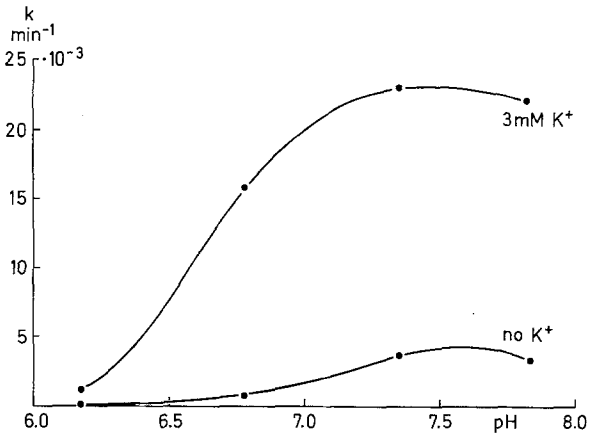

Fig. 8. Effect of external K^+ on the pH dependence of the rate constant for net K^+ efflux in the presence of external Ca^{++}. The ghosts contained 70 mM KCl, 70 mM choline chloride, and 20 mM Tris-HCl and were suspended in media containing 40 mM NaCl, 80 mM choline chloride, 8 mM $CaCl_2$, and 40 mM Tris-HCl. When KCl was added to the medium, it replaced an osmotically equivalent amount of choline chloride. The temperature was 37 °C and the hematocrit was 0.25%

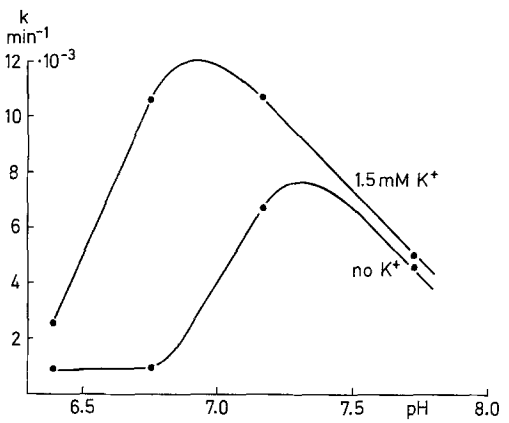

Fig. 9. Dependence of the rate constant for net K^+ efflux from intact cells on external pH in the presence of NaF with and without external K^+. The cells were suspended in media containing 40 mM NaF, 0.05 mM $CaCl_2$, 4 mM inosine, 10 mM glycylglycine, and either 112 mM NaCl or 110.5 mM NaCl and 1.5 mM KCl. The temperature was 37 °C and the hematocrit 0.25%. The rate constant for net K^+ efflux, k, was calculated from the maximal slopes as described in Materials and Methods. In contrast to all other experiments, except those in Fig. 7*a* and 7*b*, the maximal slopes used in this Figure are not identical to the initial slopes

These findings illustrate again that the Ca^{++}-K^+-stimulated transfer system exhibits characteristic differences if activated under different experimental conditions.

Table 2. Effect of external Na^+ in the absence of external K^+ on the rate constant for net K^+ efflux from K^+-loaded red blood cell ghosts

Na^+ concentration (mM)	Rate constant (min^{-1})
0	4.15×10^{-3}
10	6.40×10^{-3}
30	6.95×10^{-3}
60	8.90×10^{-3}
120	7.15×10^{-3}

Hematocrit 0.25%; Temp, 37 °C; pH 7.52. Ca^{++} in the medium 8.0 mM. The external Na^+ concentration was varied by isosmotic replacement of choline chloride with sodium chloride. The ghosts contained 70 mM KCl, 70 mM choline-Cl and 20 mM Tris-HCl.

