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Volume Regulatory Activity of the Ehrlich Ascites Tumor Cell and Its Relationship to Ion Transport

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Summary. The volume regulatory response of the Ehrlich ascites tumor was studied in KCl-depleted, Na^+-enriched cells. Subsequent incubation in K^+-containing NaCl medium results in the reaccumulation of K^+, Cl^-, water and the extrusion of Na^+. The establishment of the physiological steady state is due primarily to the activity of 2 transport systems. One is the Na/K pump (K_M for K_o^+ = 3.5 mM; J_{max} = 30.1 mEq/kg dry min), which in these experiments was coupled $1K^+/1$ Na^+. The second is the Cl^--dependent ($Na^+ + K^+$) cotransport system (K_M for K_o^+ = 6.8 mM; J_{max} = 20.8 mEq/kg dry min) which mediates, in addition to net ion uptake in the ratio of $1K^+ : 1Na^+ : 2Cl^-$, the exchange of K_i^+ for K_o^+. The net passive driving force on the cotransport system is initially inwardly directed but does not decrease to zero at the steady state. This raises the possibility of the involvement of an additional source of energy. Although cell volume increases concomitant with net ion uptake, this change does not appear to be a major factor regulating the activity of the cotransport system.

Key Words Ehrlich tumor cells · volume regulation · Na^+, K^+, Cl^- transport

Introduction

There is abundant evidence suggesting that a large fraction of the ouabain-insensitive cation flux in the Ehrlich cell [3, 10], as well as numerous other vertebrate cells [9, 32, 37], is mediated by an energetically passive, electroneutral ($Na^+ + K^+$) cotransport system which is Cl^- dependent. This system is often operationally characterized by its sensitivity to inhibition by the "loop diuretics," bumetanide and furosemide. Evaluations of the interaction between the diuretic-sensitive net ion fluxes (Na^+, K^+ and Cl^-) in the Ehrlich cell and avian erythrocytes indicate a coupling stoichiometry that most often approximates $1Na^+ : 1K^+ : 2Cl^-$ [13, 14]. While this transport system may be ubiquitous in vertebrate cells, its physiological importance in the overall regulation and maintenance of cellular electrolyte composition is unclear.

In certain cells, particularly avian erythrocytes [22, 23, 35, 36], this system is volume sensitive and under certain conditions can play a role in the regulation of cell volume. For example, cell shrinkage in response to an increase in osmolality of the extracellular medium activates a diuretic- sensitive cotransport system resulting in the net uptake of KCl. Volume increases (regulatory volume increase; RVI) and upon restoration of the initial cell volume, cotransport activity is reduced. This sequence occurs only when the extracellular K^+ concentration exceeds 2.5 mM. In contrast to the avian erythrocyte, Ehrlich cell volume does not return to normal after cells are subjected to a hyperosmotic stress [10, 15, 17]. Nonetheless, furosemide-sensitive K^+ transport, as assessed by unidirectional ^{86}Rb influx, is greatly stimulated [12]. It has been suggested that, although these cells are capable of RVI, the driving force(s) is not of sufficient magnitude to affect net ion uptake under these conditions [18].

On the other hand, preincubation of Ehrlich tumor cells in hypotonic medium results in swelling with the subsequent loss of K^+ and Cl^- through separate conductive pathways and return toward normal volume (regulatory volume decrease, RVD) [19]. Subsequent resuspension of these cells in isotonic medium results in osmotic cell shrinkage followed by a rapid return of cell volume to its original value; that is, RVI [20]. Regulatory volume increase is also observed when K^+-depleted, Na^+-enriched cells are incubated in a K^+-containing NaCl medium [13]. The restoration of cell volume in both cases is due to activation of a diuretic-sensitive cotransport pathway with resultant reaccumulation of electrolytes and water. Thus, although not as clear cut, the Cl^--dependent cation cotransport system of the Ehrlich cell does appear to play a role in the regulation of cell volume. In this regard Geck and coworkers recently proposed a feedback control model for cell volume regulation [11, 12]. The cen-

tral idea is that the diuretic-sensitive cotransport system forms part of a feedback loop whose activity is influenced by the status of the cell volume. According to this model, transport is activated when cell volume drops below a predetermined set point and is inactivated when the set point is reached or exceeded. The implication is that cell volume plays a key role in the regulation of the diuretic-sensitive cotransport pathway.

The purpose of the present investigation was to define more clearly the volume regulatory behavior of the Ehrlich cell and in particular to explore the relationship between cell volume and the activity of the Cl^--dependent cation cotransport system. Some of the present data have been presented in abstract form [28].

