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INCUBATION OF HK AND LK SHEEP RED CELLS *IN VITRO* FOR LONG PERIODS

G. R. KEPNER* AND D. C. TOSTESON

The Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N.C. 27706 (U.S.A.)

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SUMMARY

HK and LK sheep red cells have been maintained *in vitro* at 37 °C for periods of up to 3 weeks. During the incubation, periodic measurements of cell Na^+, K^+, ouabain-sensitive and ouabain-insensitive K^+ influxes were made. The effects of lowering the *in vitro* incubation temperature and of reducing the K^+ concentration in the medium on cell cation content and volume were also investigated. The results of these experiments were, in general, consistent with previous measurements of the effects of these parameters on K^+ and Na^+ fluxes in short term experiments. *In vitro* incubation for several days was also used to show that exposure to anti-L serum produces a net uptake of K^+ and extrusion of Na^+ in LK sheep red cells. A new procedure for measuring the active and passive influxes of K^+ was developed. The early time course of K^+ influx inhibition by ouabain was also studied by this procedure.

INTRODUCTION

An important problem in cell physiology is how the processes of cation transport are integrated to regulate cell cation composition and volume. Earlier studies led to the formulation of a model for such regulation in sheep red cells[1,2]. However, changes in cation composition in normal sheep red cells have long time constants and thus the predictions of the model regarding long term cation and volume regulation have not been subjected to experimental test for lack of a system to maintain red cells *in vitro* at 37 °C over periods of weeks.

This paper presents results with a system that allows *in vitro* studies to be carried out with HK and LK sheep red cells under defined conditions. Observations have been made on cell Na^+ and K^+ as well as ouabain-sensitive (pump) and ouabain-insensitive (leak) K^+ influxes in normal HK and LK red cells maintained *in vitro* for several weeks. Experiments were performed in which the incubation temperature or the K^+ concentration in the medium was varied to assess effects on cell function and survival[3].

* Present address: Department of Physiology, University of Minnesota Medical School, Minneapolis, Minn. 55455, U.S.A.

By means of the *in vitro* system it has been possible to demonstrate unequivocally that the several-fold stimulation of ouabain-sensitive K^+ uptake in LK red cells by anti-L serum is, in fact, an enhancement of active transport[4-6]. Net K^+ accumulation against an electrochemical gradient was observed when anti-L serum-treated LK cells were incubated *in vitro* for 1 week.

In the course of developing the *in vitro* technique, a new approach to measuring pump and leak K^+ influxes was developed. A method is described which permits K^+ influx measurements to be made on a few microliters of red cells.

TABLE I

MINIMUM ESSENTIAL MEDIUM (JOKLIK-MODIFIED) + SUPPLEMENTS (NON-ESSENTIAL AMINO ACIDS AND CALF SERUM)

	Compound	*Concentration*	
		mg/l	*mM*
(I) Balanced salt solution	NaCl	6500	111.3
	KCl	400	5.3
	$MgCl_2 \cdot 6\ H_2O$	200	0.9
	$NaH_2PO_4 \cdot H_2O$	1327	9.6
	$NaHCO_3$	2000	24.0
	Dextrose	2000	11.1
(II) Essential amino acids	L-Arginine·HCl	105	0.5
	L-Cystine·2 HCl	32	0.1
	L-Glutamine	294	2.0
	L-Histidine	31	0.2
	L-Isoleucine	52	0.3
	L-Leucine	52	0.3
	L-Lysine	58	0.3
	Methionine	15	0.1
	L-Phenylalanine	32	0.1
	L-Threonine	48	0.4
	L-Tryptophan	10	0.04
	L-Tyrosine (disodium)	47	0.2
	L-Valine	46	0.4
(III) Vitamins	Choline chloride	1	
	Folic acid	1	
	Inositol	2	
	Nicotinamide	1	
	D-Calcium pantothenate	1	
	Pyridoxal·HCl	1	
	Riboflavin	0.1	
	Thiamine·HCl	1	
(IV) Antibiotics	Potassium penicillin G	75 units/ml	
	Streptomycin sulfate	50 μg/ml	
(V) Non-essential amino acids	L-Alanine	8.9	0.1
	L-Asparagine·H_2O	15	0.1
	L-Aspartic acid	13.3	0.1
	L-Glutamic acid	14.7	0.1
	L-Proline	11.5	0.1
	L-Serine	10.5	0.1
	Glycine	7.5	0.1
(VI) Fetal calf serum	50 ml per l of medium		

The method also avoids those conditions of the conventional technique that necessitate several washings to separate cells from ^{42}K-containing medium. It was used to study the early time course of ouabain action on active K^+ transport.

