Intracellular Potassium

A Determinant of the Sodium-Potassium Pump Rate

ANNE B. KNIGHT and LOUIS G. WELT

From the University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514. Dr. Knight's present address is the Department of Physiology, Duke University School of Medicine, Durham, North Carolina 27710. Dr. Welt died 13 January 1974. At the time this article was submitted he was chairman, Department of Internal Medicine, Yale University.

ABSTRACT Normal human red cells which have had their intracellular sodium (Na_c) reduced have a diminished Na-K pump rate, but only if intracellular potassium (K_c) is high. If most of the K_c is replaced by tetramethylammonium or choline, both ouabain-sensitive Na efflux and K influx are significantly increased even with Na_c below normal. Cells with reduced Na_c and high K_c have an unchanged Na efflux if external potassium (K_{ext}) is removed. In contrast, low-Na, low-K cells have a large ouabain-sensitive Na efflux which shows a normal response to removal of K_{ext}. Neither low-K nor high-K cells have an altered ouabain-sensitive K efflux. Measurement at constant low Na_c and varying K_c shows the pump Na efflux to be an inverse function of K_c. Thus, in low-Na cells, K_c appears to act as an inhibitor of the pump. Inhibition by high K_c can be seen even when Na_c is normal. The effects attributed to K_c are distinguished experimentally from other variables such as cell volume, adenosine triphosphate concentration, effects of the replacement cations, and the method used to alter intracellular cation concentrations. A role is proposed for K_c, in cooperation with Na_c, in regulating the pump rate of normal human red cells.

INTRODUCTION

The human red blood cell maintains a high intracellular potassium (K_c) and low sodium (Na_c) apparently by balancing passive movements of the ions with their active transport in the opposite direction. Thus the cell extrudes Na and accumulates K in a linked process which is sensitive to ouabain and other cardiac glycosides (Schatzmann, 1953). The component of Na-K transport sensitive to ouabain is termed the Na-K pump, is dependent on adenosine triphosphate (ATP) in the cell (Hoffman, 1960), and is associated

with a ouabain-sensitive, Na-K-Mg-stimulated adenosine triphosphatase (ATPase) located in or on the cell membrane (Post et al., 1960; Skou, 1965).

Pump activity is known to be a function of its substrate ions, reaching maximal rates at approximately 40 mM Na/liter cells (Post and Jolly, 1957) and an external K (K_{ext}) of 10 mM (Glynn, 1956). Graphs of ouabain-sensitive Na efflux or K influx vs. Na_c or K_{ext} give sigmoidal relationships (McConaghey and Maizels, 1962; Sachs and Welt, 1967), although the deviation from Michaelis-Menten behavior is only apparent at very low Na_c or K_{ext}.

The sigmoidal behavior of the fluxes as a function of K_{ext} has been interpreted both as evidence for a requirement for more than one K for inward transport (Sachs and Welt, 1967) and as evidence that external Na (Na_{ext}) competes with K_{ext}, since the curve becomes a rectangular hyperbola when Na_{ext} is replaced by choline, tetramethylammonium (TMA), Mg, or Tris (Garrahan and Glynn, 1967 *b*; Sachs, 1967). The actual explanation may be a combination of the two. Precisely the same behavior has been observed when the Na-K-stimulated ATPase activity of fragmented membranes (ghosts) is measured as a function of K with constant high or low Na (Post et al., 1960).

In contrast to the two interpretations given to the behavior of the pump in response to K_{ext}, the sigmoidal response of the fluxes to varied Na_c has been interpreted only as evidence that more than one Na is required for outward transport (Sachs, 1970). It has been shown, however, that the Na- K-stimulated ATPase activity of fragmented ghosts graphed as a function of Na becomes a rectangular hyperbola when the K of the medium is reduced (Post et al., 1960), suggesting that the behavior of K_c could resemble that of Na_{ext}. Such an inhibitory effect of internal K on ATPase and K influx rates has been looked for in resealed ghosts with conflicting results, Hoffman (1962 *b*) concluding that high internal K affects the pump and Whittam (1962) that it does not.

Possible effects of K_c on the pump have not been studied previously in intact human cells, however, and it will be demonstrated in this paper that the pump is inhibited by high K_c particularly when the Na_c is low, in a manner analagous to the inhibitory effect of Na_{ext}.

METHODS

Blood for all experiments was obtained in heparinized syringes from normal human males, and the cells were separated by centrifugation and aspiration of the supernatant plasma. The cells were always washed three times before use by resuspension in isotonic tetramethylammonium chloride (300 mosm TMACl) solution (170 mM), centrifugation, and aspiration of the supernatant wash.

For most experiments the cation concentrations of the cells were altered by treatment with *p*-chloromercuribenzene sulfonic acid (PCMBS), which increases the

cell's permeability to Na and K (Garrahan and Rega, 1967). Washed cells were incubated at a 5% hematocrit for about 20 h at 4°C in isotonic solutions containing (mM): phosphate 20, chloride 150, adenine 3, inosine 1, glucose 14, PCMBS 0.1, varying concentrations of Na, K, and TMA to a total of 170 mM, and kanamycin sulfate (100 mg/liter). The PCMBS-treated cells were restored to approximately normal permeability by incubation for 30 min at 37°C in identical solutions except that 2 mM cysteine replaced the PCMBS. The cysteine-treated cells were triply washed with isotonic TMACl before further use. The above procedure is the method of Garrahan and Rega (1967) with these modifications: an increased amount of buffer, the inclusion of energy substrates, and the use of TMA to replace some Na or K when desired.

For some experiments, the cation concentrations of the cells were altered by cold storage for 1 wk at a 5% hematocrit in similar solutions except for the absence of PCMBS and cysteine. During this storage at 4°C the cells were resuspended in fresh solutions three or four times.

Na efflux measurements were made as follows: PCMBS-cysteine-treated or cold-stored red cells were incubated at approximately a 50% hematocrit in solutions with the same composition as those in which they had been pretreated, with ^{22}Na present in the media. The isotope-loaded cells were washed five times with isotonic TMACl and were placed in prewarmed (37°C) flasks in a gyrorotary water bath at a 0.02% or less hematocrit in media containing (mM) Mg 6, carbonate 6, phosphate 1.2, Cl 140, glycylglycine 29, glucose 11, Na 130, and K 10, with a pH of 7.4 at 37°C. When it was desired to decrease either the Na or K in the medium, portions of these cations were replaced by osmotically equivalent amounts of TMA. Some of the flasks also contained 10^{-4} M ouabain. After a 15–20-min equilibration period, four samples were taken from each flask over a 1-h period, and after centrifugation, 5 ml of the supernatant solutions were quantitatively transferred to counting tubes. A further sample was taken (at any convenient time), the cells lysed with two drops of nonionic detergent (Acationox), and 5 ml of these hemolysates were also transferred to counting tubes. Counting was done on a Packard Auto-gamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), usually to a minimum of 10,000 counts above background, for a maximum counting error of 1%. The leftover supernatant solutions and hemolysates were used for hemoglobin estimation by measuring absorption at 420 nm in a Coleman Jr. II Spectrophotometer (Coleman Instruments Div. Perkin Elmer Corp., Maywood, Ill.). Dividing the absorption of each supernate by that in its hemolysate gave an estimate of the fractional hemolysis (usually less than 3%). Then, $^{0}k_{Na}t = -$ [ln $(1 - Na^*_s/Na^*_h]/(1 -$ fractional hemolysis), where $^{0}k_{Na}$ = the rate constant for Na efflux (h^{-1}), t = time (h), Na^*_s = activity of the supernatant (counts per minute per milliliter), and Na^*_h = activity of the whole-suspension hemolysate (counts per minute per milliliter). The rate constants were determined by calculating the slope of the best line (by least squares) of values for the right-hand side of the above equation vs. t. Then the flux, $^{0}M_{Na} = (^{0}k_{Na})(Na_c)$. Since a measurement of Na_c was also necessary, isotope-loaded cells were also incubated in media identical to the flasks used for measurement of $^{0}k_{Na}$ but at a slightly higher hematocrit. These flasks were sampled exactly midway through the flux measurement, and Na_c and K_c were determined on the cells, to be used in the above equation for calculation of $^{0}M_{Na}$.