Materials and Methods

Cell Suspensions

Ehrlich ascites tumor cells (hyperdiploid strain) grown in Ha/ICR white male mice were harvested and washed as previously described [26]. The wash solution had the following composition (mM): 150 NaCl, 6 KCl, 10 HEPES-NaOH (pH 7.3–7.45, 290–300 mOsM). This, as well as all other media, was filtered (Gelman Metricel; 0.45 μm) prior to use. Cells were washed twice in this solution and subsequently incubated at a density of 18–20 mg dry wt/ml for 30 min at 21–23°C.

Following the preincubation period aliquots of cell suspension were washed twice and resuspended in cold, K^+-free Na gluconate (140 mM, buffered to pH 7.35 with 10 mM HEPES-NaOH). The cell suspension was then incubated 60 min in an ice bath at a density of 5–7 mg dry wt/ml. As a result of this procedure, cell K^+ and Cl^- were reduced from steady-state values of 550 ± 70 and 200 ± 15 to 250 ± 20 and 50 ± 10 mEq/kg dry wt, respectively. Na^+, which is normally 90 ± 12 mEq/kg dry wt, was increased to 275 ± 16. Cell volume decreased by 15–20%.

Ion Fluxes

The KCl-depleted, Na^+-enriched cells were then resuspended in a small volume of K^+-free Na gluconate and at time zero added to 10–15 ml of media (O_2 equilibrated, 37°C, pH 7.35) whose composition varied depending on the experiment. In most studies the medium contained 15 mM KCl + 130 mM NaCl and a trace amount of ^{86}Rb (0.3 μCi/ml cell suspension). In some cases, as indicated in the figures, the media contained furosemide, ouabain or both, while in other experiments the medium was made hypoosmotic by dilution with KCl/10 mM HEPES-NaOH buffer such that the final osmolality ranged between 295 and 230 mOsM. Periodically during the next 25 min, 0.5-ml aliquots of cell suspension were removed and added to preweighed 1.5-ml microcentrifuge tubes containing 0.8 ml ice-cold choline dihydrogen citrate solution (CDHC; [39]). The samples were then centrifuged for 15 sec at 15,000 × g, the supernatant was removed, and the tubes were weighed before the addition of 1 ml of 1% (vol/vol) ice-cold perchloric acid (PCA). At the end of the sampling period duplicate samples of cell suspension were removed for the determination of the dry cell weight [4].

The unidirectional influx of K^+ was determined from the uptake of ^{86}Rb. In the Ehrlich cell ^{86}Rb serves as an analog for K^+ and, as such, faithfully traces K^+ movements [38]. In preliminary experiments the suitability of this tracer was checked with ^{42}K and no differences were detected between tracer. The initial influx of K^+ (Rb^+), expressed as mEq/(kg dry) min, was calculated from the incorporation of ^{86}Rb, which was in all cases linear during the first few minutes of uptake. Influx was estimated from $(d^{86}Rb/dt)/SA$, where $(d^{86}Rb/dt)$ is the incorporation of ^{86}Rb per kg dry cell wt per min, that is, the slope of the time-dependent uptake and SA is the specific activity (cpm/μEq) of the extracellular K^+ (Rb^+). Net fluxes of K^+, Na^+ and Cl^- were estimated as the slopes of the initial time-dependent change in cellular electrolytes. In general, the slopes were constant during the first 5 min of change. Potassium efflux was not measured directly but rather was determined as the difference between the concurrently measured ^{86}Rb influx and net K^+ flux. The change in the ratio of cell water/dry weight was used to determine the initial net influx of water. Errors are reported as ±SEM or, in the case of kinetic parameters, of the estimate.

Analytical Methods

The cell pellets were extracted with PCA in an ice bath for 60 min and subsequently centrifuged 2 min at 15,000 × g to remove the PCA-insoluble residue. Aliquots of the PCA extracts and medium were used to determine Na^+ and K^+ by emission flame photometry using Li^+ as an internal standard and Cl^- with an autotitrator [29]. Aliquots of the PCA extract were assayed for ^{86}Rb with a Packard gamma counter. Direct determinations of extracellular water associated with the cell pellet were not conducted in every experiment. Rather, this was determined in each type of experiment using ^{86}Rb. Briefly, 0.5 ml cell suspension was added to 0.8 ml ice-cold CDHC solution containing the appropriate amount of tracer and centrifuging immediately. The radioactivity associated with the cell pellet was assumed to be confined to the extracellular phase and thereby served as a measure of the trapped fluid. In over 100 determinations conducted at different cell volumes, as well as varying cellular and extracellular ionic compositions, the extracellular water of the cellular layer (ECS) ranged between 19–22% with a mean of 20.1 ± 1.8% of the weight of the cell pellet. In the results presented here cell volume is reported in terms of g cell water (corrected for ECS) per g dry cell wt. During the normal physiological steady state (290–300 mOsM; pH 7.3–7.4) cell volume is equivalent to 3.45 ± 0.3 g water/g dry wt.

