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EFFECT OF MEMBRANE POTENTIAL AND INTERNAL pH ON ACTIVE SODIUM-POTASSIUM TRANSPORT AND ON ATP CONTENT IN HIGH-POTASSIUM SHEEP ERYTHROCYTES

A. MARTIN M. ZADE-OPPEN *, JAMES M. SCHOOLER, Jr. **, PAUL COOK *** and DANIEL C. TOSTESON ‡

From the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, NC (U.S.A.)

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Summary

Ouabain-sensitive Na^+ and K^+ fluxes and ATP content were determined in high potassium sheep erythrocytes at different values of membrane potential and internal pH. Membrane potential was adjusted by suspending erythrocytes in media containing different concentrations of $MgCl_2$ and sucrose. Concomitantly either the external pH was changed sufficiently to maintain a constant internal pH or the external pH was kept constant with a resultant change of internal pH. The erythrocytes were preincubated before the flux experiment started in a medium which produced increased ATP content in order to avoid substrate limitation of the pump.

It was found that an increased cellular pH reduced the rates of active transport of Na^+ and K^+ without significantly altering the ratio of pumped Na^+/K^+. This reduction was not due to limitation in the supply of ATP although ATP content decreased when internal pH increased. Changes of membrane potential in the range between −10 and +60 mV at constant internal pH did not affect the rates of active transport of Na^+ or K^+.

* Present address: Department of Physiology and Medical Biophysics, Uppsala University, Uppsala, Sweden.

** Present address: Department of Chemistry, North Carolina University, Durham, NC, U.S.A.

*** Present address: Department of Physiology, University of Arizona School of Medicine, Tuscon, AZ, U.S.A.

‡ Present address: Department of Physiology, Harvard Medical School, Boston, MA, U.S.A.

Abbreviations: TES, *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; Hb, hemoglobin.

Introduction

The Na^+-K^+ pump of erythrocytes is apparently electrogenic, the ratio of pumped Na^+/K^+ being 3/2 [1,2]. This means that to some extent the pump contributes to the potential difference over the cell membrane. This paper describes our attempts to determine whether the pump fluxes in high potassium (HK) sheep erythrocytes are dependent upon the membrane potential. Such a dependence was postulated by Rapoport [3,4]. In frog striated muscle, Horowicz and Gerber [5,6] reported experiments from which they concluded that a depolarisation of the membrane stimulated the Na^+ pump and Sjodin and Beauge [7] suggested that variations in the coupling ratio of pumped Na^+/K^+ might be due to a depolarization of the membrane. On the other hand Beauge et al. [8] found that the K^+ activation of the Na^+ pump in frog muscle was independent of the membrane potential. Similar results have been reported in squid axons [9] and in molluscan neurons [10]. Cotterrell and Whittam [11] investigated human erythrocytes and reported a decrease of ouabain-sensitive potassium influx and sodium efflux by not more than 20 and 40%, respectively, on reversing the chloride concentration difference to a degree corresponding to a change in membrane potential from −9 to +30 mV and a change in internal pH of about 0.7 unit. They regarded this difference as being of doubtful significance.

In the experiments described in this paper we varied membrane potential between −10 and +60 mV both under conditions when internal pH remained constant and under conditions when it changed. A preliminary report of this work has been presented in abstract form [12].

Materials and Methods

Unless otherwise stated, all chemicals were analytical grade.

Experimental procedures

Venous blood from one particular HK sheep was used in all flux measurements. In other types of experiments blood was obtained from HK or low potassium (LK) Dorset sheep. The blood was collected into a flask containing some heparin. It was kept on ice during transport to the laboratory and then processed without further delay.

Packed erythrocytes were obtained by centrifuging about 0.6 ml of blood in a nylon tube (internal diameter 3.17 mm) [13] for 15—30 min at about 17 000 × *g*. The part of the tube containing the supernatant and the top of the cell layer was cut off with a razor blade. The tubes were closed and stored in a refrigerator for the later determination of wet and dry weights, Na^+, K^+ and hemoglobin (Hb) concentrations in erythrocytes of natural volume in their own plasma. The remainder of the erythrocytes were used in the experiments after being washed 2—3 times by centrifugation and dilution, the plasma and buffy coat being removed by careful aspiration. 1 vol. of flux medium 1 (Expt. 13) or preincubation medium (see below) was used for each washing.

All procedures below were performed at or near 0° C unless otherwise stated.

ATP-generating system. In order to increase the ATP concentration of the

erythrocytes to values considerably above normal, the cells were incubated at 37° C in Erlenmeyer flasks rotated continuously at about 75 rev./min, in a 'pre-incubation medium' containing 5 mM glucose, 5 mM adenosine, 10 mM KCl, 120 mM NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.4. The incubation time was 1.5 or 2 h and the hematocrit was 20%.

Loading cells with $^{22}Na^+$. In experiments to determine $^{22}Na^+$ effluxes the pre-incubation also served for isotopic loading of the cells. A small volume of a high specific activity solution of $^{22}NaCl$ was added to the preincubation medium.