METHODS

Procedure for long term incubations

All operations were carried out with sterile solutions, equipment and technique. Sheep were bled into heparinized flasks and 20–30 ml were transferred to a centrifuge tube. After centrifuging, the buffy coat and plasma were removed by aspiration, and the packed cells resuspended in wash medium and washed three times. Typically the wash medium was the balanced salt solution component of the complete medium used for the *in vitro* incubation (Table I: components obtained from GIBCO Long Island). For experiments with low concentrations of K^+ in the incubation medium, the K^+ in the balanced salt solution was replaced, mole for mole, with Na^+. Aliquots of washed resuspended cells were transferred to stoppered bottles previously loaded with the *in vitro* incubation medium. The resulting cell suspension ($1–3 \cdot 10^{-3}$, v/v) was equilibrated with CO_2–air (5:95, v/v) to maintain the pH at 7.35 ± 0.1 and then incubated at the desired temperature with subsequent gassing every 1–2 days. In some experiments, suspensions were stirred continuously by a magnetic stirring bar while in others the cells were resuspended daily *via* swirling the bottle by hand. Aliquots of the suspension were drawn from the bottles for the periodic determinations of cell Na^+ and K^+ concentrations, hemoglobin in the medium, and K^+ influx. Cell lysis in the cultures was calculated from the hemoglobin measurements. Samples obtained without exposure to room air were used to determine the pH of the suspensions in the bottles.

In experiments with LK cells treated with anti-L serum (kindly provided by Dr B. Rasmusen) a 20% suspension of washed LK cells was incubated with the iso-immune anti-L serum (complement inactivated by heating at 56 °C) obtained from a homozygous HK sheep, which had previously been immunized with whole blood from a heterozygous LK donor. After a 1-h incubation at 32 °C, aliquots of the cell suspension: serum mixture (1:1, v/v) were transferred to bottles and the resulting 10^{-3} (v/v) cell suspension incubated as described above at 37 °C for 7 days. A control experiment used LK cells incubated with serum from a non-immunized HK sheep (HKNI serum).

Procedure for flux measurement

A 10-ml aliquot of cell suspension from each bottle was centrifuged at 1100 × *g* for 1 min (Sorvall RC2-B, 4 °C) and about 9 ml of supernatant removed by aspiration. Gentle agitation by hand resuspended the cells in the remaining supernatant, yielding a 10^{-2} (v/v) cell suspension. At the start of the flux measurement, 30–50 μCi of ^{42}KCl (Cambridge Nuclear) were added to each milliliter of 10^{-2} (v/v) suspension. In a typical experiment, samples were taken at three subsequent times (t_1, t_2 and t_3). Immediately after the second sample was removed, ouabain was added in sufficient amount to make a concentration of 10^{-4} M in the remaining cell suspension. In control experiments, it was established that 10^{-4} M ouabain acted very rapidly to inhibit the pump component of K^+ influx. This allowed

estimation of total K^+ influx from analyses of aliquots taken at times t_1 and t_2 and the leak influx from aliquots taken at times t_2 and t_3. All aliquots were prepared for analysis as follows.

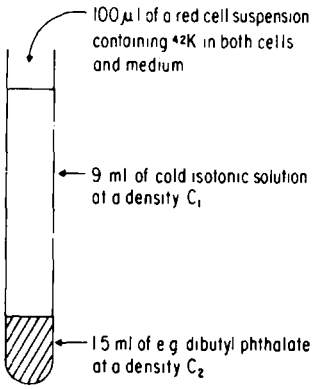


Fig. 1. Typical loading for a centrifuge tube to be used in separating red cells from ^{42}K-containing medium by means of the density separation technique. C_1, C_2 and C_3 are the densities of the upper and lower phases in the tube and of the red cells, respectively. Note that $C_1 < C_2 < C_3$.

The basic approach is illustrated in Fig. 1. Note that the ^{42}K in the cell suspension medium is diluted about 90-fold and the cells are rapidly cooled in the large volume of cold, non-radioactive solution. During centrifugation at 20000 × *g* for 5 min the cells sediment to the bottom since the density (C_2) of the fluid at the bottom of the tube was chosen to be less than C_3, the density of the cells. The volume of extracellular fluid is 1–3% of the volume in the cell pellet (unpublished observations). The density barrier at the interface between Phases 1 and 2 restricts movement of ^{42}K-containing medium and inhibits mixing. In these experiments, Phase 1 was isotonic $MgCl_2$ (0.12 M, buffered with $MgCO_3$ at 5 mg/l). Phase 2 was either dibutyl phthalate (Baker Chem. Co., New Jersey), an organic ester immiscible with water and non-lytic to the red cells[7], or isotonic $MgCl_2$ *plus* dextran (10–15%, w/w). Several factors influence the choice for any particular application, *e.g.* ease of handling, cost, density barrier stability. All flux studies reported here were carried out with dibutyl phthalate unless specified otherwise. Note that with dibutyl phthalate there is a phase as well as a density difference at the interface between Phases 1 and 2. After the 5-min centrifugation, solution Phase 1 was aspirated and the tube set aside to allow the tube walls to drain before further aspiration of the tube walls and the surface of Phase 2. About 9 ml of isotonic $MgCl_2$ were then squirted into the tube to rinse the tube walls and surface of Phase 2 free of ^{42}K. The supernatant was then totally aspirated, leaving the tightly packed red cell pellet. 3 ml of hemolysing fluid (made by adding 25 ml conc. NH_4OH, 0.5 ml non-ionox detergent and 10.78 g CsCl to 16 l distilled water) were added and the tube vortexed to aid in lysing the cells.