Reagents

Furosemide was kindly supplied by Hoechst Pharmaceuticals. Ouabain was a product of Sigma Chemical Co., St. Louis, Mo., while ^{86}Rb was purchased from New England Nuclear, Boston, Mass. All other reagents were of the highest quality obtainable.

Results

We have adopted standard functional definitions of the transport activities. For the Na/K pump, the

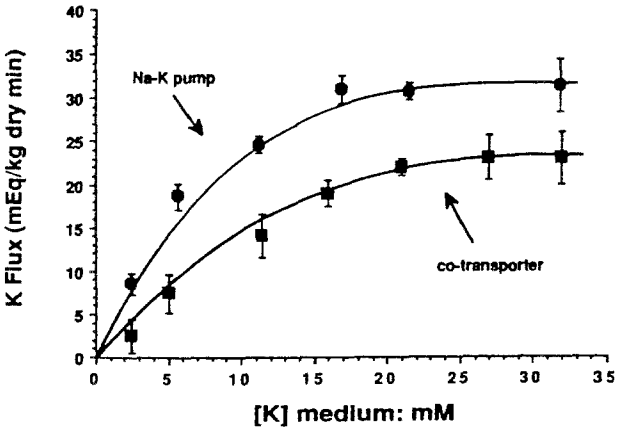

Fig. 1. Dependence of the ouabain-sensitive (Na/K pump) and furosemide-sensitive (cotransport) net K^+ fluxes on the extracellular $[K^+]$. Tumor cells were incubated for 60 min in cold, K^+-free Na gluconate to deplete K^+ and Cl^- and to load with Na^+. After centrifugation the cells were resuspended in media containing 2.5–35 mM KCl + 140–110 mM NaCl in the presence and absence of 1 mM ouabain or furosemide. Net K^+ flux was estimated from the initial rate of K^+ uptake. The curves are the best-fit lines of the data to Michaelis-Menten kinetics. Points and bars are the means + SE of at least five determinations

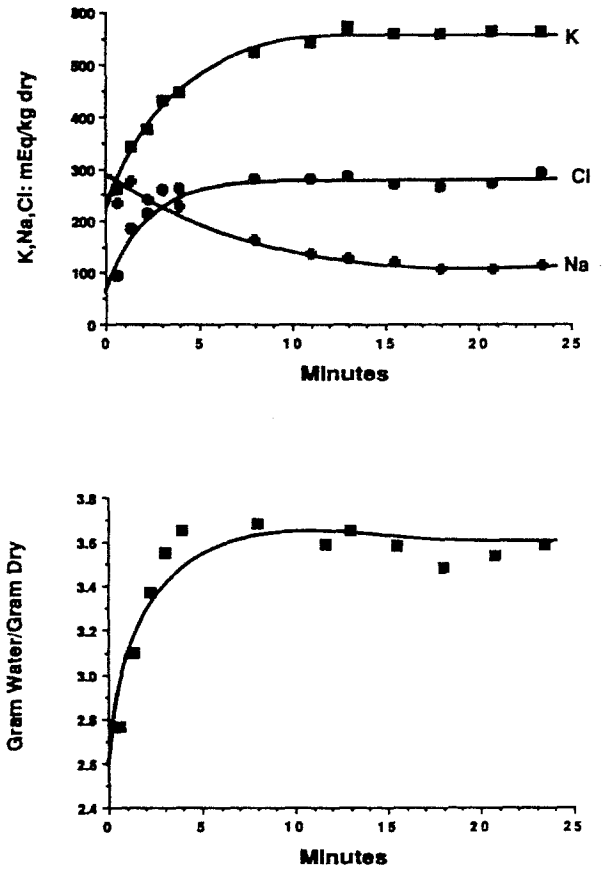

Fig. 2. Time-dependent changes in cellular electrolytes and water in Ehrlich tumor cells. *Upper panel:* Na^+, K^+, Cl^- content expressed as mEq/kg dry cell wt; *lower panel:* cell water content in g/g dry cell wt. Cells were prepared as described in Fig. 1. They were then resuspended in isotonic medium (300 mOsM, pH 7.32–7.40): 15 mM KCl + 130 mM NaCl containing a trace amount of ^{86}Rb