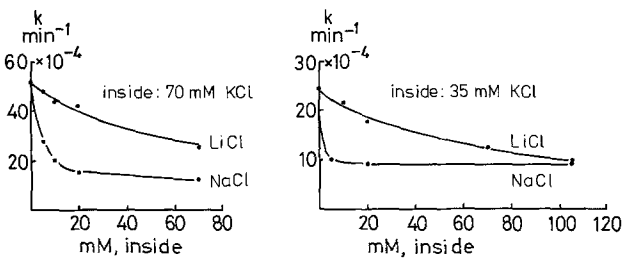

Fig. 10. Rate constant for net K^+ efflux in the presence of Ca^{++} as a function of Na^+ or Li^+ concentration inside the ghosts at two different values of internal K^+. The ghosts contained 20 mM Tris-HCl, the indicated concentration of K^+, the Na^+ or Li^+ concentration given on the abscissa, and enough choline chloride so that the total Na^+ or Li^+ plus K^+ plus choline concentration was 140 mM. They were suspended in media containing 140 mM choline chloride, 8 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4. The temperature was 37 °C and the hematocrit 5%. Although no K^+ was added to the medium, at this hematocrit there is sufficient external K^+ to partially activate K^+ efflux (*see* Fig. 2)

II. Interactions Between Sodium and Potassium

In the absence of external K^+, increasing the external Na^+ concentration leads to a slight activation of K^+ efflux (Table 2), although the activation is much less pronounced than with external K^+.

At stimulatory concentrations of external K^+, internal Na^+ (or Li^+) inhibits the Ca^{++}-K^+-induced K^+ efflux (Fig. 10). Increasing the external K^+ concentration up to 0.5 mM has little or no effect on this inhibition. However, if the external K^+ concentration is further increased, the inhibition by internal Na^+ decreases and at sufficiently high K^+ concentrations may completely disappear (Fig. 11). Conversely, the results could be de-

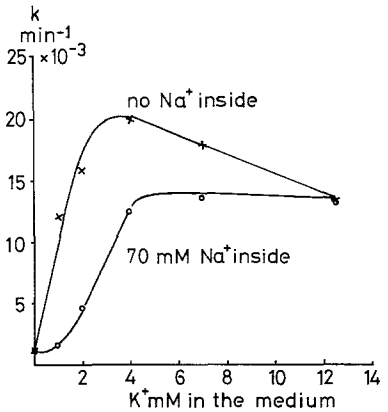

Fig. 11. Effects of internal Na^+ on the rate constant for net K^+ efflux in the presence of Ca^{++} as a function of external K^+ concentration. The ghosts contained 70 mM KCl, 70 mM choline chloride or NaCl, and 20 mM Tris-HCl. They were suspended in media containing 120 mM choline chloride, 8 mM $CaCl_2$, and 40 mM Tris-HCl. The external KCl concentration was varied by isosmotic replacement of choline chloride. The pH was 7.2, temperature 37 °C, and hematocrit 0.25%

scribed by stating that the effect of external K^+ is modified by internal Na^+. In other words, K^+ and Na^+ mutually interact across the membrane.

In the net flow experiments described above, the interactions between K^+ and Na^+ may be affected by variations of the membrane potential. Fig. 12 shows that, at low concentrations of external K^+, inhibition by Na^+ is also seen when K^+/K^+ equilibrium exchange is measured by means of ^{42}K at identical concentrations of K^+ and Na^+ on both sides of the membrane. At high K^+ concentrations in ghosts and medium, in five out of nine experiments increasing Na^+ concentrations on both sides of the cell membrane led to a stimulation of K^+ efflux by about 20%, except in one experiment, where the stimulation was 80%. In the other four experiments there was no effect of Na^+.

Table 3 supplements these findings by showing that a sodium gradient across the membrane does not affect the K^+/K^+ exchange at equal K^+ concentrations of 70 mM in ghosts and medium.

III. Effects of Magnesium and a Complexing Agent (EDTA)

It is apparent from Fig. 13 and Table 4 that varying the internal or external Mg^{++} concentration is without significant effect on the Ca^{++}-K^+-stimulated K^+ transfer. The Table further shows that external EDTA