Analytical procedures

The ^{42}K radioactivity in the lysed cells was counted in an automatic well-type scintillation counter (Packard). Na^+ and K^+ in lysed cells or in supernatants were

measured, after suitable dilution with hemolysing fluid, by atomic absorption spectrophotometry (Perkin–Elmer Model 303). Hemoglobin was determined using Drabkin's solution (containing EDTA at a final concentration of 1 mM) and measuring absorbance at 540 or 418 nm (Gilford Spectrophotometer Model 300-N). Before taking an aliquot of the hemolysed cells for hemoglobin determinations, it was necessary to centrifuge the lysed cell suspension at 20000 × *g* for 5 min. This procedure sedimented the red cell membrane fragments to which dibutyl phthalate was adhering and prevented interference with the hemoglobin determination. A thermostatted radiometer micro-sample pH meter was used to measure pH.

Calculations

$$\left(\frac{\Delta K^*}{\Delta t}\right)_c \cdot \frac{[K]_o}{K_o^*} = \text{Flux} \qquad \text{mmoles/(original l of cells) per h} \tag{1}$$

$(\Delta K^*/\Delta t)_c$ is the rate of uptake of ^{42}K into the cells, corrected for back flux which was less than 5% of K^*_c. $[K]_o$ is the concentration of potassium in the flux medium in mmoles/l. K^*_o is the activity of ^{42}K in the flux medium in cpm/l.

$$V_t/V_o = (Na + K)_t/(Na + K)_o \tag{2}$$

Measurements of cell (Na + K) in mmoles/original l of cells refer to that number of cells occupying, initially, a volume of 1 l. Total cell cation concentration, essentially the [Na + K] in mmoles/l of cell water, is assumed constant. Cell volume consists of cell solids (fixed) *plus* cell water (variable). If the cell (Na + K) content changes the cell water volume must change to keep [Na + K] constant and the volume occupied by the original l of cells must change. Changes in cell (Na + K) with time are thus related to V_t/V_o, the relative volume of osmotically responsive cell water at time "*t*" after initiating the *in vitro* incubation, and indicate the relative change in cell volume.

$$[Na] \text{ mmoles/l of cell water} = \frac{(Na) \text{ mmoles/original l of cells}}{(0.68)\cdot(Na + K)_t/(Na + K)_o} \tag{3}$$

Eqn 3 is used to convert measured values of cell cation contents to units of cation concentration. The value 0.68 was used for the fraction of total cell volume in osmotic equilibrium with the extracellular fluid at the start of the experiment.

RESULTS

K^+ influx technique

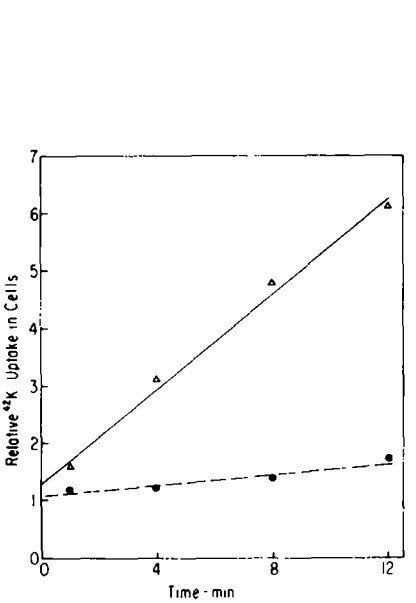
The data presented in Table II were from an experiment designed to compare flux measurements made by the technique described above with those made by several other procedures including our previous technique for total K^+ influx in HK sheep red cells (1). These data suggested that Procedures 2 and 3 gave values for the K^+ influx not significantly different from those obtained by Procedure 1, while also yielding a higher cell recovery. Procedure 4 was most unreliable because

TABLE II

K^+ INFLUX IN HK SHEEP RED CELLS AS DETERMINED BY FOUR DIFFERENT PROCEDURES FOR SEPARATING ^{42}K-LOADED CELLS FROM THE ^{42}K-CONTAINING MEDIUM