transport activity is that persisting in the presence of 1 mM furosemide but is inhibited by 1 mM ouabain. The cotransport system is defined operationally as transport that occurs in the presence of 1 mM ouabain but is inhibitable by 1 mM furosemide. In preliminary experiments we found the activities of both systems to be relatively independent of the extracellular $[Na^+]$ and $[Cl^-]$ so long as these exceeded about 100 mM. It also became clear early in the investigation that increasing extracellular $[K^+]$ resulted in significant increases in both Na/K pump and cotransport activity. The hyperbolic relationship between extracellular $[K^+]$ and net K^+ transport mediated by these pathways is shown in Fig. 1. These data were fit to the Michaelis-Menten equation by the weighted linear regression procedure of Wilkinson [40] as described previously [4]. The apparent K_M is 3.5 ± 0.4 mM K^+ for the pump and 6.8 ± 0.7 mM K^+ for the cotransporter. The corresponding maximal fluxes, expressed as mEq/(kg dry) min, are 30.1 ± 2.6 and 20.8 ± 1.5, respectively. Based on these findings, all subsequent experiments were performed with the extracellular $[K^+]$ maintained at between 15 and 18 mM. It is important to point out that at physiological concentrations of K^+ (3–5 mM) both transport systems exhibited measurable activity although only about 40% of that observed at 15–18 mM K^+.

An example of the time course of the restoration of cellular electrolytes and water is shown in Fig. 2. Upon the addition of the KCl-depleted, Na^+-enriched cells to the 15 mM KCl/130 mM NaCl medium there was a rapid reaccumulation of KCl and extrusion of Na^+ that was essentially complete within 10 minutes. Cell volume increased and returned to the normal steady-state level (3.5 g water/g dry cell wt) during this same time interval.

The effects of ouabain and furosemide on these processes are shown in Figs. 3 and 4. Incubation in the presence of ouabain (Fig. 3) resulted in the net uptake of K^+, Na^+ and Cl^- during the first 4–5 min. The accumulation of K^+ plus Na^+ was 156 mEq/kg dry wt, which was identical to the uptake of Cl^-. Since the cell water content over the same time interval increased by 0.92 g/g dry wt, the calculated osmolality of the transported solution is 339 mOs/kg water. In six other experiments conducted in the same way the osmolality ranged between 305–345 mOs/kg with the mean of 325 ± 15 mOs/kg, in good agreement with the results of others [12, 13]. At longer incubation times, that is, greater than about 7

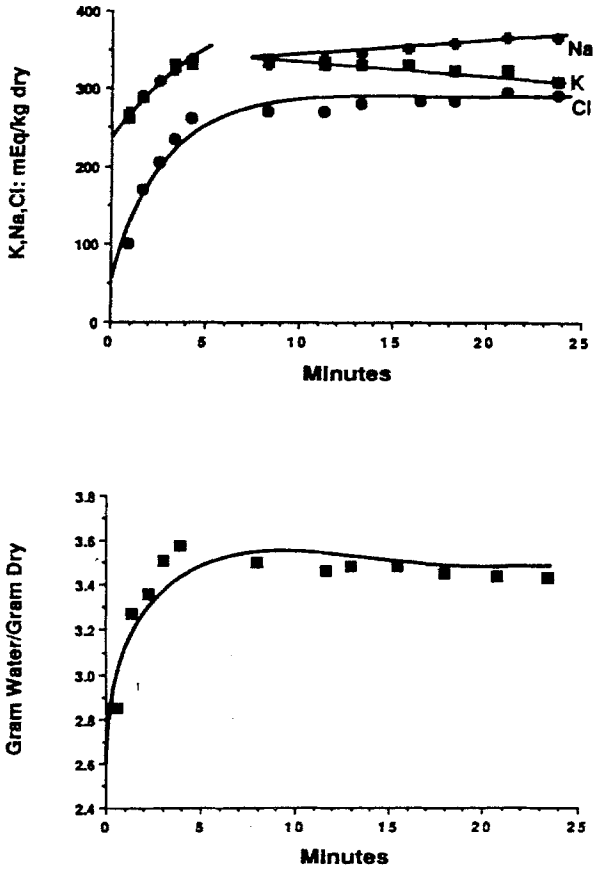

Fig. 3. Effect of ouabain on the time-dependent changes in cellular electrolytes and water in Ehrlich tumor cells. *Upper panel:* Na^+, K^+, Cl^- content expressed as mEq/kg dry cell wt; *lower panel:* cell water content in g/g dry cell wt. Cells were prepared as described in Fig. 1. They were then resuspended in isotonic medium: 15 mM KCl + 130 mM NaCl + 1 mM ouabain + ^{86}Rb