The above procedure differs from the method of Sachs and Welt (1967) only in the istotope-loading media used and in the inclusion of midpoint samples for Na_c and K_c. The former modification was necessary to maintain the abnormal cation content of the cells before the flux measurement, and the latter was added because red cells tend to gain or lose ions in flux media, particularly when their Na_c has been raised or lowered. Therefore, a midpoint sample was considered to be more representative of the actual Na_c during the flux measurement than an initial Na_c would be.

K efflux was measured by the same method as for Na efflux, except the cells were loaded with ^{42}K for 3 or 3½ h.

K influx was determined by the method of Sachs and Welt (1967) with minor modifications necessitated because K influx was always measured simultaneously with Na efflux determination. Therefore, cells to be used for K influx were first incubated for 3 h in media exactly like the ^{22}Na-loading media except for the absence of ^{22}Na. Flasks containing solutions as described for Na efflux, but containing ^{42}K, were prewarmed in a gyrorotary water bath, and washed cells were added to the flasks to approximately a 5% hematocrit. Two samples were taken 1 h apart; the supernatant solutions were saved and diluted appropriately for counting; the cells were washed rapidly three times with cold isotonic TMACl solution and lysed with two drops of nonionic detergent. The lysed cells were diluted to a convenient volume (usually 6 ml) and exactly 5 ml of each hemolysate were transferred to counting tubes, the remainder being used for hemoglobin determination by the cyanomethemoglobin method. The diluted supernatant solutions and the hemolysates were counted as for the Na efflux method, with correction made for counts lost by decay made when necessary. Then, ${}^{i}M_K = (\Delta K^*_c)/(K^*_s/K_s)$, where ${}^{i}M_K$ = the K influx (millimoles per liter cells per hour), ΔK^*_c = the increase in activity (counts per minute per milliliter) in the cells over exactly 1 h, and K^*_s/K_s = the specific activity of the supernatant solution. For the calculation it was necessary to know the volume of cells hemolyzed and counted; therefore, hemoglobin (by the cyanomethemoglobin method) and hematocrit were determined on a sample of cell suspension used for the flux, and $v = (vol_h \cdot hgb_h)/(hgb_{rs}/hct_{rs})$, where v = ml of cells counted, vol_h = ml of hemolysate counted, hgb_h = g/ml of hemoglobin in the hemolysate, hgb_{rs} = g/ml hemoglobin of the reference suspension of cells, and hct_{rs} = fractional hematocrit of the reference suspension.

For all flux measurements, the pump was taken to be the flux in the absence of ouabain minus the flux in the presence of 10^{-4}M ouabain. Na and K were measured simultaneously on an Instrumentation Laboratories flame photomometer (Instrumentation Laboratory, Inc., Lexington, Mass.) with Li as the internal reference. For determination of Na_c and K_c, the cells were always washed three times with Na- and K-free isotonic TMACl solution, and Na, K, (in duplicate), and hematocrit (in triplicate) were measured on the washed-cell suspension. Then, millimoles per liter cells of Na (or K) = mM Na (or K) in the suspension ÷ hematocrit of the suspension.

All results presented are the means of at least duplicate flux measurements which differed from each other by no more than 5%. The single exception is Fig. 5 in which single data points from three entirely separate experiments are given.

Intracellular ATP concentration was measured on a cell hemolysate by the method

of Dufresne and Gitelman (1970), a semiautomated procedure in which ATP is reacted with a luciferin-luciferase firefly extract, and the resulting light flashes are counted in a Packard Tri-Carb liquid scintillation spectrometer.

Percent cell water was usually determined by weighing red cell suspensions of known hematocrit both wet and after being dried for 24 h at 100°C. Then, % cell water = 100 [wet-dry)/(wet-tare) 100 − (100 − % hct)]/ % hct.

Cell chloride concentration was measured on cells packed at about ×30,000 *g* for 5 min in specially constructed lucite tubes with a 1-cm diameter well (the remaining packed cells being used for water determination). The cells were hemolyzed and diluted 20-fold with water: 1 ml of this hemolysate plus 3 ml water was treated with 0.5 ml of 0.7 N $ZnSO_4$ − 0.25 N H_2SO_4 and 0.5 ml of 0.75 N NaOH to deproteinize the hemolysate, and 1 ml of the resulting clear supernatant solution was reacted with 3 ml chloride reagent (0.1 N HNO_3 − 10% acetic acid) and titrated on a Buchler Cotlove chloridometer automatic titrator (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N. J.) with appropriate standards. Chloride was also determined on the solution in which the cells had been packed (0.1 ml supernate plus 4 ml chloride reagent). The chloride ratio (Cl_c per kilogram cell water/Cl_{ext} per kilogram external water) was then used to calculate the membrane potential using the Nernst equation, $E_m = -0.058 \log (Cl_c/Cl_{ext})$, where E_m is the transmembrane potential in volts.

Oxmolality was measured by freezing-point depression using an Advanced osmometer (Advanced Instruments, Inc., Needham Heights, Mass.). ^{22}Na and ^{42}K were obtained as the chlorides in water from International Chemical & Nuclear Corporation, Burbank, Calif. or from Cambridge Nuclear Radiopharmaceutical Corporation, Cambridge, Mass. Choline chloride (Nutritional Biochemicals Corporation, Cleveland, Ohio) was purified before use by recrystallization from 5% ethanol. Sigma Chemical Co., St. Louis, Mo. was the source of adenine, inosine, PCMBS, L-cysteine, and ouabain. Kanamycin sulfate solution was from Bristol Laboratories, Syracuse, N. Y.

RESULTS

Effect of Intracellular K on Na Efflux and K Influx in Low-Na Cells

Normal cells were divided into two groups, and both were treated with PCMBS solutions containing either 170 mM K or 30 mM K plus 140 mM TMA. Both groups of cells thus became very low in Na, but with intracellular K either slightly above normal or about 60% below normal. Ouabain-sensitive Na efflux was then measured on five such paired groups of cells, and K influx was measured simultaneously in three of the experiments. The results are shown in Fig. 1. Both ouabain-sensitive Na efflux and K influx are significantly reduced in low-Na, high-K cells. This reduction in both halves of the coupled pump cannot be explained by the lowered Na_c alone because low-Na cells with the K_c also reduced show a very large pump flux. Thus both aspects of the coupled pump are reduced when only the Na_c is lowered, and increased when the K_c is also lowered. The ouabain-sensitive Na efflux from low-Na cells shows an inverse response as K_c is varied (Fig. 2) both in the presence of saturating K_{ext} (10 mM) and in the absence of K_{ext}.