Interwash. Between preincubation and the flux experiment, the cells were washed in flux medium 1 (see below). The number of washes was 2 (no isotope during preincubation) or 5 and in each wash the volume added was never less than the cell volume.

Flux incubation. Flux determinations were made with a hematocrit of 1% or less. The cells were suspended in Erlenmeyer flasks continuously rotated at about 75 rev./min in a water bath at 37° C. For the isotope flux determinations 5 or 10-ml samples were taken in duplicate at three times; at 'zero' time (within 5 min after the addition of packed cells to the flux medium), at 30 or 50 or 60 min and at 90 or 100 or 120 min. Also 35-ml samples were taken, the cells collected in the nylon tubes and centrifuged for wet and dry weight determination. In addition 10-ml samples were taken for the determination of cellular ATP.

The samples used for isotope flux determination were collected in pre-cooled test tubes and immediately centrifuged at 0° C. The Sorvall RC 2 B centrifuge was accelerated to reach 17 000 × *g* and turned off after about 10 s at this rate. This gave a total running time of 1—2 min. The cells (0.05 or 0.1 ml) were washed twice in 2 ml of ice-cold wash medium containing 285 mM sucrose, 5 mM $MgSO_4$ to which 1 N HCl was added to reduce the pH to 3.8. The whole washing procedure usually required just over 5 min. Finally, between 1 and 3 ml of water were added to the cell pellet, the volume being the same as the size of the supernatant sample used for counting the radioactivity. The same samples were then used for determination of radioactivity, Na^+, K^+, Cl^- and Hb concentrations.

The flux experiments. (1) Principles. The chloride ratio was varied by replacement of $MgCl_2$ in the buffer medium by sucrose. This induces a shift in pH because of the following relations between the internal (i) and external (0) ionic concentrations

$$\frac{[Cl]_i}{[Cl]_0} = \frac{[OH]_i}{[OH]_0} = \frac{[H]_0}{[H]_i} \tag{1}$$

The membrane potential was tentatively assumed to be that given by the chloride or hydrogen ratios.

$$E_{Cl} = \frac{RT}{F} \ln \frac{Cl_i}{Cl_0} = \frac{RT}{F} 2.303 (pH_i - pH_0) \tag{2}$$

Since both the membrane potential and pH are presumably important variables, the pump fluxes were measured as functions of both the potential, keeping pH_i constant, and pH_i, keeping the potential constant.

To change the membrane potential, cells were transferred from a medium with a high to one with a low Cl^- concentration, the H^+ concentration being concomitantly increased by the same factor. Under these conditions the internal pH should not change, at least with a low hematocrit (1%) and a well-buffered medium. To keep the potential under the latter chloride conditions constant, the Cl^- concentration was kept at the same low value but the medium was buffered at a normal pH; thus resulting in the cell interior becoming more alkaline.

If erythrocytes are suspended in an electrolyte medium at elevated pH, the cellular ATP is reduced [14,15]. Further, erythrocytes suspended in media of normal pH containing mainly non-electrolytes (or if electrolytes, impermeable anions) have an increased turnover of ATP and a decreased ATP content [16]. In order to avoid substrate limitation of the pump. The cellular ATP was increased artificially as described above to a value considerably above normal before starting the flux measurements.

(2) Experimental details. The flux media were varied so as to obtain the following three conditions. Flux medium 1: A high Cl^- or reference condition (200 mM Cl^-). The medium contained 5 mM KCl, 97.5 mM $MgCl_2$, 0.8 mM sucrose, 5 mM adenosine, 5 mM glucose, and 20 mosM Mg/TES buffer, pH 7.4. The latter was obtained by dissolving solid $MgCO_3$ in a solution of TES (Sigma Chemical Co., St. Louis, MO, U.S.A.) to the desired pH and filtering off undissolved material. Flux medium 2: An altered membrane potential without changing the internal pH. The Cl^- concentration of medium 1 was reduced to 20, 10 or 5 mM and concomitantly the H^+ concentration was increased by the same factor. For example, flux medium 2 could have the following composition: 5 mM KCl, 2.5 mM $MgCl_2$, 248 mM sucrose, 5 mM adenosine, 5 mM glucose and 20 mosM Mg/TES, pH 6.2. Flux medium 3: An altered membrane potential without changing the external pH. Under these conditions the Cl^- concentrations were the same in flux media 2. A flux medium 3 thus had a similar composition to medium 2 except that the pH was the same as in medium 1.

Analytical methods

Determination of wet and dry weights. Erythrocytes were packed by centrifugation in nylon tubes as described above and stored. Later the tube bottom was cut off and the contents emptied on to an aluminium planchet with the aid of a plastic plunger and weighed as quickly as possible. The cells were dried at +95 to +98°C for at least 15 h and then weighed again. Duplicate samples were taken from each batch.