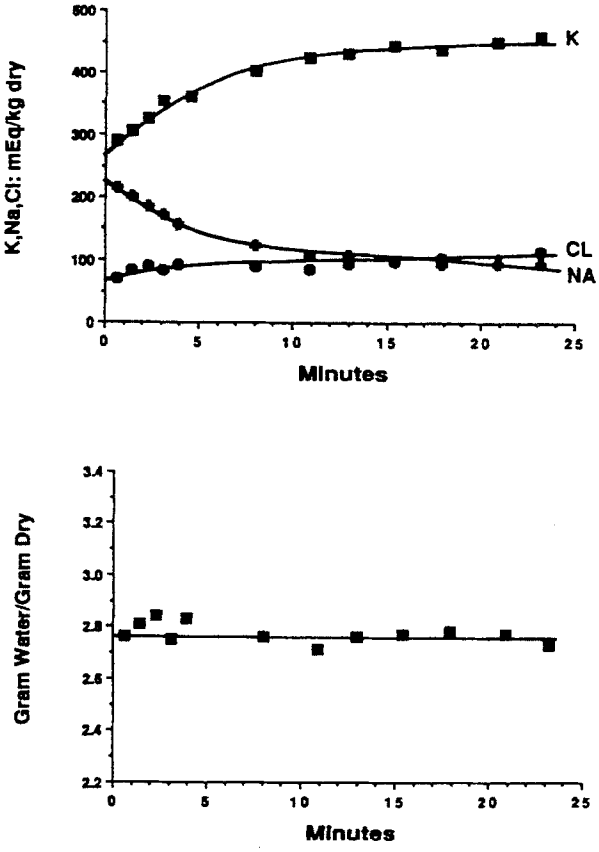

Fig. 4. Effect of furosemide on the time-dependent changes in cellular electrolytes and water in Ehrlich tumor cells. *Upper panel:* Na^+, K^+, Cl^- content expressed as mEq/kg dry cell wt; *lower panel:* cell water content in g/g dry cell wt. Cells were prepared as described in Fig. 1. They were then resuspended in isotonic medium: 15 mM KCl + 130 mM NaCl + 1 mM furosemide + ^{86}Rb

min, there was a progressive loss of K^+ which was balanced by an equivalent gain in Na^+. As a consequence, both the Cl^- content and cell volume remained almost constant.

In contrast to ouabain, incubation in the presence of furosemide (Fig. 4) resulted in the extrusion of Na^+, which quantitatively equalled the accumulation of K^+. There was virtually no change in Cl^- content or cell volume. Pump activity continued until the intracellular Na^+ and K^+ concentrations attained their normal steady-state values, that is, 30 mEq Na^+/kg water and 160 mEq K^+/kg water. Two points are noteworthy. First, cell volume did not increase and, in fact, remained about 20% below the normal steady-state value. This suggests that the pump can restore Na^+ and K^+ to normal levels even in the absence of volume restoration. Second, the reaccumulation of Cl^- was almost completely inhibited by furosemide, indicating that the cotransport pathway represents a major route for Cl^- entry. Furthermore, without Cl^- reaccumulation cell volume does not increase.

Table 1 summarizes the results of 14 separate experiments. The initial net K^+ flux is almost completely accounted for by the sum of ouabain- and furosemide-sensitive components. Less than 10% persists when both of these transport pathways are inhibited. The unidirectional $K^+(Rb^+)$ influx in both control and ouabain-treated cells greatly exceeds the net K^+ flux, indicating the existence of a significant K^+ backflux. This occurs even though K^+ was vigorously accumulated and indicates an exchange of cellular (*i*) for extracellular (*o*) K^+ superimposed on the net K^+ flux. K^+ efflux is possibly mediated by the cotransport system since in the presence of furosemide or absence of Cl^- (data *not shown*) the net K^+ flux and $K^+(Rb^+)$ influx are identical. That is, in the absence of cotransport activity K_i^+–K_o^+ exchange is zero. Alternatively K_i^+–K_o^+ exchange may be mediated by another system that is Cl^--

Table 1. Net and unidirectional transport in KCl-depleted, Na^+-enriched cells measured in isoosmotic medium (15 mM KCl, 130 mM NaCl)[a]

	Net K^+ flux	K^+ (Rb^+) influx	K^+ (Rb^+) efflux	Net Na^+ flux	Net Cl^- flux
		(mEq/(kg dry) min)			
Control	49.2 ± 4	68.9 ± 3.2	20.1 ± 4	−17.9 ± 2.4	35.2 ± 3.6
Furos-sensitive	20.3 ± 2.1	49.3 ± 3	26.7 ± 3.8	+18.8 ± 1.5	38.3 ± 4.2
Ouab-sensitive	30.9 ± 2.8	27.9 ± 3.5	0	−28.5 ± 1.9	0