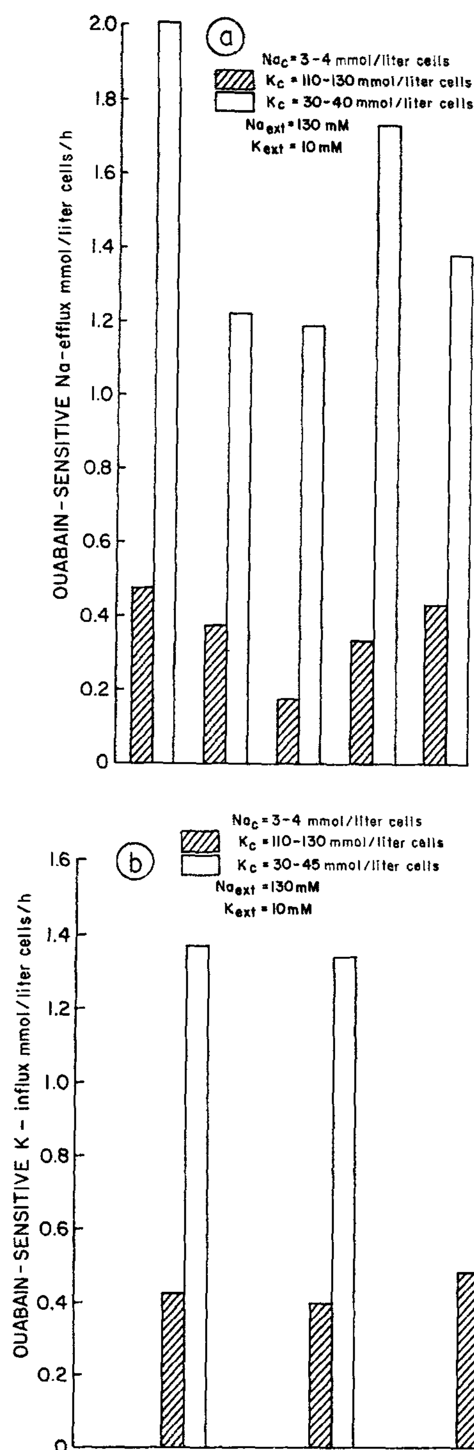
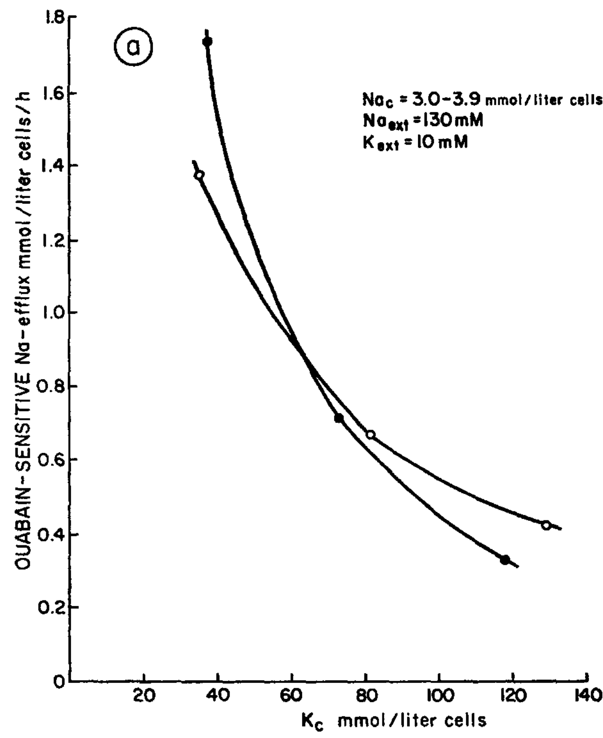


FIGURE 1. Effec of intracellular K (K_c) on ouabain-sensitive Na efflux (1 *a*) and K influx (1 *b*). Cells were treated with PCMBS, followed by cysteine, to alter intracellular cation concentrations, as described under Methods. TMA was used to replace K_c in the low-K cells. In this and the following figures, the standard flux medium was used, with only the Na, K, and TMA of the media being varied. This standard medium contained (millimoles per liter) 6 $MgCO_3$, 1.2 phosphate, 29 glycyl-glycine (pH 7.4), 140 Cl, 11, glucose, and Na, K, and TMA as described in each figure.



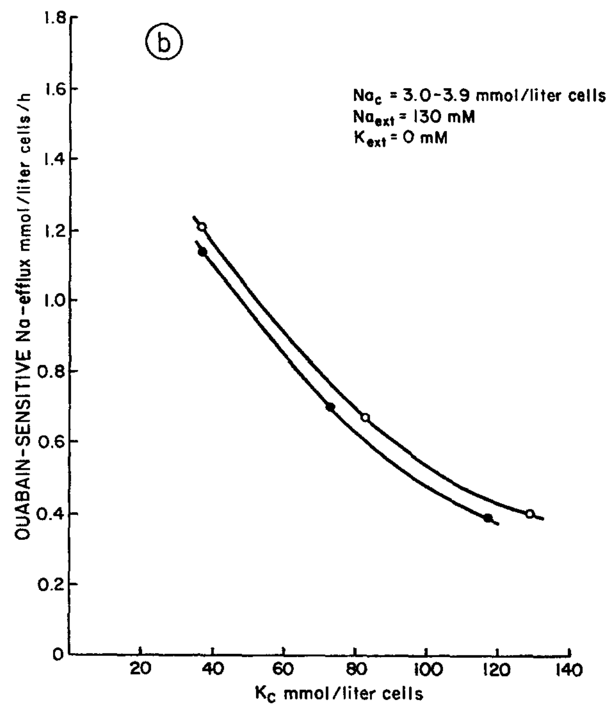


FIGURE 2. Response of ouabain-sensitive Na efflux to K_c at constant low Na_c, with saturating K_{ext} (2 *a*) or zero K_{ext} (2 *b*). Cells were PCMBS-treated to alter intracellular cation concentrations, TMA being used to replace K_c.

Response of Low-Na Cells to External K

The ouabain-sensitive Na efflux from normal red cells, as reported by Garrahan and Glynn (1967 *a*) is reduced by about one-third when K_{ext} is reduced from saturating concentrations to zero as long as Na_{ext} is kept high. With zero K_{ext}, the pump is believed to perform Na-Na exchange instead of Na-K exchange, and the unidirectional efflux of Na is one-third slower when the cells are exchanging Na than when K is available externally.

Comparison of Fig. 2 *a* and 2 *b* suggested that the K_c has some effect on this sensitivity of the cells to K_{ext}. Data relevant to this point are shown in Table I. Both groups of cells in this table have a low Na_c, but those with a high K_c show the same low ouabain-sensitive Na efflux whether or not K_{ext} is present. In contrast, low-Na cells with a low K_c show a sensitivity to K_{ext} typical of normal cells.

Table I also includes cells in which K_c was replaced by choline, with exactly the same results as when TMA was used.

The very small ouabain-sensitive Na efflux of low-Na, high-K cells, com-

TABLE I

EFFECT OF REMOVAL OF K_{ext} ON ${}^{o}M_{Na}^{o-s}$

Na_c	K_c	${}^{o}M_{Na}^{o-s}$		Difference
		K_{ext} = 10 mM	K_{ext} = 0 mM	
(mmol/liter cells)				
I High K_c cells				
2.3	117	0.11	0.19	−0.08
2.9	129	0.10	0.14	−0.04
2.9	127	0.26	0.27	−0.01
3.4	129	0.43	0.40	0.03
3.4	120	0.27	0.33	−0.06
3.7	117	0.33	0.39	−0.06
4.2	125	0.46	0.44	0.02
II Low K_c cells				
3.2	36	1.38	1.22	0.16
3.4	37	1.50	1.12	0.38
3.5	38	1.73	1.14	0.59
4.2	50	1.74	1.08	0.66
III				
2.8	23	1.61	0.71	0.90
3.4	28	1.72	1.06	0.66

Cells were treated in PCMBS solutions, as described under Methods, with either high K (group I), high TMA (group II), or high choline (group III), to obtain cells with low Na_c and high or low K_c. Na-efflux measurements (millimoles per liter cells per hour) were carried out in the standard flux media, with Na_{ext} = 130 mM and either K_{ext} or TMA_{ext} = 10 mM.

bined with the lack of response to K_{ext} of such cells, suggests the possibility that the active Na-K transport of the cells is almost entirely turned off by the high K_c and the small transport ability remaining is equally as capable of performing Na-Na exchange as Na-K exchange.