HK cells were incubated as described for the experiments of Fig. 2 and 100 or 200-μl aliquots of the 2% cell suspension were withdrawn at 1, 40 and 120 min after addition of ^{42}K. The aliquots were then added to centrifuge tubes containing only isotonic $MgCl_2$ ((1) and (4)) or to tubes with isotonic $MgCl_2$ *plus* either dibutyl phthalate (2) or dextran in isotonic $MgCl_2$ solution (3). The tubes were centrifuged 5 min at 20000 × *g* in the cold and the tubes for Expts 2, 3 and 4 were treated as described in Methods, except the tubes for Expt 4 were treated to a direct rinse which attempted to avoid disturbing the packed cell button. The tubes for Expt 1 were washed in the normal fashion by resuspending the cells and then centrifuging. Cells were then hemolysed by addition of hemolysing fluid and ^{42}K counted and hemoglobin determined. Appropriate samples of hemolysate were analysed for ^{42}K and K^+ content. Each of the three points on the uptake curves was done in duplicate. Duplicates were within 3% of the mean value for all but the 1-min point where they were within 10% of the mean. Fluxes were calculated from the slopes of the uptake curves.

Separation procedure	*Cells recovered* (%)	*Total K^+ influx (mmoles/(original l of cells) per h)*
(1) Cells washed 3× in isotonic $MgCl_2$	84	0.60
(2) Dibutyl phthalate used for Phase 2 (see Fig. 1)	93	0.59
(3) Dextran (12%, w/w) in isotonic $MgCl_2$ used for Phase 2	92	0.59
(4) Packed cell button rinsed once (isotonic $MgCl_2$)	79	0.65



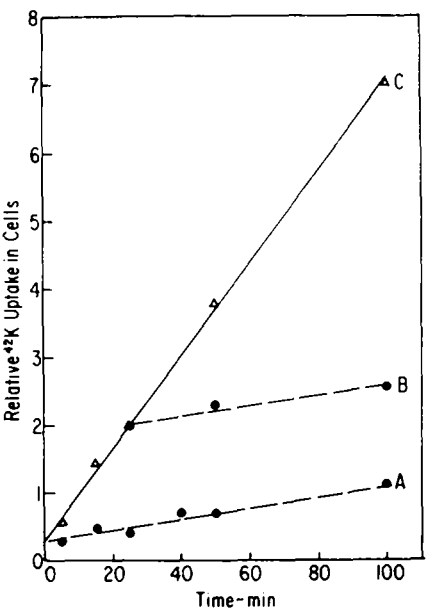


Fig. 2. Study of the initial response of ^{42}K uptake to ouabain. HK sheep red cell suspensions (about 5%, v/v) were incubated at 37 °C and pH 7.4 in a medium containing the balanced salt solution of Table I. The solid circles indicate flasks to which ouabain was added at time zero, final concentration 10^{-4} M. Each point is the mean of 4 replicates. All replicates were within 12% of the mean value for a point.

Fig. 3. ^{42}K influx experiment with HK sheep red cells. The 3% cell suspensions were incubated at 37 °C and pH 7.4 in a medium containing the balanced salt solution of Table I. Ouabain, final concentration 10^{-4} M, was added at 20 min before time zero (Curve A) or at 25 min after time zero (Curve B). Each point is the mean of 4 replicates. All replicates were within 10% of the mean value for a point.

the rinse technique removed some cells from the packed button. Employing Procedure 2 facilitated studies on the initial time course of the ouabain inhibition of K^+ influx. The data of Fig. 2 established that ouabain, at a concentration giving maximum inhibition of active transport, acted quite rapidly after addition to intact cell suspensions. Based on the above results, the standard procedure for K^+ influxes was revised (see Methods) and a comparison of the data obtained in the new procedure with that of the conventional technique is presented in Fig. 3. Previously, separate flasks were used to generate the points for Curves "A" and "C". Thus, the total K^+ influx was determined from two experimental points on the "C" curve and the ouabain-insensitive K^+ influx from any two points lying on the "A" curve. It is necessary to obtain two points for each curve to adequately characterize the linear portion of each of these uptake curves because of the non-zero intercept. In the new procedure, one flask sufficed because ouabain could be added at the time of sampling for the second uptake point and a third point then enabled one to calculate the ouabain-insensitive K^+ influx directly, as illustrated in Curve "B". Similar experiments with LK sheep red cells and with human red cells gave comparable results.