Hemoglobin determination. To cell samples or supernatants suitably diluted in a hemolysing solution consisting of a detergent (0.5 ml non-ionox/16 l) and CsCl (10.78 g/16 l) in water, a reagent containing 80 g $NaNO_2$, 2.08 g KCN and 40 g $NaHCO_3$/l was added. The proportions were 1 vol. reagent to 40 vols. of sample [17]). The absorbance of the resulting cyanomethemoglobin was determined at a wavelength of 540 nm or 420 nm on a Gilford 300-N spectrophotometer (Gilford Instr. Lab. Inc., Oberlin, OH, U.S.A.). In Expt. 13 and those shown in Figs. 1 and 3 the hemolysing solution also contained 25 ml conc. NH_4OH, no reagent was added and Hb was determined without prior transformation to cyanomethemoglobin.

Other determinations. Determination of Na^+ and K^+ in lysed cells or supernatants was made by atomic absorption spectrophotometry (Model 303 Perkin Elmer Corp., Norwalk, CT, U.S.A.) after suitable dilution with hemolysing solution.

Cl^- was determined coulombmetrically on a Buchler-Cotlove Chloride Titrator (Buchler Instruments Inc., Fort Lee, NJ, U.S.A.). The determinations were made on supernatants or hemolysates without prior protein precipitation. No correction was made for possible systematic errors from unspecific titration of cellular components.

$^{42}K^+$ and $^{22}Na^+$ activities were determined using a Packard Autogamma System with a scintillation well for determination of γ-emission. Care was taken to have a constant geometry in all samples.

ATP was determined on cells hemolysed in water. Samples were deproteinated with perchloric acid and neutralised with a K_2CO_3 solution. The disappearance of NADH fluorescence was measured on a ratio fluorimeter (Farrand Optical Co., Inc., New York) after addition of usually 0.1 ml sample to 1.0 ml of a reagent solution. This contained the necessary enzymes and substrates (except, of course, ATP) for the conversion of 3-phosphoglyceric acid to glyceraldehyde 3-phosphate and inorganic phosphate (P_i) (McManus, ●.●., personal communication).

pH determinations were made with a pH meter equipped with a thermostated microelectrode chain (PHM 22, Radiometer, Copenhagen, Denmark).

Osmolarities of solutions were measured by freezing point depression (Osmette Precision Osmometer, Precision Systems, Framingham, MA, U.S.A.).

Calculations

Concentrations. Concentrations of Na^+, K^+, Cl^- and ATP were calculated in mmols in the number of erythrocytes which originally occupied the volume of 1 l:

$$\text{mM (original cells)} = \text{mM (observed)} \times \frac{A_c}{A_s}$$

where A_s is the Hb absorbance in the sample and A_c that in the packed erythrocytes of the original whole blood after correction for dilution.

The concentrations in mmol/l of cell water was obtained from values obtained this way by multiplication with a correction factor:

$$\frac{1}{1.1\,(1-f_0)\,\frac{f_s}{1-f_s}}$$

where f_0 is the water fraction in the original cells and f_s that in the cells of the sample and the density of the original cells is assumed to be 1.1.

The membrane potential (E_{Cl}) and pH_i. These were calculated from the chloride ratio and the known pH of the medium according to Eqn. 2.

Correction necessitated by hemolysis. In Na^+ efflux experiments, the radioactivity of the medium was corrected for the increase due to hemolysis of some of the cells

$$\text{cpm} = \text{cpm}_s - \frac{A_m}{A_s} \times \text{cpm}_0$$

where cpm_s is the activity in the supernatant sample and cpm_0 that of the hemolysed cell sample at zero time. A_s is the Hb absorbance in the sample having cpm_0, and A_m is the Hb absorbance of the medium.

Ionic fluxes. These were calculated as follows

$$i_{M_K} = \frac{\Delta cpm/l \text{ cells}}{(\Delta time) \times (\text{specific activity in medium})}$$

and

$$o_{M_{Na}} = \frac{\Delta cpm/l \text{ supernatant}}{(\Delta time) \times (\text{specific activity in cells})} \times \frac{1 - \text{hematocrit}}{\text{hematocrit}}$$

Pump fluxes were defined as ouabain-sensitive fluxes and obtained as the difference in flux values between two cell suspensions run in parallel, one containing 10^{-4} M ouabain and the other none.

Results

The cell washing procedure

The estimation of cellular ionic concentrations by direct measurement on packed cells with correction for trapped plasma has the disadvantage of compounding errors in several measurements. We therefore elected first to wash the cells free of extracellular Na^+, K^+, Cl^- and radioactivity. However, in order to be useful, it is a requirement that a washing procedure itself does not cause any loss from the cells. The essential feature of the washing solution was the use of sucrose to maintain isotonicity together with some electrolyte to minimize disturbance to the Na^+ and K^+ permeability of the cells [18—22]. We tested the washing procedure as described below.