Studies of Net Na Movement

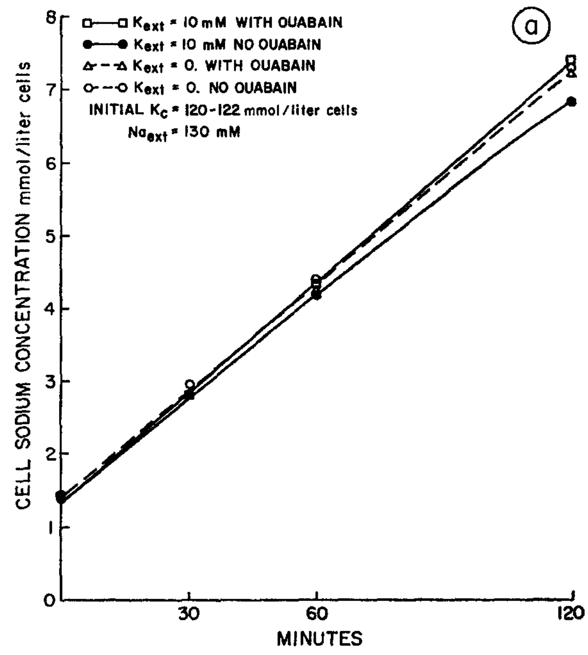
Low-Na cells are not in a steady state, and since the determination of Na efflux rate by tracer measurements is based on the assumption that the midpoint Na_c represents the average Na_c during the flux measurement period, a number of nonisotopic measurements of Na movement were made on low-Na cells, both to check the validity of the results already presented, and to confirm that a midpoint Na_c measurement represents a true mean in such cells.

Fig. 3 *a* shows such a study on cells with an initially low Na_c and high K_c, where the total Na_c was measured in samples taken over a 2-h period. In these cells neither the removal of K_{ext} nor the presence of ouabain significantly affected the initial rate of accumulation of Na, which agrees with the ^{22}Na measurements. As the Na_c increased, the net Na accumulation rate showed a slight ouabain-sensitive decrease in the presence of K_{ext}, indicating that the pump is accomplishing a small net Na extrusion as the Na_c approaches normal. Furthermore, the gain of Na is linear under all conditions for the full 2-h period, making a midpoint measurement of Na_c a valid estimate of the mean concentration during the flux period. As previously noted, isotopic fluxes were measured over 1 h, starting 15 min after addition of the cells to the media.

Fig. 3 *b* shows similar data for cells with an originally low Na_c and low K_c, and it can be seen that both ouabain and K_{ext} have significant effects on these cells. For uninhibited cells, the removal of K_{ext} increases the rate of Na accumulation, and the addition of ouabain increases it further. In the absence of K_{ext} the cells show no effect by ouabain, as expected, since Na-Na exchange does not result in net Na extrusion. Thus, these results agree with the ^{22}Na measurements on low-K cells. In addition, the gain of Na is linear for the full 2 h, except in the case of uninhibited cells in the presence of K_{ext}, and in this case linearity is approximated over the period of concern for tracer measurements.

Effect of K_c on K Efflux

Although the K efflux observed from normal human red cells is movement along its electrochemical gradient, a small portion of this efflux is inhibited by ouabain and has been attributed to K-K exchange through the pump (Glynn and Luthi, 1968). This ouabain-sensitive K efflux can also be observed in the absence of K_{ext} in a high-Na medium, and under these condi-



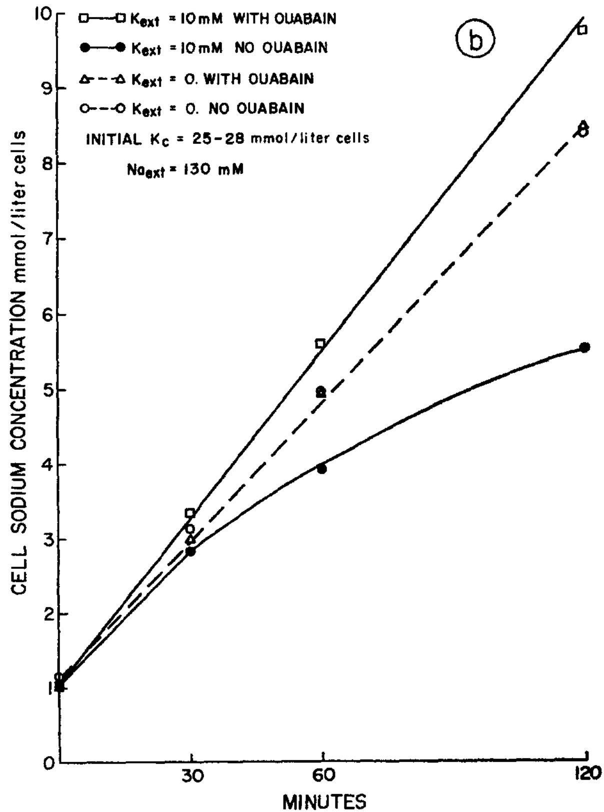


FIGURE 3. Comparison of net Na gain by low-Na cells, with either high (3 *a*) or low (3 *b*) K. PCMBS-treated cells. Each graph is representative of three experiments.

tions it is thought to represent an entire reversal of the pump (Glynn and Lew, 1970). In both cases, the K efflux has been demonstrated to be a specific one and not simply a lack of discrimination between Na and K by the carrier (Glynn et al., 1970). This observation makes it unlikely that the inhibitory effects of K_c observed here are due to a simple substitution and transport by K at Na sites on the carrier. In line with this idea, Fig. 4 shows that the ouabain-sensitive portion of the K efflux from low-Na cells is not decreased when K_c is lowered to 24 mM cells.

It may be that a much larger reduction of K_c would affect the ouabain-sensitive K efflux from low-Na cells, but it seems most likely that a high K_c simply interferes with the outward transport of Na from such cells without itself being carried at a faster rate.

Effect of K_c at Normal and High Levels of Na_c

The question next arises whether the high K_c of human red cells is inhibitory to the pump at Na_c levels in the normal range. All prior investigations of the effect of varying Na_c on the pump rate in human cells have been done by simultaneously varying the K_c in a reciprocal manner. Thus, in previous work, the K_c has been low only when the Na_c has been high.

Fig. 5 shows experiments measuring ouabain-sensitive Na efflux as a function of Na_c, with the K_c held constant at two levels. The Na_{ext} was largely replaced by TMA in order that the Na_c might remain low throughout the period of measurement. The K_c of cells represented by the solid line is the same as in normal cells and the Na_c's include the entire normal range. Fig.