	Cell Volume		
	Initial	Final	Net water flux
	(g water/g dry)		[g/(g dry) min]
Control	2.87 ± 0.3	3.75 ± 0.4	0.239 ± 0.04
Furos-sensitive	2.85 ± 0.2	3.65 ± 0.5	0.219 ± 0.04
Ouab-sensitive	2.79 ± 0.3	2.71 ± 0.3	0

[a] Direction of net Na^+ flux: (+) refers to uptake and (−) to extrusion.
Ouabain-sensitive: fluxes that are inhibited by 1 mM ouabain but are not inhibited by 1 mM furosemide.
Furosemide-sensitive: fluxes that are inhibited by 1 mM furosemide but are not inhibited by 1 mM ouabain.
Errors are expressed as ±SE of each individual experiment, which was replicated at least four times.

dependent and shares furosemide sensitivity. When the cotransport system is inhibited by furosemide the net fluxes of Cl^- and water are reduced to zero. The magnitude of the furosemide-insensitive net fluxes of Na^+ and K^+, however, are essentially the same, although oppositely directed. This suggests that under the conditions of these experiments, the pump does not contribute to net solute movement.

Activity of the cotransport system is most clearly evident when the pump is inhibited. In this case, the sum of the net cation fluxes ($K^+ + Na^+$ = 39.1 mEq/kg dry wt min) equals the net Cl^- flux (38.3 mEq/kg dry wt min), giving an electroneutral cotransport with an apparent stoichiometric relationship of $1K^+ : 1Na^+ : 2Cl^-$. The net uptake of electrolytes, which persists for only 4–5 min (Fig. 3), induces a water flow resulting in a 20–25% increase in cell volume. In the absence of pump activity, however, the cotransport system is unable to maintain the internal electrolyte composition and the cells begin to lose K^+ and gain Na^+ (Fig. 3). Cell volume, however, does not change.

Thus, incubation of KCl-depleted, Na^+-enriched cells in isotonic 15 mM KCl/130mM NaCl medium results in rapid accumulation of K^+, Cl^- and extrusion of Na^+, as well as restoration of volume to the steady-state level. How this process is regulated is unclear. The recent model of Geck and coworkers [10, 12] proposes that in the Ehrlich cell volume is sensed and if below a predetermined set point the cotransport pathway is activated. With volume restoration, the normal set point is reached and cotransport is inactivated. The implication is that net ion transport mediated by the cotransport pathway is controlled by cell volume.

To test this hypothesis KCl-depleted, Na^+-enriched Ehrlich cells whose volume was 15–20% below normal were subsequently incubated in hypoosmotic (260 ± 4 mOsM) medium containing 15 mM KCl/110 mM NaCl + ^{86}Rb. The tonicity was adjusted (by trial and error) so that the cell volume would rapidly be returned as close as possible to its normal physiological steady state. Figure 5 illustrates the results of a representative experiment. Upon resuspension in 260 mOsM medium, cell volume rapidly increased from 2.82 to 3.35 g water/g dry wt. Since the movement of water in response to an osmotic gradient is extraordinarily rapid [15, 16], the cells were osmotically equilibrated within a few seconds. The pattern of accumulation of K^+, Cl^- and the extrusion of Na^+ is essentially the same as that observed in the 300 mOsM medium (Fig. 2), suggesting that the activity of the Na/K pump and cotransporter is similar in the two cases (*see* Table 2). Although the absolute amount of water taken up is comparable in both the isosmotic and hypo-osmotic conditions, the cell volumes at the conclusion of the two experiments were very different. In the case of the hypo-osmotic medium, cell volume initially approximated the physiological steady state (3.35 g water/g dry wt) but rapidly increased by more than 20% to 4.15 g water/g dry wt concomitant with the redistribution of Na^+, K^+ and Cl^-.

Table 2 summarizes the results of 10 separate experiments in which net and unidirectional fluxes were measured after the cells were resuspended in

Table 2. Net and unidirectional transport in KCl-depleted, Na^+-enriched cells measured in hypo-osmotic medium (15 mM KCl, 110 mM NaCl)[a]

	Net K^+ flux	K^+ (Rb^+) influx	K^+ (Rb^+) efflux	Net Na^+ flux	Net Cl flux
		[mEq/(kg dry) min]			
Control	52.9 ± 1.8	72.5 ± 2.8	20.3 ± 1.3	−13.2 ± 0.4	42.4 ± 3.7
Furos-sensitive	25.5 ± 2.5	48.1 ± 2	22.7 ± 2.2	+21.2 ± 3.3	44.7 ± 3.9
Ouab-sensitive	25.4 ± 3.1	25 ± 2.1	0	−23.8 ± 0.5	1.2 ± 0.8