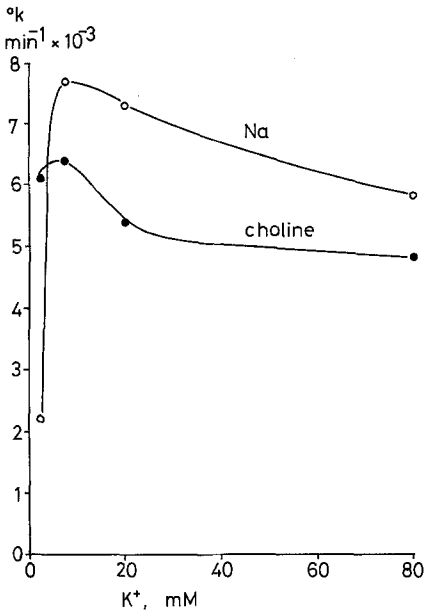

Fig. 12. Rate constant for ^{42}K exchange in the presence of Ca^{++} as a function of internal and external K^+ concentration. Both ghosts and media contained identical concentrations of K^+, Na^+ and choline, along with 20 mM Tris-HCl, pH 7.5–7.6. The media also contained 8 mM $CaCl_2$. The K^+ concentration was varied by replacement of NaCl (open circles) or choline chloride (closed circles), so that the sum of the concentrations of sodium or choline plus potassium was 140 mM. The temperature was 37 °C and the hematocrit 2.5%

Table 3. Effect of sodium on rate constant (^0k) of Ca^{++}-induced ^{42}K exchange at equal potassium concentrations inside the ghosts and in the media[a]

Exp. No.	Concentrations in the ghosts (mequiv/liter)			Concentrations in the media (mequiv/liter)			$^0k \times 10^3$ (min^{-1})
	KCl	NaCl	Choline-Cl	KCl	NaCl	Choline-Cl	
1	70	0	70	70	0	70	12.2
	70	70	0	70	0	70	11.7
	70	0	70	70	70	0	10.2
2	70	0	70	70	0	70	16.4
	70	70	0	70	0	70	20.1
	70	0	70	70	70	0	14.4
3	70	0	70	70	0	70	14.8
	70	10	60	70	0	70	14.0
	70	30	40	70	0	70	16.5
	70	70	0	70	0	70	18.7

[a] In contrast to the experiments presented in Fig. 12, sodium is not equally distributed between inside and outside. Other experimental conditions as in Fig. 12.

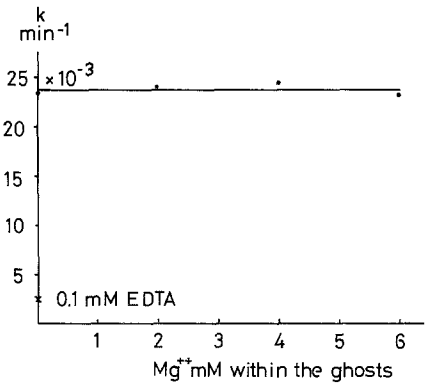

Fig. 13. Effect of Mg^{++} or EDTA on the rate constant for net K^{+} efflux as measured in the presence of external Ca^{++}. The ghosts contained 70 mM KCl, 70 mM choline chloride, 20 mM Tris-HCl and the indicated concentrations of $MgSO_4$ (●) or EDTA (×). They were suspended at 0.25% hematocrit in media containing 120 mM choline chloride, 8 mM $CaCl_2$, 2.5 mM KCl, 40 mM Tris-HCl, pH 7.5. The temperature was 37 °C

Table 4. Effect of external and internal Mg^{++} and EDTA on rate constant for Ca^{++}-induced net K^{+} efflux from human red blood cell ghosts

Exp. No.	Inside (mM)		Outside (mM)		$k \times 10^3$ (min^{-1})
	Mg^{++}	EDTA	Mg^{++}	EDTA	
1	8.0	0	0	0	24.4
	0	0	8.0	0	29.8
2	0	0	0	0	17.6
	0	0	8.0	0	15.7
3	4.0	0	0	0	16.7
	4.0	0	0	0.1	15.3
4	0	0.1	0	0	2.6
	0	0	0	0	23.5