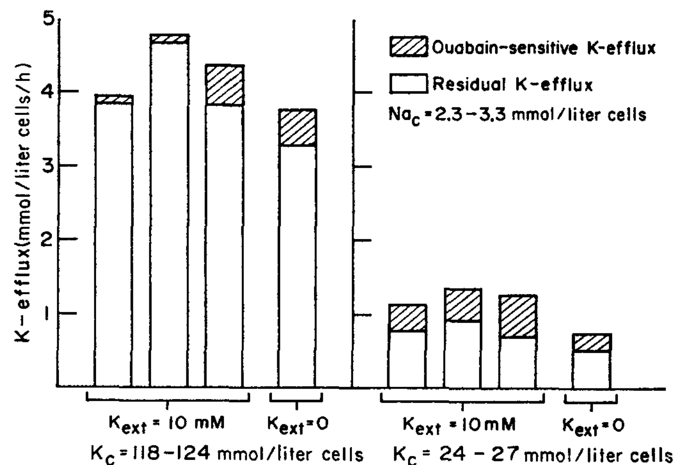
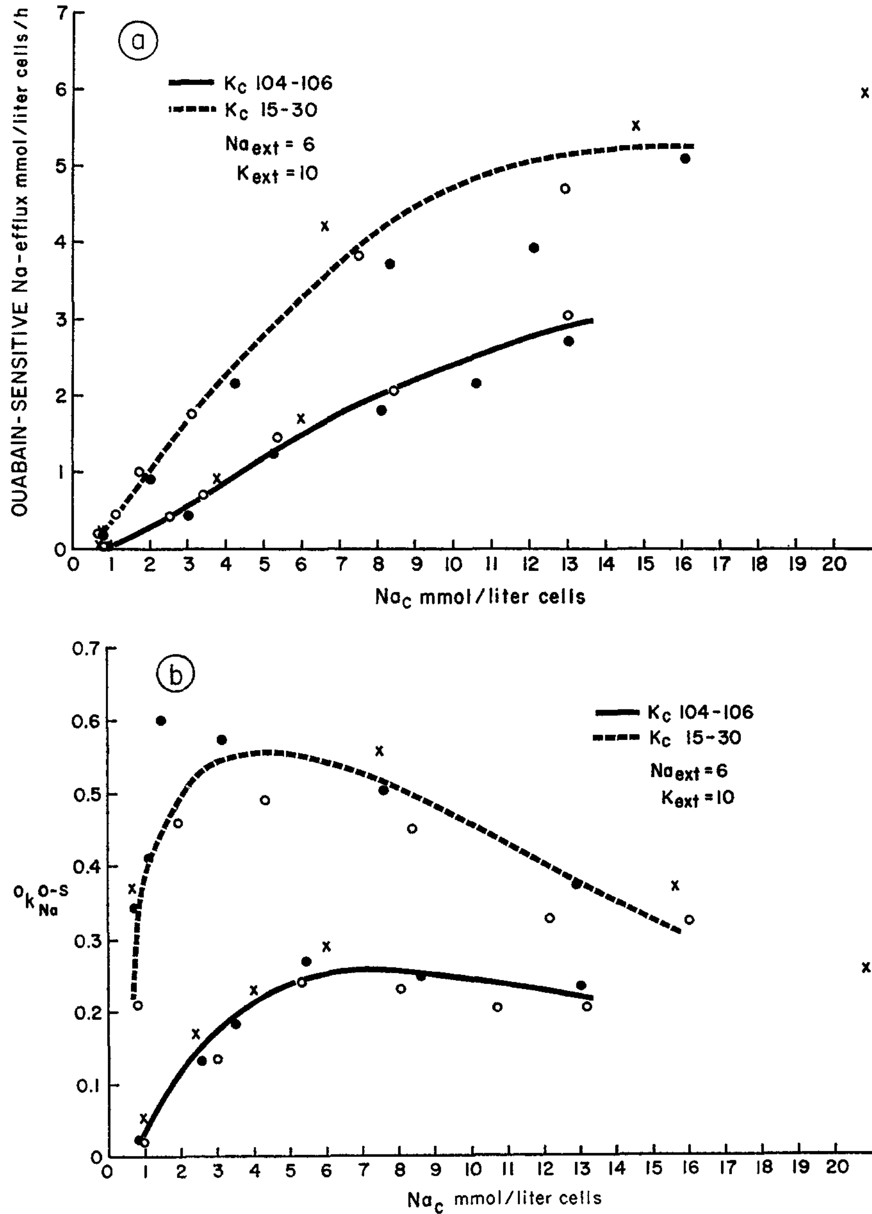


FIGURE 4. K efflux from low-Na cells with high (left group) or low (right group) K_c. Na_{ext} = 130 mM. Each bar represents total K efflux, with subdivisions for ouabain-sensitive and ouabain-insensitive components. PCMBS-treated cells.

5 *a* thus indicates that the normally high K_c of human red cells is inhibitory to the pump when compared to cells with a normal Na_c and a reduced K_c. In Fig. 5 *b*, the rate constant for ouabain-sensitive Na efflux is plotted against Na_c, and it can be seen that the peak of the curve is shifted to the left in low-K cells, as one expects when the concentration of an inhibitor is reduced.



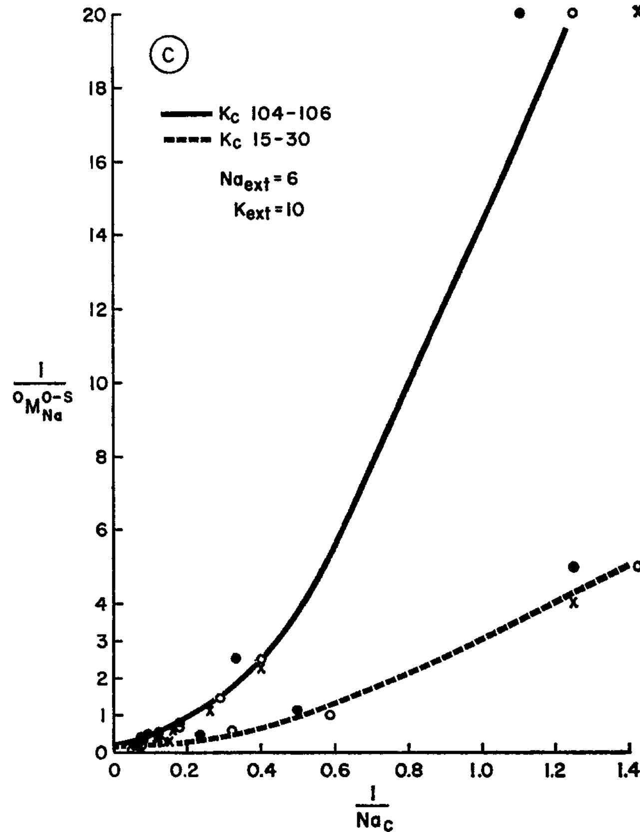


FIGURE 5. Ouabain-sensitive Na efflux from PCMBS-treated cells with varying Na_c and two levels of K_c (millimoles per liter cells). Results (of three experiments) expressed as efflux (5 *a*), as the rate constant (h^{-1}) for efflux (5 *b*), and as a double-reciprocal plot (5 *c*).

Fig. 5 *c* is a double reciprocal plot of the data of Fig. 5 *a*: 1/flux versus $1/Na_c$ at two K_c levels. The line for high-K cells has a much larger slope and curvature than that for low-K cells.

Several measurements were made of the ouabain-sensitive Na efflux with Na_c between 49 and 97 mmol/liter cells, well above the level needed for maximal velocity of the pump (Post and Jolly, 1957). At the same time, the K_c was varied from 4 to 55 mmol/liter cells. The results (Table II) show no significant effect on V_{max} by K_c for any K_c greater than 4 mmol/liter cells. When the K_c is four or less, however there is an apparent reduction in V_{max}. In no case is V_{max} increased by lowering K_c in the range studied.