(A) Washed erythrocytes suspended in an unbuffered solution of 295 mM sucrose were washed two or four times in unbuffered media containing either 295 mM sucrose or 285 mM sucrose and 5 mM $MgSO_4$ in order to investigate if this treatment would alter the cellular concentrations of Cl^-, Na^+ and K^+. $MgSO_4$ was chosen because Mg^{2+} are effective in avoiding increased cation leakage in electrolyte-free media [20,21], and SO_4^{2-} exchanges more slowly than Cl^- by several orders of magnitude [23] especially at the acid pH [24] used in the washes reported here. The concentration of $MgSO_4$ was chosen as a compromise between a high value to avoid cation leakage and a low value to minimise Cl^--SO_4^{2-} exchange. The pH was not measured in these cell suspensions. However, in other experiments where erythrocytes were washed in unbuffered media, the pH measured in the suspension approached a value of 8 after a few washes. The results are shown in Fig. 1. There was a small cation loss in both media. The Cl^- loss was about 20 mM in four washes. Most of the Cl^- loss must thus have been due to an exchange with the extracellular SO_4^{2-} and other anions inadvertently present such as OH^- and HCO_3^-. The latter is derived from CO_2 in the solution. HCO_3^- probably contributed most since SO_4^{2-} permeates slowly and OH^- was present at extremely low concentrations.

(B) Cells were washed in an air-equilibrated medium buffered at pH 6.2 or with the same medium after it had been degassed by being subjected to a partial vacuum. Cl^- losses where somewhat but not sufficiently reduced by this

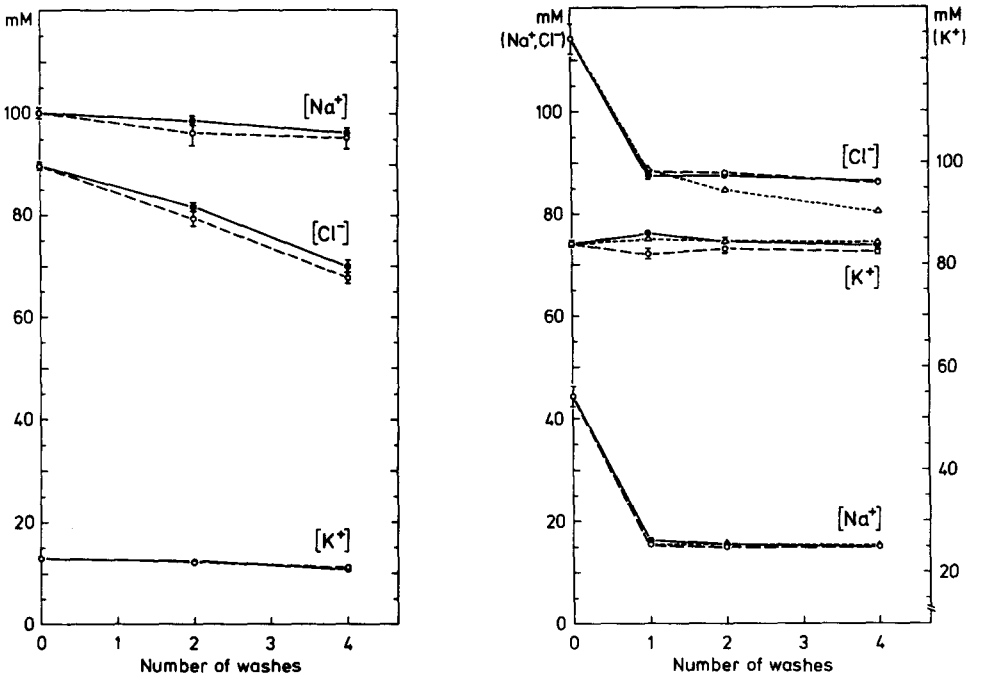

Fig. 1. Ion concentrations during washing in sucrose or sucrose/$MgSO_4$. Pretreatment: LK sheep cells were stored 24 h as packed cells. Cells were then washed three times in unbuffered 295 mM sucrose. Then the cells were diluted 100 times in the same medium, distributed into 20 centrifuge tubes and well packed. Cells treated this way = 'unwashed' in the diagram. Washes: 0.1 ml cells were now washed two or four times with 7 ml each time of unbuffered solutions of either 285 mM sucrose, 5 mM $MgSO_4$ (- - - - - -) or with 295 mM sucrose (———). The pH in the suspension was approximately 8. Vertical bars for standard error of the mean (n = 4). The washing procedure was accomplished within about 85 min. In the four washes there was a decrease in cation concentration of about 6 mM and a Cl^- loss about 20 mM. There was no significant difference between the media.

Fig. 2. Ion concentrations during washing in sucrose/$MgSO_4$ with an without acid. Pretreatment: Fresh HK sheep cells were once washed in 0.17 M NaCl then diluted 50 times in the same medium, distributed into 40 centrifuge tubes and well packed. Cells treated this way = 'unwashed' in the diagram. Washes: 0.1 ml cells were washed with 1 ml (●———●) or 7 ml (○- - - - -○) of 285 mM sucrose, 5 mM $MgSO_4$, ▪ N HCl to pH 3.8 or 7 ml of a similar medium but without the acid (triangles and broken lines). Vertical bars for standard error of the mean (n = 4). Between pretreatment and the first wash there is a decrease by 28 mM Na^+ and 26 mM Cl^- presumably due to removal of extracellular medium. During the subsequent washes there is a significant decrease in Cl^- but only when the washing solution was not acidified.

means of reducing the HCO_3^- concentrations, presumably because no strict precautions were taken to avoid re-entry of gas during the washings.