The ghosts contained 70 mM KCl, 70 mM choline-Cl, 20 mM Tris-HCl, and the concentrations of EDTA and Mg^{++} indicated in the Table. The media contained 120 mM choline-Cl, 40 mM Tris-HCl, 8 mM $CaCl_2$, 2.5 mM KCl, and EDTA or $MgSO_4$ at the concentrations listed above. The internal concentration of $MgSO_4$ was varied by isosmotic replacement with choline-chloride. pH 7.5. Temperature 37 °C. Hematocrit 0.25%.

at a concentration which is much lower than the external concentration of Ca^{++} also exerts no effect. However, if EDTA is incorporated at this same low concentration into the ghosts in the absence of Mg^{++}, the Ca^{++}-K^{+}-stimulated K^{+} transfer is virtually abolished. The concentration dependence of the inhibitory effect of internal EDTA is presented in Fig. 14.

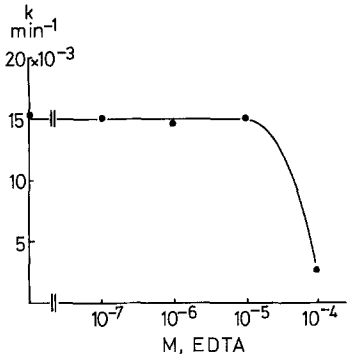

Fig. 14. Rate constant for net K^+ efflux in the presence of external Ca^{++} as a function of internal EDTA concentration. The ion concentrations were the same as in Fig. 13, except that the K^+ concentration in the media was 3 mM. The pH was 7.2, temperature 37 °C and hematocrit 0.25%

Discussion

It has been reported previously that low concentrations of external K^+ stimulate $^{42}K^+$ efflux from metabolically depleted red cells or ghosts, while high concentrations inhibit (Blum & Hoffman, 1971; Riordan & Passow, 1973). The present results confirm these findings and show in addition that the action of external K^+ does not simply reflect an enhancement of K^+/K^+ exchange by the Ca^{++}-activated transfer system, but instead an activation of that system which, in the presence of a K^+ concentration difference across the membrane, results in an increased net K^+ efflux. They demonstrate that external K^+ is an absolute requirement for the activation of the K^+ transfer system. It may be stated, therefore, that in the presence of Ca^{++}, K^+ induces the observed permeability change.

The described findings with red cell ghosts apply to a variety of other experimental situations in which certain divalent metal ions produce a similar net K^+ efflux in intact cells. In addition to lead poisoning these conditions include the action of Ca^{++} in the presence of fluoride, iodoacetate plus adenosine, or propranolol. The permeability change brought about under these diverse circumstances shows the following common features: (1) It is highly specific for K^+, while Na^+ permeability is little if at all affected (Passow, 1961; Kregenow & Hoffman, 1962; Ekman, Manninen & Salminen, 1969); (2) it can be inhibited by oligomycin (Blum & Hoffman, 1971; Riordan and Passow, 1971); (3) in all cases the rate of net K^+ efflux is one or two orders of magnitude faster than one would

expect from an inhibition of active transport; (4) under most experimental conditions, such as in intact cells after poisoning with Pb^{++} (Passow & Tillmann, 1955) or with Ca^{++} in the presence of triose reductone (Passow & Vielhauer, 1966) or propranolol (Manninen, 1970), or in ghosts after addition of Ca^{++} alone (Riordan & Passow, 1971), the time course of K^+ loss can be subdivided into an initial fast phase and a subsequent slow phase. The transition from the fast to the very much slower rate of K^+ loss may occur while the system is still far from equilibrium. The amount of K^+ lost during the rapid phase varies with the concentration of the divalent metal ion, while the rate constants of fast and slow K^+ loss are only slightly dependent on that concentration (*see* Riordan & Passow, 1971)[1]. The demonstration of similar effects of external K^+ under a variety of conditions adds another common feature which suggests that the described changes of K^+ permeability induced by divalent metal ions in intact cells in the presence of drugs or metabolic poisons are due to changes of the same K^+ transfer system which is modified by Ca^{++} in the experiments with ghosts described above[2].