TABLE II

EFFECT OF K_c ON V_{max}

	Na_c	K_c	${}^oM_{Na}^{o-s}$
	(mmol/liter cells)		(mmol/liter cells/h)
I	56	55	4.39
	53	31	5.09
	49	20	5.02
	57	4	4.35
II	56	52	4.63
	50	27	4.34
	45	17	4.33
	52	3	3.26
III	64	47	5.24
	84	28	5.52
	97	4	3.96
	78	4	4.72
IV	59	48	6.11
	83	27	6.56
	95	4	4.41
	76	4	4.78

Ouabain-sensitive Na efflux from PCMBS-treated cells with Na_c above 45 mM cells. Na_{ext} = 130 mM, K_{ext} = 10 mM. Groups I^c and III were from the same donor. Mean of all cells with K_c 30 or above = 5.08 ± 0.3 SEM. Mean of cells with K_c less than 10 = 4.26 ± 0.22 SEM. $P = 0.025$ that cells with $K_c \leq 4$ are different from cells with $K_c \geq 17$.

Effects of Other Variables

It was considered possible that any of several other variables might actually be responsible for the inhibitory effects which we have been attributing to the K_c. Possible effects of the replacement cation and the nonsteady-state situation have been mentioned previously. Remaining possibilities include (*a*) effects of PCMBS or cysteine, (*b*) cell volume or membrane potential changes, and (*c*) different levels of ATP in high- and low-K cells.

The first-listed variable is excluded by the data shown in Fig. 6. This figure differs from Fig. 5 in that the cation concentrations in the cells were altered by cold storage instead of by PCMBS treatment: The ouabain-sensitive Na efflux is plotted against Na_c at two K_c levels in Fig. 6 *a* and as a double reciprocal plot in 6 *b*. Cold storage is not as effective as PCMBS treatment in reducing the K_c, but the effect of the K_c on the Na efflux is the same in either case. Thus the inhibitory effect of a high K_c cannot be attributed to effects of PCMBS or cysteine. It should also be noted that the behavior of the flux as a function of Na_c is unaffected by the presence of high (130 mM) or low (6 mM) Na_{ext}, given a constant K_{ext} of 10 mM (cf. Figs. 5 and 6), although the absolute magnitude of the flux may be affected.

The second variable which might mimic an inhibitory effect of high K_c, that a coincident volume change in low-K cells might increase the pump rate, is considered in Fig. 7. Cell shrinkage, of necessity, would increase the effective Na_c, tending to stimulate the pump. It was, in fact, observed that for cells with the same Na_c (per liter cells), the cell water was higher in high-K cells and was reduced in low-K cells (this is shown in Table III). However,

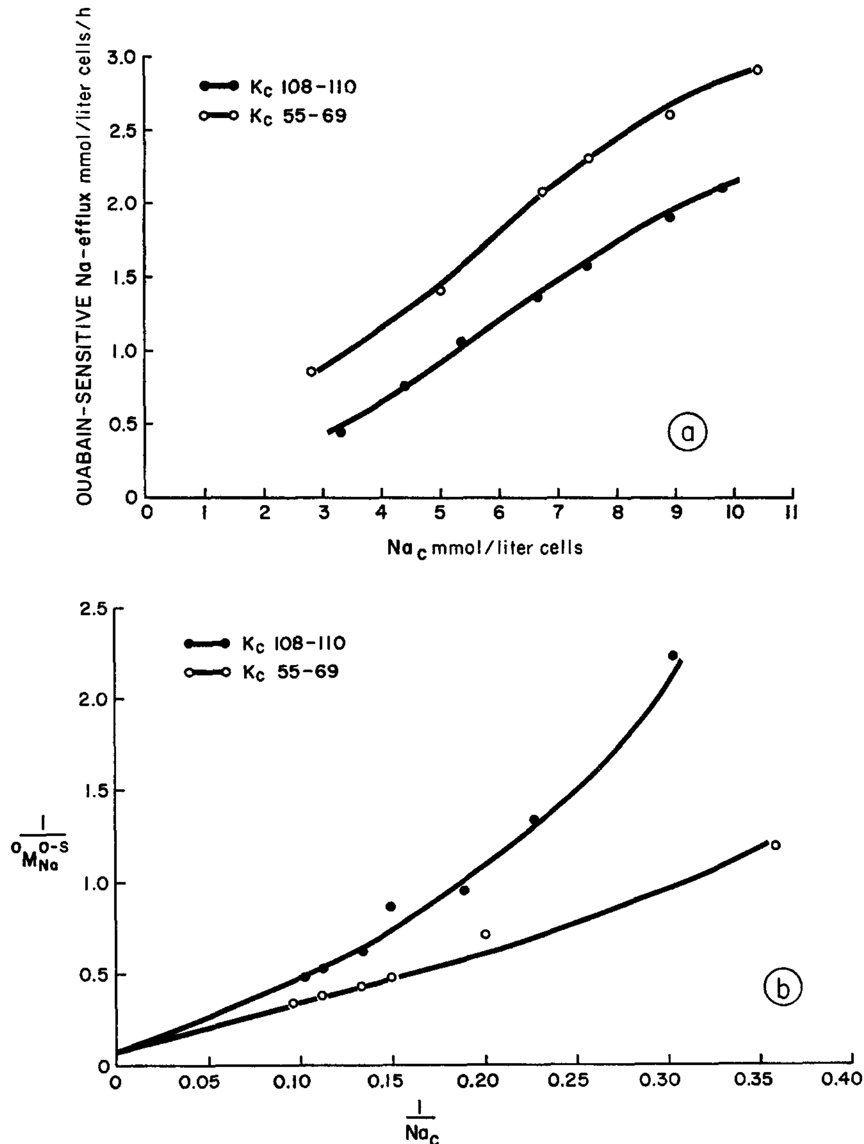


FIGURE 6. Ouabain-sensitive Na efflux from cold-stored cells with varying Na_c and two levels of K_c (millimoles per liter cells). Results expressed as efflux (6 *a*) and as a double-reciprocal plot (6 *b*).

when the ouabain-sensitive Na efflux and the Na_c are expressed in terms of the cell water (Fig. 7), it can be seen that the inhibitory effect of a high K_c on the pump is still apparent.

Paralleling the observed volume changes, it was also found that there was a difference in intracellular chloride between low- and high-K cells. Table III shows the chloride ratios of normal cells and of low-Na cells with high and low K_c, the membrane potentials calculated from the Cl ratios, and the % cell water. It can be seen that the Cl ratio is reduced in low-K cells and the membrane potential is correspondingly more negative.