(C) The following experiment was made on HK sheep erythroyctes which had been suspended in an unbuffered NaCl solution. It was therefore expected that measured Na^+ and Cl^- values would decrease during the washings because the cell fraction would initially contain some trapped NaCl solution. Loss of cellular cations would be detected by K^+ concentration changes; exchange loss of cellular Cl^- would be shown up by the difference between Cl^- and Na^+ concentration changes. The washing media chosen were unbuffered 285 mM sucrose, 5 mM $MgSO_4$ and the same solution brought to pH 3.8 by addition of normal HCl. This low pH was chosen in order to decreased HCO_3^- and OH^- con-

centrations to minimize Cl^--OH^- and Cl^--HCO_3^- exchange during the washing (see Principles of flux experiments). SO_4^{2-} exchanges very slowly with Cl^- in the absence of HCO_3^- [25,26]. 0.1 ml cells were washed with either 1 or 7 ml solution in each wash. The result is shown in Fig. 2. The loss of Na^+ (28 mM) and Cl^- (26 mM) in the first wash were the same within the errors of the method and were presumably due to extracellular trapped NaCl being washed away. Na^+, K^+ and Cl^- remained constant during the subsequent washes. In the non-acidified wash medium, Cl^- decreased measurably with each wash. Acidification of the medium apparently reduced the loss of cellular Cl^-. The washing procedure involving the acidified medium was therefore adopted for the following experiments.

Changes of ATP concentration in the various conditions

Washed but not preincubated erythrocytes were incubated at 37° C in the various flux media without adenosine. The cellular ATP was determined on cells samples just before and at the end of the incubation period (Table I). Both in the reference conditions, and with the low chloride and low pH_0, the cellular ATP content decreased either not at all or only slightly during the incubation period. However, in the low chloride and normal pH_0 medium, there was a large fall in ATP (from 0.86 to 0.10 mmol/l or original cells). In this medium the membrane potential is about 60 mV, inside positive, as calculated from the chloride ratio, and the internal pH is about 8.4.

The results in Table I contain values taken from incubations both with and without ouabain. The results shown in Table II indicate that ouabain has only a very minor effect on ATP content, confirming the observations of Minakami and Yoshikawa [15].

The result shown in Table I was confirmed in another experiment, summarized in Fig. 3. Washed erythrocytes from four HK sheep were incubated at 37° C in glucose-containing media with either a high (200 mM) or a low (5.5 mM) chloride concentration. The pH_0 in both cases was 7.7. The figure shows that in all four cases the cellular ATP in the low chloride medium (alkaline cells) fell to values considerably below those in the high chloride concentration media.

Low cellular ATP values during flux incubations were undesirable because it is known that the pump fluxes decrease when there is a limitation of the substrate ATP [27]. Table III shows the results of an experiment in which we attempted to increase cellular ATP concentrations to values well above normal by incubation at 37° C in a suitable medium containing glucose, adenosine and inorganic phosphate. It is clear from Table III that the cellular ATP was increased by this treatment. The average increase after a 2 h incubation was 80%.

Table IV presents ATP contents in the erythrocytes after preincubation and incubation in the three different flux media all of which contained adenosine. The results confirms the observations described in Table I and in Fig. 1, i.e. preincubated cells incubated in an adenosine-containing medium suffer a net loss in ATP when the medium is low in chloride and has a normal pH (the cells having an altered membrane potential and an alkaline interior) in comparison with either a high chloride concentration and normal pH medium (cells of

TABLE I

AMOUNTS OF ATP IN ERYTHROCYTES BEFORE AND AFTER 120 min INCUBATION AT 37°C IN MEDIA OF DIFFERENT Cl^- CONCENTRATION AND pH

Fresh HK sheep cells, washed twice in 0.17 M NaCl and once in 0.34 M sucrose, were diluted 100-fold and incubated at 37°C for 120 min in media of the following composition: (a) 0.5 or 5 mM KCl, 100 or 97.75 mM $MgCl_2$, 41 or 37.8 mM sucrose, 5 mM glucose, 20 mOsM Mg/TES, pH 7.3 (37°C). (b) 0.5 or 5 mM KCl, 4.75 or 2.5 mM $MgCl_2$, 276 or 275 mM sucrose, 5 mM glucose, 20 mOsM Mg/TES, pH 7.3 (37°C). (c) 0.5 or 5 mM KCl, 4.75 or 2.5 mM $MgCl_2$, 276 275 mM sucrose, 5 mM glucose, 20 mOsM Mg/TES, pH 6.2 (37°C). The pairs of figures are duplicate values from the same cell suspension. In the set (a) incubation shows no significant effect. In (b) ATP is reduced to 74%, and in (c) to only 12% of the value before incubation.