	Cell Volume		
	Initial	Final	Net water flux
	(g water/g dry wgt)		[g/(g dry) min]
Control	3.25 ± 0.4	4.09 ± 0.4	0.257 ± 0.04
Furos-sensitive	3.26 ± 0.2	3.99 ± 0.4	0.256 ± 0.04
Ouab-sensitive	3.28 ± 0.2	3.19 ± 0.3	0

[a] Direction of net Na^+ flux: (+) refers to uptake and (−) to extrusion.
Ouabain-sensitive: fluxes that are inhibited by 1 mM ouabain but are not inhibited by 1 mM furosemide.
Furosemide-sensitive: fluxes that are inhibited by 1 mM furosemide but are not inhibited by 1 mM ouabain.
Errors are expressed as ±SE of each individual experiment which has replicated at least three times.

Table 3. Steady-state cell volume and electrolyte concentration following incubation of KCl-depleted, Na^+-enriched Ehrlich cells in media of varying tonicity[a,b]

Osmolality (mOs/kg water)	Cell volume (g water/g dry)	$[K^+]$	$[Na^+]$ (mEq/kg water)	$[Cl^-]$	$\Delta\mu_{net}$ (kcal/mol)
300 ± 5	3.41 ± 0.33	156 ± 16	32 ± 5	75 ± 8	0.237
260 ± 4	4.08 ± 0.14	129 ± 11	26 ± 4	65 ± 8	0.363
230 ± 2	4.32 ± 0.22	128 ± 13	21 ± 6	62 ± 6	0.311

[a] The composition of the media was: 300 mOsM = 15 mM KCl, 130 mM NaCl; 260 mOsM = 15 mM KCl, 110 mM NaCl and 230 mOsM = 15 mM KCl, 95 mM NaCl.
Data are the mean ± SE of at least four separate experiments.
[b] $\Delta\mu_{net}$ is the sum of the chemical driving forces exerted on Na^+, K^+ and Cl^- in the steady state.

hypo-osmotic medium (260 ± 4 mOsM). The magnitude of the initial net fluxes of K^+, Na^+, Cl^- and water and the sensitivity to inhibition by ouabain and furosemide is comparable to that observed under isotonic conditions (Table 1). The behavior of the unidirectional K^+ (Rb^+) fluxes is also similar and, like those measured under isotonic conditions, indicates the existence of a furosemide-sensitive K_i^+–K_o^+ exchange. Quantitatively, the efflux amounts to about 50% of the influx. The results of this type of experiment, as well as those shown in Table 1, indicate that net water movements are not only directly dependent on the activity of the co-transport system, but that the final volume attained is secondary to the reestablishment of the normal electrolyte composition, transmembrane gradients, or both. This point is illustrated by the results summarized in Table 3. Incubation of KCl-depleted, Na^+-enriched cells for 25 min in 15 mM KCl/NaCl media of differing osmolalities (300, 260 and 230 mOsM) results in the establishment of a characteristic steady state. Although there are some differences between the patterns of cellular $[K^+]$, $[Na^+]$ and $[Cl^-]$ they are, in general, comparable. Cell volumes, however, are not. The physiological steady-state volume is exceeded by 19 ± 3% in the 260 mOsM medium and by 27 ± 2% in the 230 mOsM medium. This lends support to the idea that restoration of the ionic composition and not volume *per se* limits net transport of K^+, Na^+ and Cl^- by the co-transport system. If this is indeed the case then initially there should be a substantial inward driving force favoring net ion uptake that in time would decrease towards zero as the net fluxes of K^+, Na^+ and Cl^- approached zero. The passive driving force operating on this system can be estimated by calcu-