TABLE III

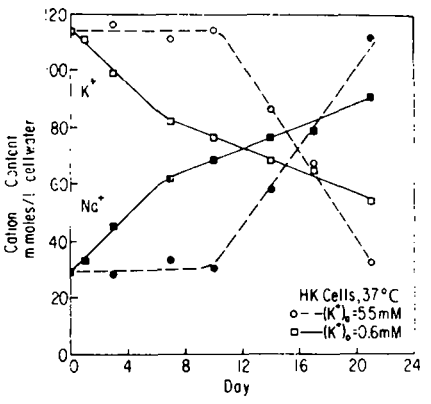
CATION CONTENT AND LYSIS OF SHEEP RED CELLS INCUBATED *in vitro*

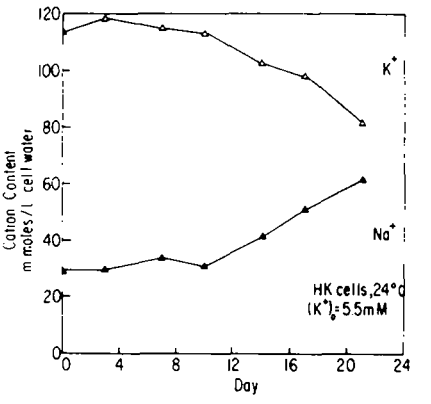
HK or LK cells were incubated *in vitro* at 37 or 24 °C and with 5.5 or 0.6 mM K^+ in the culture medium. Cell content of Na^+ or K^+ in mmoles/original l of cells is related to relative changes in cell volume by the expression for V_t/V_0 presented in the calculations section. The cell concentrations of Na^+ and K^+ in mmoles/l of cell water are calculated using Eqn 3 and presented in Figs 4–7. The data are from one study encompassing four experiments at each condition excepting 0.6 mM K^+ (two experiments). Each value for cation content in the table is the mean of 6–12 determinations. Replicates were within 10% of the mean for data taken through day 10. Replicates for subsequent data points were within 30% of the mean.

Temperature (°C)	$[K^+]_0$ (*mM*)		*Cell content of* ($Na^+ + K^+$) (*mmoles/original l of cells*) *Hemolysis* (%)						
		Day:	*0*	*3*	*7*	*10*	*14*	*17*	*21*
(a) HK cells									
37	5.5	Cations	98	93	79	75	109	80	113
		% Lysis	0	0	0	6	13	31	58
37	0.6	Cations	98	94	99	89	92	65	79
		% Lysis	0	0	0	3	9	27	25
24	5.5	Cations	98	88	93	83	85	70	76
		% Lysis	0	0	0	4	5	29	16
(b) LK cells									
37	5.5	Cations	100	95	86	92	137	112	132
		% Lysis	0	0	0	4	8	—	30
37	0.6	Cations	100	99	97	97	126	—	143
		% Lysis	0	0	0	2	3	42	41
24	5.5	Cations	100	89	90	101	124	109	128
		% Lysis	0	0	0	9	9	43	46

Long term incubation in vitro

HK and LK red cells were tested for their capability to function *in vitro* over periods of at least 1 week. Table III summarizes the data on the ($Na^+ + K^+$) content and lysis of the cells during one such study. The results from these experiments, at least over the first 10 days, were typical of the behavior exhibited by HK and LK red cells incubated in this system for 1–3-week periods. In most of the experiments shown in Table III, the cells developed increased cation permeability and content with some cellular lysis after day 10. Although no lysis had been detected at day 7, approximately 3–9 % of the LK cells and 5–13 % of the HK cells had lysed by day 14.





K+ Influx	m moles/(orig l Cells)/h			
○ pump	.65	.46	.51	.24
leak	.05	.03	.11	.8
□ pump	-	.26	.28	.25 (.90)
leak	-	.01	.01	.01 (.02)

K+ Influx	m moles/(orig l cells)/h			
△ pump	.29	.27	.40	.34
leak	.03	.01	.02	.03

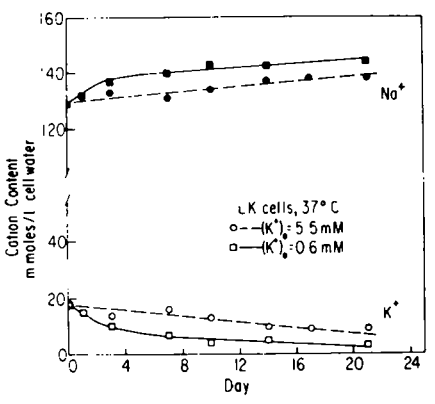
Fig. 4. HK cells were incubated *in vitro* at 37 °C, pH 7.35, approx. $3 \cdot 10^{-3}$, v/v, in the medium of Table I ($[K^+]_o$ is the K^+ concentration in the medium). Influx measurements were conducted as described in Methods. For the K^+ influx measurements on the cells incubated *in vitro* with 0.6 mM K^+ in the medium, the K^+ concentration actually present in the flux medium was 1.0 mM because of the non-radioactive K^+ carrier in the ^{42}K used. The K^+-influx data in parentheses were taken at day 21 with 6.0 mM K^+ in the flux medium. The other flux values were determined on days 0, 7, 14 and 21. Each flux was done by the three-point method with 2–4 replicates at each time point. Fluxes were calculated from the slopes of the lines between appropriate time points. The normal leak fluxes for HK cells are typically low (0.03–0.05) and the range of values observed was (0.005–0.060), excepting on days 14 and 21 for the cells with $[K^+]_o$ = 5.5 mM. Replicates for the flux values were always within 75 % of the mean flux values. Replicates varied from flux to flux in all the *in vitro* studies.