The final potential source of an apparent effect of K_c on the pump is the cells' ATP concentration: If high- and low-K cells had significantly different ATP levels it could account for the observed differences in their ouabain-sensitive Na efflux rates. Table IV shows ATP measurements on high- and low-K cells with various Na_c's, including Na_c above that needed for V_{max}. It is evident that the energy substrates provided are sufficient for the cells to maintain ATP within the normal range irrespective of their Na and K

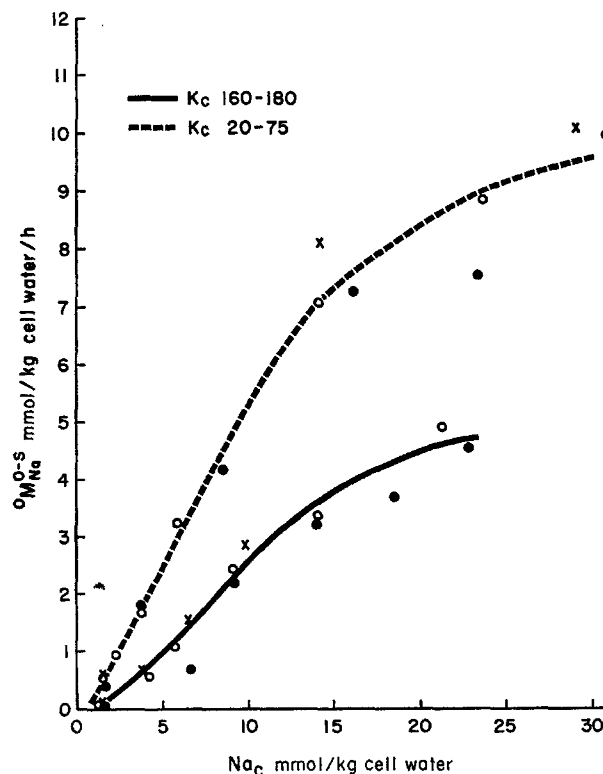


FIGURE 7. Replot of results shown in Fig. 5, with the flux and Na_c expressed in terms of intracellular water.

TABLE III

CHLORIDE RATIO, CELL WATER, AND MEMBRANE POTENTIAL OF NORMAL AND LOW-Na CELLS

	Cl_c/Cl_{ext}	E_m	H_2O
		(mV)	(%)
Untreated cells Na_c = 6-7.8 K_c = 98-104	0.63±0.01	−11.8±0.06	63.2±0.4
Low-Na, high-K cells Na_c = 0.6-4.0 K_c = 114-123	0.64±0.04	−11.2±1.0	68.1±0.2
Low-Na, low-K cells Na_c = 0.6-3.6 K_c = 20-40	0.43±0.03	−21.2±1.3	57.0±0.5

Chloride ratios (millimoles Cl per kilogram cell water ÷ millimoles Cl per kilogram extracellular water), calculated membrane potentials, and percent cell water for normal, high-, and low-K cells. Each number represents mean ± SEM of eight determinations. Cl and H_2O measured on PCMBS-treated cells packed in 170 mM TMA Cl. Cl ratio and E_m not significantly different for untreated cells compared with high-K cells ($P > 0.5$). Cl ratio and E_m significantly different for low-K cells compared with either normal or high-K cells ($P \leq 0.001$). Cell water values for all three groups are significantly different from each other ($P \leq 0.001$). Na_c and K_c expressed as mmol/liter cells.

concentrations. Therefore, a difference in ATP cannot be responsible for the observed differences in pump activity.

DISCUSSION

The behavior of the Na-K pump of intact human red cells in response to changes of K_c can best be explained by interpreting the K_c as a competitive inhibitor of the pump. Such an interpretation fits in well with the previously demonstrated inhibition of the pump by Na_{ext} (Sachs, 1967). Consideration of K_c as a similar (but not identical) inhibitor is further supported by the response of the Na- K-stimulated ATPase of ghosts to variations of Na and K, which have been observed by previous investigators.

The results of studies with the Na_c and K_c varied independently (Figs. 5 and 6) distinguish the known stimulatory effect of increasing Na_c from the inhibitory effect of high K_c: either an increase of Na_c or a decrease of K_c raises the pump rate. Conversely, while a decreased Na_c reduces the pump rate, a simultaneous reduction of K_c raises the pump rate significantly, whether Na efflux or K influx is being measured (Fig. 1). In fact, if the Na_c is made low and held constant while the cell K is varied, the flux rate is an inverse function of the K_c (Fig. 2). If the Na_c is held low, only when the K_c is reduced such that the ratio of K_c to Na_c is approximately the same as

TABLE IV

ATP CONTENT OF HIGH- AND LOW-K CELLS

Group	Na_c	K_c	ATP	Group	Na_c	K_c	ATP
	(mmol/liter cells)				*(mmol/liter cells)*		
I	1.3	119	1.2	IV	56	55	1.5
	0.9	46	1.7		53	31	1.7
					49	20	1.7
II	2.8	127	1.6		57	4	1.5
	3.0	27	1.8		56	52	1.4
	2.8	128	1.6		50	27	1.2
	3.0	24	1.7		45	17	1.5
					52	3	1.3
III	1.0	100	2.0		64	47	1.6
	5.5	104	2.3		84	28	1.7
	11.0	104	2.3		97	4	1.6
	0.9	29	2.4		78	4	1.5
	5.0	37	2.1				
	12.0	37	2.3	V	3.5	118	1.2
					3.5	118	1.3
					4.5	57	1.5
					4.2	56	1.6

ATP concentrations in cells with varied Na_c and K_c. Flux measurements for cells in group II are included in Fig. 4, for group III in Fig. 5, and for group IV in Table II. Cation content in all groups was altered by PCMBS treatment, except group V, which was cold stored. Mean of high-K cells = 1.69 ± 0.16 SEM (groups I, II, III, IV). Mean of low-K cells = 1.89 ± 0.12 SEM. $P > 0.15$.

cells with a normal K_c and Na_c (about 10 to 1) does the pump rate increase to approximately the rate seen in normal cells.

A combination of a very low Na_c and a high K_c appears to shut off the pump to such an extent that the normal response of the Na efflux to stimulation by K_{ext} disappears (Table I). Nor do such cells have an increased K efflux (Fig. 4). Since ouabain-sensitive K efflux represents reversal of at least part of the normal pump operation (Glynn and Lew, 1970), this can be interpreted as another indication that the pump itself is very nearly inactivated in all of its operations. The cells in these experiments probably contained adequate orthophosphate to allow K efflux to occur, since they had been exposed to 20 mM phosphate and only 1 mM glucose. Such pretreatment maintained ATP levels (Table IV) and should have allowed intracellular phosphate to remain at least normal, although it was not measured. This is of interest because Glynn et al. (1970) showed that orthophosphate and high Na_{ext} or K_{ext} were the major requirements for K efflux to occur. These minimal requirements were apparently met here since there was an observable K efflux, but it is possible that to show an increased K efflux it would be necessary to actually elevate the internal phosphate. In any case, the ouabain-sensitive K efflux is not affected by K_c in the same range and in

similar conditions to those in which it obviously affects the Na efflux and K influx of low-Na cells.

The following were shown not to be responsible for the evident inhibitory effect of high K_c on the pump: (*a*) volume changes that occur when the K_c is changed (Fig. 7); (*b*) the intracellular ATP concentration (Table IV); (*c*) nonsteady-state situation (Fig. 3); (*d*) effects of PCMBS or cysteine (Fig. 6); or (*e*) the choice of a replacement cation. This last point is based not only on the use of two replacement cations in these experiments (Table I), but also on the observations of other investigators studying the effects of various cations on the pump and ATPase. The response of the pump to K_{ext} is of the same form in media containing high TMA, Mg, choline, or Mg-sucrose (Sachs, 1967; Sachs, 1970; Garrahan and Glynn, 1967 *a*; Priestland and Whittam, 1968). Similarly, the ATPase of resealed ghosts has similar properties when the external medium contains Mg, Tris, choline, or TMA (Hoffman, 1962 *a*; Whittam and Ager, 1964; Schatzmann, 1965; Whittam, 1962). In the case of the ATPase of fragmented ghosts, it has been found that the response of the ATPase to variations of Na and K is no different if TMA or choline is used to bring the medium to isotonicity or if the medium is left hypotonic (Smith and Welt, 1971; Robinson, 1970). Furthermore, there have been several direct demonstrations of an inhibitory effect of high K on the ATPase (as well as a complementary inhibition by high Na) on fragmented red cell ghost ATPase (Post et al., 1960; Dunham and Glynn, 1961) and no nerve and kidney microsomes (Skou, 1957, 1960; Green and Taylor, 1964; Robinson, 1970; Ahmed et al., 1966); in all of these experiments, apparently no replacement cation was used as the Na and K of the media were varied.