Condition	Ouabain	KCl (mM)	ATP (mmol/original l of cells)	
			Before	After
(a) $[Cl]_0$ = 200 mM; pH_0 = 7.3; E_{Cl} = −8 mV; pH_i = 7.2	—	0.5	0.55	0.82
			0.52	0.70
	—	5	0.94	0.67
			0.95	0.74
	+	5	0.98	0.83
			0.94	0.87
Average			0.81	0.77
(b) $[Cl]_0$ = 10 mM; pH_0 = 6.2; E_{Cl} = +75 mV; pH_i = 7.4	—	0.5	0.88	0.68
			0.95	0.65
	—	5	0.82	0.57
			0.88	0.63
	+	5	0.90	0.72
			0.92	0.71
Average			0.89	0.66
(c) $[Cl]_0$ = 10 mM; pH_0 = 7.3; E_{Cl} = +60 mV; pH_i = 8.4	—	0.5	0.81	0.18
			0.88	0.07
	—	5	0.84	0.08
			0.90	0.06
	+	5	—	0.10
Average			0.86	0.10

TABLE II

CELLULAR ATP AFTER INCUBATION WITH 0.1 mM AND WITHOUT OUABAIN

Average values and the standard errors of the means (S.E.) are presented. The values were combined independently of whether the media contained 0.5 or 5 mM KCl. The media used are those specified in Table I except that the media used on preincubated cells also contained 5 mM adenosine. Preincubation was performed for 2.5 h at 37°C in a medium containing 5 mM glucose, 5 mM adenosine, 10 mM KCl, 120 mM NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.4.

	ATP (mmol/original l of cells)		
	Without ouabain	With ouabain	Difference
Not preincubated cells, no adenosine	0.53 ± 0.12	0.53 ± 0.12	0 ± 0.04
Preincubated cells, adenosine	1.29 ± 0.09	1.27 ± 0.09	−0.03 ± 0.01

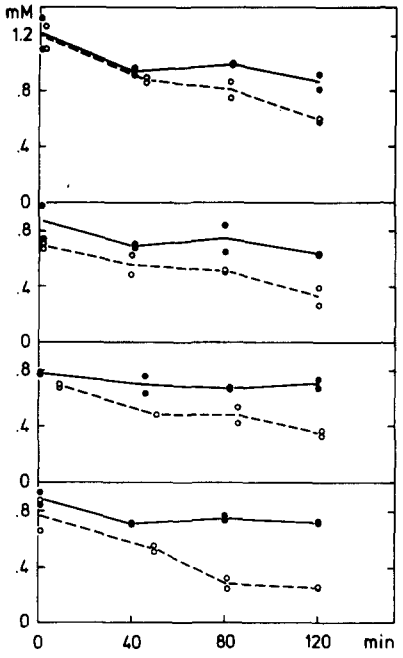

Fig. 3. Amounts of ATP in erythrocytes from four HK sheep during incubation at 37°C in media with high and low Cl^- concentrations, $pH_0 \approx 7.7$ (no adenosine). Erythrocytes washed once in 0.170 M NaCl and once in 0.340 M sucrose, were suspended in either a medium with 200 mM Cl^- (0.5 mM KCl, 99.8 mM $MgCl_2$, 5 mM glucose, 29 mM sucrose, 20 mosM Mg/TES, ph 7.6) or 5.5 mM Cl^- (0.5 mM KCl, 2.5 mM $MgCl_2$, 5 mM glucose, 280.5 mM sucrose, 20 mosM Mg/TES, pH 7.7). In the low (Cl^-) medium the erythrocytes will have an internal pH close to 9.0 and E_{cl} about +75 mV. In the high (Cl^-) medium pH_i = 7.4 and $E_{Cl} \approx -10$ mV. In all four cases ATP decreases more in the low than in the high (Cl^-) medium.

TABLE III

EFFECT ON CELLULAR ATP OF THE PREINCUBATION MEDIUM AFTER 2 h INCUBATION AT 37°C

Erythrocytes were incubated at a hematocrit of 15—20% in a medium containing 5 mM glucose, 5 mM adenosine, 10 mM KCl, 120 mM NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.4. Each figure is the average from duplicate samples. Incubation at 37°C in this medium resulted in a significant increase in cellular ATP.