Fig. 5. Both incubation *in vitro* and flux measurements on these HK cells were carried out at 24 °C. Each flux was done with 4 replicates at each time point. The range of values observed for leak fluxes was (0.01–0.05). The replicates for pump fluxes were always within 30 % of the mean flux values.

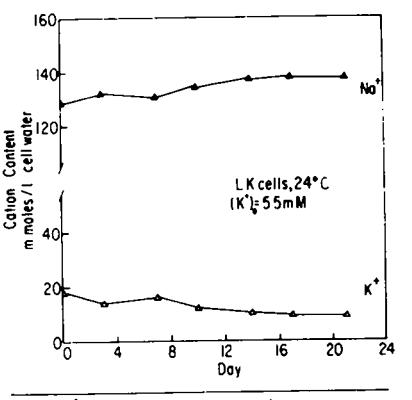
In Figs 4 and 5 the K^+ influxes and the concentrations of Na^+ and K^+ in HK cells have been plotted as a function of time for the various experiments. Under normal *in vivo* conditions, *i.e.* 37 °C and 5 mM external K^+, the concentrations of Na^+ and K^+ in these HK cells incubated *in vitro* were maintained constant up to day 10. There was, however, a tendency for the HK cells to shrink as seen in

the data of Table III and in other experiments under these conditions. After day 10, the HK cells in this experiment lost K^+ and gained Na^+ (Fig. 4) while the cell volume was increasing (Table IIIa). At day 7, the K^+-pump and leak influxes in this experiment were the same as in fresh cells. On day 14, the leak influx was up by a factor of three. The leak influx of K^+ was increased further at day 21. The interpretation of the K^+-pump data is complicated by the fact that a decrease in cell K^+ and an increase in cell Na^+ concentration produces an increased pump activity in HK and LK sheep red cells[6]. The value for the K^+ pump at day 21, was well below that characteristic of fresh HK cells with the same internal K^+ concentration (55 mM).

When the HK cells were in a medium containing 0.6 mM K^+, their volume remained fairly constant (Table IIIa) for 7–10 days and then decreased progressively. As expected the cell Na^+ and K^+ concentrations changed substantially because of the reduced K^+ influxes, both leak and pump, caused by the lower concentration of K^+ in the medium. However, as the cell K^+ concentration decreased, the K^+-pump activity increased (Fig. 4). Note that when the flux medium contained 6 mM K^+, the K^+-pump influx in cells incubated in 0.6 mM K^+ was elevated well above the value of about 0.6 mmole/(original l of cells) per h observed in cells incubated in 5.5 mM K^+ (see the day 21 pump influx values in parentheses in Fig. 4). The K^+-leak influx in these HK cells, incubated with low external K^+, apparently stayed constant during the incubation. No dramatic increase in the leak influx was seen after day 10 though cell lysis began to be detected after that time.



K^+ influx m moles/(orig l cells)/h				
○ pump	.17	.18	.29	.32
leak	.20	.07	.26	.27
□ pump	-	.10	.12	.10 (.37)
leak	-	.02	.05	.05 (.25)



K^+ influx m moles/(orig l cells)/h				
▲ pump	.03	.04	.08	.09
leak	.06	.04	.07	.10

Fig. 6. LK cells were incubated *in vitro* at 37 °C, pH 7.35, $1.7 \cdot 10^{-3}$, v/v, in the medium of Table I. Influx studies and replicates were done as in the experiments in Fig. 4. The replicates for the flux values were always within 50% of the mean flux values.

Fig. 7. Both incubation *in vitro* and flux measurements on these LK cells were carried out at 24 °C (replicates as in Fig. 5). The replicates for the flux values were always within 50% of the mean flux values, excepting day 14 replicates which were within 90%.

The results of an experiment with HK cells incubated at 24 °C are presented in Fig. 5. The changes in cell Na^+ and K^+ concentrations were slower than for the HK cells incubated at 37 °C. The volume of cells incubated at 24 °C decreased particularly after the 7th day (Table IIIa). The K^+-pump and leak influxes remained constant except for some increase in pump influx as the cell K^+ concentration decreased.