Although volume changes leading to changes in the effective concentration of Na or K in the cells have been excluded per se as an explanation for the effects of K_c (Fig. 7), it was observed that low-K cells were not only shrunken but also had a reduced chloride ratio, and therefore a more negative calculated membrane potential (Table III). A possible explanation for both the reduced Cl in the cells and the reduced volume is that, while PCMBS increases the cells' permeability to Na and K (Garrahan and Rega, 1967) so that they lose K in a low-K medium, they apparently do not take up enough TMA to replace all of the lost K. The cells thus lose water, packing the hemoglobin more closely. Gary-Bobo and Solomon, (1968) have observed that the net charge on the hemoglobin molecule becomes more negative as its concentration increases; thus shrunken cells tend to lose Cl, decreasing the Cl ratio. The resulting more negative membrane potential cannot account for the increased pump rate in low-K cells since the Na efflux portion of the reaction goes against the electrical gradient. However, the reduced Cl ratio also indicates an intracellular pH reduction, and the

resulting pH gradient cannot be discounted as being the actual mechanism for the increased pump rate of low-K cells.

As referred to above, there have been several direct demonstrations of inhibition of the ATPase by high Na or K. In addition, several investigators have postulated that the high K_c of intact cells would be found to be inhibitory to the pump, at least in low-Na cells, either on the basis of indirect evidence (Maizels, 1968; Skou, 1965) or on the basis of models of the pump-ATPase system (Post et al., 1960; Opit and Charnock, 1965), or on both (Hoffman and Tosteson, 1971). An attempt to demonstrate an effect of internal K on the ATPase of resealed ghosts was made by Whittam (1962). However, since resealed ghosts undergo a pronounced volume change during their preparation, it is difficult to compare the internal ion concentrations in his experiments to concentrations in intact cells. The internal sodium concentrations in the ghosts were rather high, though still in the range where the internal Na did affect the activity, and no influence of internal K was seen over a fivefold range. He concluded that the ATPase activity was a function only of the internal Na and the external K.

That same year, however, Hoffman (1962 *b*) measured Na efflux from resealed ghosts which had been hemolyzed either in water or in hypotonic KCL, and observed a higher strophanthidin-sensitive Na efflux from ghosts prepared in the former manner. Although the experiment was complicated by tonicity effects and by heterogeneity of the ghost preparations, it strongly suggests an inhibitory effect by internal K.

The fact that the inhibitory effect of K_c on the pump is modified by a sufficiently high Na_c is the probable explanation for Whittam's failure to observe inhibition by internal K in ghost preparations. In fact, the Na_c must be normal or slightly below normal before the inhibitory effect of a high K_c becomes readily observable (Fig. 5). At the other end of the scale, with Na_c well above saturating levels of about 40 mmol/liter cells, reducing the K_c too far also lowers the flux rate (Table II). This apparently anomalous effect of K_c recalls the apparent reduction of the flux in media containing saturating K_{ext} which occurs when all or most of the Na_{ext} is replaced by other ions (Hoffman and Kregenow, 1966; Lubowitz and Whittam, 1969). Sachs (1970) has suggested that most of the pump increase due to Na_{ext} can be explained by an increasing Na_c during the flux measurement in the presence of high Na_{ext}. Because K_{ext} is already saturating, a similar explanation cannot be applied to the apparent V_{max} alteration by K_c. It is, however, possible that a minimal amount of K_c and Na_{ext} are necessary for the pump to retain its normal responsiveness to K_{ext} and Na_c. It appears that both the ratios of K_c to Na_c and of Na_{ext} to K_{ext}, as well as their absolute amounts, determine the pump rate.

At a primary level, the data presented here suggest that the inhibition of

the pump by high K_c is of the competitive type (affecting the slope of a double-reciprocal plot), and, in fact, that it resembles the known competition by Na_{ext} (Sachs, 1970). It cannot be excluded, however, that the inhibition is of the mixed type (affecting slope and intercept); distinguishing between these alternatives by direct means may actually not be possible in intact cells, because it would be necessary to increase the Na_c above 40 mmol/liter cells, keeping the K_c high at the same time. It should be noted that reduction of K_c to 55 mmol/liter cells is enough to stimulate the pump (Fig. 6). Since a K_c of 55 was the highest obtained with Na_c over 40 (Table II), the data of Table II are not sufficient to determine conclusively whether or not K_c affects V_{max} with respect to Na_c.

Unfortunately, the mechanism of the pump is as yet too uncertain to enable one to make a decision between K_c as an allosteric effector and competitive inhibition by K_c at more than one substrate site. It seems apparent that there are at least two sites for K_{ext} (Sachs and Welt, 1967). Similarly, evidence has been presented by Sachs (1970) for at least two sites for Na_c; the form of Fig. 5 *b* supports this observation, since it would be difficult to account for the biphasic form of the curve of rate constant vs. Na_c without at least two Na_c sites. Robinson (1970) also supports a two-Na site interpretation from work on the ATPase. K_c apparently interacts either directly at the two Na_c sites, or in an allosteric manner at separate sites. Assuming that there are two sites on the carrier for the active efflux of sodium, one could suggest two possibilities for the inhibitory action of K_c. Both views imply a competition between Na and K for the Na sites and that with higher K_c more Na sites are preempted by K. The first view is that having preempted a site the K is then ejected from the cell. This appears to be unsupported by the data since the ouabain-sensitive K-K exchange and the ouabain-sensitive K efflux into a K_o medium is not different in high- and low-K_c cells. The second view is that when K preempts Na sites the carrier is deterred in such a fashion as to diminish the rate of pump activity without influencing K efflux. This view is still compatible with these data. It is, in any case, evident that K_c is an important determinant of the pump rate.

We propose that the inhibitory effect of a high K_c demonstrated here is partially responsible for controlling the physiological level of Na and K in normal red cells. An increased Na_c serves as its own regulator by directly stimulating the pump rate, reducing the Na_c. Since the normal high K_c of human red cells is in the inhibitory range, and since it exerts its inhibitory effect most strongly when the Na_c is low or normal (Figs. 5 and 6), the K_c would be important in preventing the Na_c from dropping below normal. If the Na_c were to decrease slightly, the pump rate should be reduced not only by the reduction of substrate concentration but also by inhibitory action of the K_c, allowing a gain of Na by diffusion. The cell would thus be

more sensitive to slight reductions of Na_o than if the K_o were to have no effect on the pump. A similar argument would apply to the controls exerted by the ratio and concentrations of the external ions.

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This represents work done by Dr. Knight in fulfillment of the requirements for the Ph.D. in Biochemistry at the University of North Carolina School of Medicine May, 1972.

Note Added in Proof Since the submission of this manuscript, a paper has appeared (R. P. Garay and P. J. Garrahan. 1973. The interaction of sodium and potassium with the sodium pump in red cells. *J. Physiol.* (*Lond.*). **231**:297) which also suggests that high intracellular K competes for pump sites with internal Na in the normal range.

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