In the past the similarity of the underlying mechanism was not readily apparent since the conditions under which the divalent metal ions elicit the described response show little resemblance. Among the three divalent metal ion species known to produce the effect (Ca^{++}, Mg^{++} and Pb^{++}), only Pb^{++} is capable of evoking the permeability change in freshly drawn intact cells. Ca^{++} acts only if added together with metabolic poisons such as fluoride or iodoacetate plus adenosine (Gardos, 1958, 1959; Lepke & Passow, 1960); or with certain drugs such as triose reductone (Passow & Vielhauer, 1966) or propranolol (Ekman *et al.*, 1969); or if applied to ghosts (Riordan & Passow, 1971; Blum & Hoffman, 1972). Mg^{++} can replace Ca^{++} in fluoride poisoning of intact cells, but neither in the Gardos

1 In the case of lead poisoning this behavior can be explained by an all-or-none effect in the individual cells: with increasing Pb^{++} concentration there is a shift of the rate of K^+ loss from a low to a high value in an increasing number of cells (Passow & Tillmann, 1955). It is not clear whether this all-or-none behavior also applies to the effects produced with Ca^{++} or Mg^{++}. In fluoride poisoning another type of all-or-none effect has been demonstrated (Eckel, 1958). With increasing time an increasing number of cells undergo the permeability change, until finally all of the cells of the population are modified. Perhaps in these experiments the concentration of alkaline earth ions was sufficient to modify the permeability in all of the cells, but the time at which an individual cell becomes responsive to Ca^{++} or Mg^{++} depends on the time at which its ATP concentration falls below a certain level.

2 Perhaps the inhibition of K^+ efflux by internal Na^+ may also be a common feature, since it has not only been observed in Ca^{++}-treated ghosts but also in ghosts exposed to Pb^{++} (Passow, 1969).

effect [i.e., in the presence of iodoacetate plus adenosine (Lepke & Passow, 1960)] nor in experiments with ghosts (*see* Table 4).

The examples described above suffice to illustrate that some divalent cation is always required to evoke the permeability change but that under different experimental conditions different divalent metal ion species may be needed. Hence, without additional information concerning the mode of action of these cations, it seems difficult to define the transport system in terms of the divalent cations and the corresponding experimental conditions necessary to induce the permeability change. In contrast, the features mentioned above which are unrelated to divalent cations, but common to the K^+ loss in all of these various circumstances are better suited to characterize the transport system.

Since the K^+ concentration in the medium is too low to influence directly the K^+ concentration inside the ghosts, one may ask how the extracellular K^+ concentration controls net K^+ efflux. It is unlikely that a reduction of the concentration difference between ghosts and medium is a major factor in the observed stimulation, since the variation of that difference is small. The possibility cannot be excluded that a reduction of the K^+ gradient contributes to the observed inhibition at higher K^+ concentrations, although it should be pointed out that K^+-K^+ exchange is definitely inhibited (*see* Figs. 6 and 12 and Blum & Hoffman, 1971). The transmembrane potential does not seem to be a decisive factor, because a simultaneous increase of internal and external K^+ at zero concentration difference across the membrane has essentially the same effect as increasing the external K^+ at a fixed and high concentration of internal K^+ (compare Figs. 6 and 3).

It has been suggested that Ca^{++} exerts its action at the inner membrane surface (Whittam, 1968; Lew, 1971; Romero & Whittam, 1971; Blum & Hoffman, 1972). Since Ca^{++} influx into metabolically depleted cells depends on the external K^+ concentration, it would seem conceivable that external K^+ regulates Ca^{++} influx and thus controls the Ca^{++} effect at the inner membrane surface (Lew, 1974). The stimulation by low K^+ concentrations can certainly not be explained in this manner, since at K^+ concentrations between 0.02 and 4 mM in the medium, the rate of Ca^{++} uptake is maximal and constant. At high K^+ concentrations, Ca^{++} uptake is inhibited by external K^+ with a K_i of about 40–50 mM (Lew, 1974). This could explain why higher concentrations of external K^+ inhibit net K^+ efflux. In the experiments with ghosts described in this paper and in lead poisoning of intact cells (Grigarzik & Passow, 1958) the permeability change takes place as fast as one can measure it, even if the metal

is added in the presence of a high K^{+} concentration in the medium. Thus, at least in these cases, the inhibition of divalent cation uptake does not seem sufficient to account for the inhibition of the K^{+} transfer system by high external K^{+}.