Expt. No.	ATP (mmol/original l of cells)			
	Initial	Final	Increase (mM)	Increase (%)
22	1.30	2.34	1.04	+80
23	1.40	2.42	1.02	+73
24	0.86	1.74	0.88	+102
26	0.97	1.41	0.44	+45
27	0.98	1.95	0.98	+100
28	0.83	1.60	0.77	+93
29	0.70	1.32	0.62	+89
30	1.16	1.97	0.81	+69
Average ± S.E.	1.03 ± 0.09	1.84 ± 0.14	0.82 ± 0.07	+80

TABLE IV

FINAL AMOUNTS OF ATP IN PREINCUBATED CELLS AFTER INCUBATION AT 37°C IN DIFFERENT FLUX MEDIA, ALL CONTAINING ADENOSINE

Preincubated and washed erythrocytes were incubated in media containing 5 mM KCl and 5 mM adenosine and for the rest as specified under Table I. Each ATP value is the average of four determinations, duplicate samples with and without 0.1 mM ouabain. There is no significant difference between the conditions $[Cl]_0$ = 200 mM, pH_0 = 7.5, and $[Cl]_0$ = 10 mM, pH_0 = 6.2. However, with $[Cl]_0$ = 10 mM, pH_0 = 7.5 the value is significantly less ($0.005 < P < 0.025$ if compared with $[Cl]_0$ = 10 mM, pH_0 = 6.2) but still not reduced below a normal value.

Expt. No.	Incubation time (min)	ATP (mmol/original l of cells, ± S.E.)		
	$[Cl]_0$:	200 mM	10 mM	10 mM
	pH_0:	7.5	6.2	7.5
18	90	1.52 ± 0.03	1.47 ± 0.02	1.06 ± 0.02
19	90	1.57 ± 0.02	1.49 ± 0.03	0.96 ± 0.02
21	100	1.15 ± 0.04	1.28 ± 0.01	0.62 ± 0.01
13	120	1.84 ± 0.03	1.77 ± 0.04	1.13 ± 0.02
20	120	1.42 ± 0.03	1.14 ± 0.02	0.75 ± 0.01
Average ± S.E.		1.50 ± 0.11	1.43 ± 0.11	0.90 ± 0.10

normal membrane potential and normal pH) or a low chloride concentration and low pH medium (cells with an altered membrane potential and a normal pH).

The ATP concentrations were still higher than normal at the end of the flux incubation periods except for the medium in which the cells became alkaline. In this medium the final ATP values were very close to the normal concentration of the donor sheep (Table III, initial values). An exception was Expt. 21, when the ATP at the end of the incubation in medium 3 was only 0.62 mM.

Na^+ and K^+ pump fluxes in the various conditions.

The values for pump fluxes and the conditions under which they were obtained are given in Table V. With both a low chloride concentration and low pH in the media, the K^+ pump fluxes were similar to those obtained in the reference condition. When the chloride was low and the pH_0 normal, however, the K^+ pump fluxes were reduced by about 50%.

The average values for Na^+ pump fluxes under the three conditions show a similar pattern, the significance of the difference is, however, destroyed by two values, both from the second flux period in Expt. 13. If these are disregarded, the Na^+ pump fluxes give the same result as the K^+ pump fluxes. If these values are taken into account, the Na^+ pump flux values do not contradict a reduction in pump activity for Na^+ such as is observed for the K^+. Assuming that pumping transport of Na^+ and K^+ is coupled and has a fixed Na^+/K^+ coupling ratio of 1.5 [2] the data in Table V support the conclusion that the pump activity is significantly reduced when the pH_i is high compared with the other two conditions.

Since Na^+ and K^+ pump values were obtained from separate experiments there are no paired data from which ratios of Na^+ to K^+ pump flux values can be calculated. Table V shows values for this ratio computed from the average values of the fluxes. The ratios show a relatively large variation as expected

TABLE V

Na^+ PUMP OUTFLUX (oM^P_{Na}) AND K^+ PUMP INFLUX (iM^P_K) OF ERYTHROCYTES IN THE DIFFERENT MEDIA

Washed, preincubated and washed erythrocytes were incubated in media of different chloride concentrations and pH but all in 5 mM KCl, 5 mM glucose and 5 mM adenosine. Osmolarity was kept constant by replacement of $MgCl_2$ with sucrose. Cellular concentrations in samples taken during the flux incubations were for Na^+ 20 (range 16—22) and for K^+ 144 (range 131—158) mmol · l^{-1} water. In each incubation two successive flux periods were used: the first being 30 min and the second 60 min in all K^+ flux experiments. In the Na^+ flux experiments they were either 50 min (Expt. 21) or 60 min (Expt. 13). Flux values were computed from duplicate samples. The two values from each experiment in the table are those from the first and second flux period. The flux values are expressed in mmol of ions entering or leaving the number of erythrocytes that had the volume of 1 l when originally in their own plasma.

Condition	Expt. No.	oM^P_{Na}	Expt. No.	iM^P_K	oM^P_{Na}/iM^P_K
$[Cl]_0$ = 200 mM; pH_0 = 7.3; $E_{Cl} \approx -10$ mV; $pH_i \approx 7.2$	13	0.43	18	0.37	
		0.33		0.33	
	21	0.64	19	0.38	
		0.52		0.31	
Average		0.48		0.35	1.4
$[Cl]_0$ = 10 mM; pH_0 = 6.2; $E_{Cl} \approx +70$ mV; $pH_i \approx 7.3$	13	0.62	18	0.42	
		0.28		0.34	
	21	0.62	19	0.57	
		0.57		0.35	
Average		0.52		0.42	1.2
$[Cl]_0$ = 10 mM; pH_0 = 7.4; $E_{Cl} \approx +50$ mV; $pH_i \approx 8.2$	13	0.33	18	0.13	
		0.27		0.18	
	21	0.36	19	0.22	
		0.32		0.17	
Average		0.32		0.18	1.8

from the spread in Na^+ pump flux values and cannot therefore be taken as a proof of the expected value of 1.5, but do not on the other hand exclude such a value.