The results from corresponding experiments with LK cells are presented in Table IIIb and Figs 6 and 7. It should be noted that these particular LK cells were not typical in regard to their K^+-pump and leak influxes as measured at day 0 under normal conditions (37 °C and 5.5 mM $[K^+]_o$). The pump and the leak were higher than those customarily found for LK red cells in this laboratory, 0.15 and 0.09 mmoles/(original l of cells) per h, for pump and leak respectively[1]. The volume changes in LK cells under the differing conditions of the experiments were qualitatively similar (Table IIIb). There was only a slight decrease in cell volume over the first 10 days of the incubation and then the cells began to swell steadily. The K^+ influx data in Figs 6 and 7 revealed that the leak influx was high and increased somewhat over the course of the incubations. Pump influx values were also higher, in part due to the decreasing cell K^+ concentration. In all the LK experiments, the changes in cell Na^+ and K^+ concentrations proceeded in similar directions but the rates of change were reduced at the lower incubation temperature (Fig. 7) and decreased by a higher K^+ concentration in the incubation medium (Fig. 6).

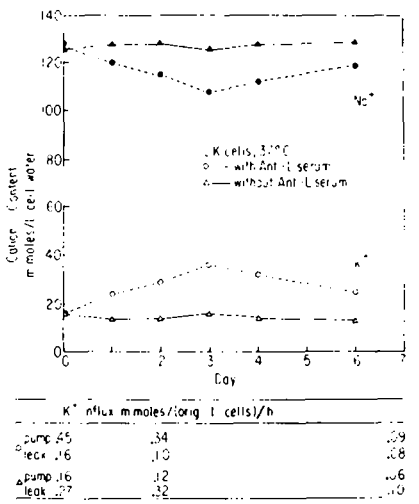


Fig. 8. Experiment with LK red cells treated with anti-L and incubated *in vitro* at 37 °C. Each flux was done with 4–8 replicates at each time point. The replicates for the flux values were always within 40% of the mean flux values.

Fig. 8 and Table IV present the results of an *in vitro* incubation with anti-L-treated LK red cells. The cell K^+ concentration increased and the cell Na^+ concentration decreased in the anti-L-treated cells over the first 3 days of the incubation. No such changes were observed in LK sheep red cells treated with non-immunized HK sheep serum. Ouabain-sensitive K^+ uptake was increased in the anti-L-treated

cells at day 0 and day 2. The decreased ouabain-sensitive K^+ uptake in the anti-L-treated cells at day 2 was probably a consequence of the increased K^+ concentration in the cells. The K^+ pump remained normal in the cells treated with non-immunized HK sheep serum at day 0 and day 2. The K^+-leak influxes were normal in the anti-L-treated cells but much higher than normal in the cells treated with non-immunized HK sheep serum at day 0 and day 2. After day 3, K^+ concentration decreased while Na^+ concentration increased in anti-L-treated cells. By day 7 the anti-L stimulation of ouabain-sensitive K^+ uptake had disappeared.

TABLE IV

CATION CHANGES IN ANTI-L-TREATED LK SHEEP RED CELLS INCUBATED *in vitro*

LK cells were treated with anti-L and incubated at 37 °C. Cell content of Na^+ and K^+ is related to V_t/V_0. The cell concentrations of Na^+ and K^+ are calculated using Eqn 3 and presented in Fig. 8. The data are from two studies, two to five experiments at each condition in each study. Each value for cation content in the table is the mean of 6–12 determinations. Replicates were within 10% of the mean.

Treatment	*Cell content of* ($Na^+ + K^+$) (*mmoles/original l of cells*)					
Day:	*0*	*1*	*2*	*3*	*4*	*6*
Anti-L	98	83	90	79	71	66
Non-immunized HK sheep serum	97	80	91	83	73	83

DISCUSSION

The observations reported here on *in vitro* incubation of red cells represent substantial progress towards the final development of an *in vitro* system for simulating the *in vivo* environment and maintaining normal functions in sheep red cells. However, additional evaluation is needed before the system will be completely satisfactory for this purpose. For example, the complexity of the incubation medium suggests that redundant components may be present. The most effective range of concentrations of essential components remains to be defined. Furthermore, we do not have complete explanations for the shifts in total cation content seen in these and other studies with both HK and LK cells incubated in media containing the same concentrations of Na^+ and K^+ as normal blood plasma. Despite these limitations, we report the system in its present form because it permits a wide range of experiments which have previously been impossible.

For example, the experiment on the stimulation of the pump in LK cells by anti-L necessitated following cation changes in LK red cells *in vitro* for several days. The data in Table IV and Fig. 8 demonstrated net K^+ accumulation against an electrochemical potential gradient in the anti-L-treated LK red cells. This established unequivocally that anti-L stimulates the active transport system of LK sheep red cells.