The discussion above suggests that the effect of external K^{+} can neither be ascribed to changes in driving forces for K^{+} movements nor to changes in divalent metal ion influx, although these factors may modulate the observed effect. We infer, therefore, that K^{+} stimulation and inhibition of net K^{+} efflux are associated with binding of K^{+} to specific control sites at the outer cell surface. The apparent dissociation constant of the activating sites is 0.7 ± 0.15 mM; that of the inhibiting sites is much higher.

Like external K^{+}, internal K^{+} can stimulate net K^{+} efflux. This stimulation does not simply represent an increase in driving force but rather an activation of the K^{+} transfer system. Our experiments were inadequate, however, to decide whether or not internal K^{+} is an absolute requirement for the K^{+} permeability increase.

In ghosts exposed to Pb^{++} or Ca^{++}, K^{+} efflux is inhibited by internal Na^{+}. One of the most remarkable features of the K^{+} transfer system is the interdependence of inhibition by internal Na^{+} and activation by external K^{+}. External K^{+} may either reduce the affinity of internal Na^{+} sites or affect their accessibility to Na^{+}. Conversely, internal Na^{+} may affect the binding of external K^{+} to the K^{+} control sites. This dependence demonstrates again that the system is capable of transferring information about the external K^{+} concentration across the membrane. It is possible that Na^{+} on the inside or K^{+} on the outside exert an allosteric effect on a molecule which spans the membrane.

The complexity of these ionic interactions as well as certain observations concerning the action of ouabain have led Blum & Hoffman (1971) to speculate that the Na^{+}-K^{+} pump mediates the K^{+} movements.

Recently, however, Lew (1974) has pointed out that the substrate-depleted red cells of certain animal species do not respond to the action of Ca^{++}, although they are capable of accomplishing active transport by means of the K^{+}-Na^{+}-Mg^{++}-activated ATPase. Similarly, it has been indicated (Dunker & Passow, 1953) that Pb^{++} or fluoride induce the K^{+} permeability change in human rabbit and rat erythrocytes but not in those of the horse or the pig, even though red cells from all of these species actively transport sodium and potassium (Shaw, 1955; Kirschner & Harding, 1958). These findings support the view that the pump is not involved. Nevertheless, the specificity and complexity of the ionic interactions lead one to suspect that the K^{+} permeability change is a manifestation

of a specific system with functional significance in the intact cell. In contrast to the action of a rigidly coupled pump flow of K^+ and Na^+, such a K^+-specific leak mechanism, operating in conjunction with the K^+-Na^+ pump, would permit independent regulation of cell volume and K^+ concentration (Passow, 1961, 1963).

The present paper is essentially confined to adding further information for an empirical description of the relationships between various variables which control the permeability change. No attempt was made to provide a discussion of specific molecular mechanisms by which these variables may exert their effects since despite the work in many laboratories the empirical description of these relationships is still incomplete and since some variables may still be unknown. The existence of as yet unknown variables is suggested by the variability of the absolute values of the rate constants reported in this paper. The ambiguities of attempts for an interpretation in terms of molecular mechanisms is easily exemplified by considering the effects of pH and complexing agents. The observed pH dependence of the net K^+ loss could be associated with a competition between H^+ ions and either Ca^{++} or K^+, with a noncompetitive effect at a third binding site, or with any combination of such effects. The inhibition by internal EDTA in the presence of external Ca^{++} would simply seem to support the theory that the K^+ permeability increase is prevented by sequestration of penetrating Ca^{++} inside the ghosts. However, EDTA inhibits at a concentration which is lower than the Ca^{++} concentration expected to obtain in the ghosts under our experimental conditions (*see* Porzig, 1972). EDTA might therefore exert its effect either by inhibiting Ca^{++} uptake or else by forming a ternary complex with a divalent metal ion normally present in the membrane, thereby preventing the displacement of that divalent metal ion by Ca^{++}. Obviously, further experimental work is needed until one could think of a theoretical interpretation of the effects of divalent cations on alkali ion permeability.

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