Discussion

Among the three conditions tested, cellular ATP content decreased during the incubation period only when the internal pH was high (8.2). This result is consistent with previously reported observations. For example, as the pH of the medium was increased from 7.4 to 8.2, Minakami and Yoshikawa [15] found that the ATP content was reduced in human erythrocytes incubated in saline solutions for 1 h. This effect was more pronounced when the concentration of inorganic phosphate was zero rather than 30 mM. Tsuboi and Fukunaga [16] studied human erythrocytes in electrolyte and non-electrolyte media at normal external pH. They found increased ATP turnover and reduced ATP concentrations in the non-electrolyte media. They did not attribute these findings to a change in intracellular pH. However, Tsuboi [28] found a stimulation of glycolytic rate and acceleration of ATP degradation in erythrocytes in a low-

electrolyte medium buffered to normal pH. His experiments support his conclusion that there is an increased ATP degradation associated with increased internal pH as well as a separate, pH-independent, activation of (ouabain-insensitive) membrane ATPase in low-electrolyte media. The influence of internal pH on glycolytic rate was confirmed by Minakami et al. [29]. The present results are in good agreement with those of Tsuboi [28] and Minakami et al. [29] regarding the effect of increased internal pH on glycolysis and ouabain-insensitive ATPase activity but do not confirm Tsuboi's suggestion that low external ionic strength stimulates ATPase activity directly independent of an increase in pH.

The variation in Na^+-K^+ pump activity found can be discussed on the assumption that either the effect is dependent on one variable alone, or two variables in combination are required. The possibility cannot be excluded that for instance the change in membrane potential stimulated the pump activity, while the simultaneously decreased external pH had a correspondingly inhibitory effect. Only if we make the plausible assumption that one variable alone is responsible for the observed effect can the results reported in this paper be taken as a support of the conclusion that changes of membrane potential between −10 and +70 mV at constant internal pH do not alter the rate of active transport of K^+ and Na^+. This conclusion agrees with those reported for human erythrocytes [11], frog striated muscle [8], squid axons [9], and molluscan neurons [10] but not those for frog muscle of Horowicz and Gerber [5,6] and or Rapoport [3,4]. If we regard the pump as a current generator, this result suggests that the internal resistance ($\Delta E/\Delta I$) of this current source is high. Assuming that we could detect a change of 10% in the normal pump fluxes for a change in membrane potential of 80 mV and assume 1/3 of the Na^+ transport constitutes electrical current, we can estimate a pump conductance of less than 10^{-9} $ohm^{-1} \cdot cm^{-2}$. Assuming a pump density of 1 μm^{-2} in HK sheep erythrocytes [30], this yields an estimate of a conductance of less than 10^{-17} ohm^{-1}/pump.

The data shown in Table V show that increased internal pH at constant membrane potential inhibits the K^+-Na^+ pump. This result is similar to those reported by Ellory and Maher [31]. Using LK goat erythrocytes they showed that the K^+ pump was considerably inhibited by increasing pH from 7.5 to 9.0 in a high chloride medium. This result could, however, be due to substrate limitation of the pump as they did not report taking any measures to avoid a decrease of ATP concentration in the alkaline cells. On the other hand, they also reported inhibition of ATPase activity by increasing pH in fragmented ghosts in media containing 2 mM ATP.

Increasing internal pH could reduce the rate of Na^+-K^+ transport either by altering the affinity of the system for some activator or inhibitor, or by changing the maximum transport rate. External K^+ stimulates and external Na^+ inhibits the pump. Since our experiments were carried out in the absence of external Na^+ and in 5 mM K^+ which is ten time greater than the concentration of K^+ required to half-maximally stimulate the system under these conditions [2] it is unlikely that the effect of pH_i is attributable to changes in the affinity of the pump for K_0^+ or Na_0^+. On the other hand, the pump is stimulated by internal Na^+ and inhibited by internal K^+. Our experiments were carried out

with HK sheep erythrocytes containing relatively high K^+ and low Na^+ concentrations so that inhibition of the pump by increased pH_i could be due to altered affinities for internal Na^+ and/or K^+. This idea is strongly supported by preliminary experiments on HK sheep erythrocyte with internal K^+ and Na^+ concentrations adjusted to 0.2 or 2 and 92 or 91 mmol/l cells, respectively, by the Nystatin method [32] prior to the flux incubation. The pump activities were the same when the membrane potential and pH_i were either 51 mV and 8.2 or −10 mV and 7.2. Further experiments will be required to resolve this point.

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