It is evident from the data in Table III and in Figs 4–7 that even such relatively substantial perturbations as decreasing $[K^+]_0$ by a factor of ten or lowering the incubation temperature to 24 °C still required several days before their effects on cell cation content, influxes and volume were readily observable. This fact illustrates another important potential use of the long term incubation system. In

biological systems, it is often difficult to control conditions so that measurements are reproducible within $\pm$ 1 %. This is particularly true of rate processes such as cation transport across membranes. On the other hand, it is clear that a 1 % change in the rate of transport of an ion such as K^+, if uncompensated for a sufficient period of time, can produce large changes in cell K^+ content. Since physiological regulation presumably maintains red cell K^+ and Na^+ content constant to $\pm$ 0–5 %, it follows that net transport rates for these components are regulated at least as precisely. Quantitative estimation of transport rates to this degree of precision in acute experiments is impossible with present techniques. By permitting temporal amplification of small rate changes in the form of relatively large and easily measurable changes in cell content of K^+ and Na^+ (or other components), long term incubation *in vitro* may allow more complete elucidation of the physiological regulation of transport processes.

It is of interest to compare the studies with a long term *in vitro* technique, as described here, with acute *in vitro* studies conventionally devoted to characterizing the transport process. For example, recent work in this laboratory has shown that active transport in both HK and LK sheep red cells increases progressively as the ratio of internal K^+/Na^+ concentrations is reduced[8]. Changes in internal ion composition in these acute experiments were effected by the *p*-chloromercuribenzene sulfonate method[9]. The results reported in this paper on the effect of reducing external K^+ concentration in the long term incubation medium are consistent with the earlier observations. Thus, both in HK (Fig. 4) and LK (Fig. 6) sheep red cells, reduction of internal K^+ concentration by long term incubation in the low K^+ medium was associated with stimulation of K^+-pump influx. Similarly, the effect of reducing the temperature of the long term incubation system from 37 °C to 24 °C is consistent with the temperature coefficients of K^+ influx and outflux measured in acute experiments in human red cells[10]. Since the temperature coefficients of these two processes are both large and approximately equal, reduction in temperature would not be expected to produce appreciable changes in cell K^+ concentration (see Figs 5 and 7).

We have been unable to find reports in the literature on studies of cation and volume regulation in mammalian red cells maintained *in vitro*. However, a study has been reported on volume changes and ion movements in mouse lymphoblasts in suspension culture[11]. It was found that in such growing cells there was a steady accumulation of cations as the volume increased during the division cycle. The doubling time of these cells (11 h) makes it rather difficult to investigate long term regulation of their cation content.

Considerable *in vitro* experience[12] has been accumulated with human red cells stored under blood banking conditions, *i.e.* 4 °C or –79 °C. At 4 °C, active transport is nearly zero and the cells proceed to lose K^+ and gain Na^+ *via* the passive leaks. The gain in Na^+ exceeds the K^+ loss and the cells swell. At 3 weeks the cell Na^+ and K^+ concentrations are about equal. At –79 °C the cells lose about one-third of their K^+ over a 3-month period. These cells can reestablish normal cation gradients if they are incubated at 37 °C in the presence of suitable substrates. The cells are viable *in vivo* as evidenced by the success with transfusion of stored blood. Studies carried out at 37 °C may further elucidate the requirements for preservation of blood at 4 °C or at lower temperatures.

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REFERENCES

1 D. C. Tosteson and J. F. Hoffman, *J. Gen. Physiol.*, 44 (1960) 169.
2 D. C. Tosteson, in J. F. Hoffman, *The Cellular Functions of Membrane Transport*, Prentice-Hall, New Jersey, 1964, p. 3.
3 G. R. Kepner and D. C. Tosteson, *Biophys. Soc. Abstr.*, 10 (1970) 224.
4 J. C. Ellory and E. M. Tucker, *Nature*, 222 (1969) 477.
5 P. K. Lauf, B. A. Rasmusen, P. G. Hoffman, P. B. Dunham, P. H. Cook, M. L. Parmelee and D. C. Tosteson, *Proc. 3rd Int. Biophys. Congr.*, 1961, p. 71.
6 G. R. Kepner and D. C. Tosteson, *Proc. 3rd Int. Biophys. Congr.*, 1969, p. 69.
7 D. Danon and Y. Marikovsky, *J. Lab. Clin. Med.*, 64 (1964) 668.
8 P. G. Hoffman and D. C. Tosteson, *J. Gen. Physiol.*, 58 (1971) 438.
9 P. J. Garrahan and A. F. Rega, *J. Physiol.*, 193 (1967) 459.
10 A. K. Solomon, *J. Gen. Physiol.*, 36 (1952) 57.
11 C. Jung and A. Rothstein, *J. Gen. Physiol.*, 50 (1967) 917.
12 T. Prankerd, *The Red Cell*, C. Thomas, Springfield, 1961, p. 94.