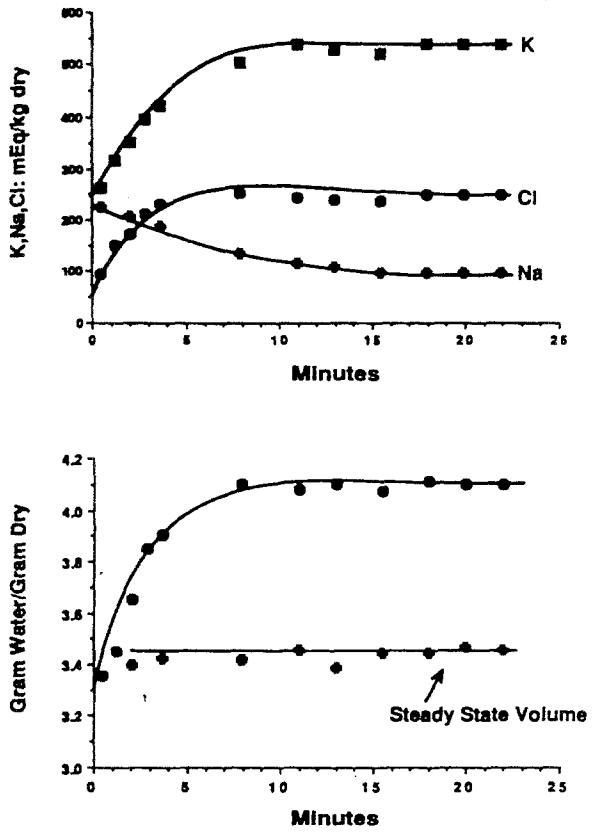

Fig. 5. Effect of hypo-osmotic medium on the time-dependent changes in cellular electrolytes and water in Ehrlich tumor cells. *Upper panel:* Na^+, K^+, Cl^- content expressed as mEq/kg dry cell wt; *lower panel:* cell water content in g/g dry cell wt. Cells were prepared as described in Fig. 1. They were then resuspended in hypo-osmotic medium (260 mOsM, pH 7.35–7.40): 15 mM KCl + 110 mM NaCl + ^{86}Rb. Steady-state volume refers to cells not exposed to K^+-free Na Gluconate medium but rather incubated in isotonic medium (15 mM KCl + 130 mM NaCl, 300 mOsM, pH 7.35)

lating the difference in the sum of the chemical potentials of Na^+, K^+ and Cl^- inside and outside the cells [14, 21, 24]. Since the stoichiometry of the cotransporter appears to be $1K^+:Na^+:2Cl^-$ and thereby electroneutral, the net driving force is given by:

$$\Delta\mu_{net} = \Delta\mu_K + \Delta\mu_{Na} + 2\Delta\mu_{Cl} = RT \ln\{[K]_o \cdot [Na]_o \cdot [Cl]_o^2/[K]_i \cdot [Na]_i \cdot [Cl]_i^2\}$$

where *o* and *i* represent extra- and intracellular concentrations, respectively; *R* and *T* have their usual meaning.

Application of this equation requires that both the internal and external concentrations of K^+, Na^+ and Cl^- be known. KCl-depleted, Na^+-enriched cells had the following ionic composition (mEq/kg cell water): $K^+ = 94 \pm 7$, $Na^+ = 85 \pm 8$ and $Cl^- = 30 \pm 5$. The corresponding extracellular concentrations (mM) for the isotonic medium were: $K^+ = 15.6 \pm 3$, $Na^+ = 130 \pm 3$ and $Cl^- = 139 \pm 4$. Therefore, the initial net driving force is inwardly directed and amounts to 1.11 kcal/mol, consistent with the rapid uptake of ions by the cotransporter. The last column of Table 3 shows that, although the values of $\Delta\mu_{net}$ calculated for cells incubated in the 3 media are similar, each is greater than zero. This suggests that a net inward driving force acts on Na^+, K^+ and Cl^- even though the net fluxes are zero.

Discussion

Three major contributors to Na^+ and K^+ transport in the Ehrlich cell have been identified. These include the Na/K pump, a Cl^--dependent cation cotransport system and diffusion. In the physiological steady state the cotransport system appears to function as a bidirectional exchanger, that is, it exchanges internal ($Na^+ + K^+ + Cl^-$) for that in the external medium [3, 27], while the Na/K pump compensates for the movements of K^+ and Na^+ down their respective electrochemical potential gradients. When the steady state is disturbed, such as when cells are KCl depleted and Na^+ loaded, the restitution of the normal distributions of electrolytes and water involves the concerted efforts of both the cotransporter and pump. Although these transport systems operate in a complementary fashion they differ kinetically. The cotransport system has an apparent K_M for extracellular K^+ twofold greater than that of the Na/K pump. The apparent K_M for the cotransport of K^+ is similar to values reported for avian [35] and human red cells [7, 8] although somewhat higher than that reported for HeLa cells [33] and 3T3 cells [2].

The contribution of each transport pathway to the restoration of the internal environment of the cell can be partitioned, at least phenomenologically, by the use of appropriate inhibitors. While this is accepted practice, it is important to point out that very few inhibitors exhibit absolute specificity. For example, although furosemide is the "classic" inhibitor of ($K^+ + Na^+ + Cl^-$) cotransport in a variety of cell types [3, 9, 21], it also inhibits other processes including Cl^-/HCO_3 exchange in human red blood cells [5] and volume-activated KCl transport in sheep red blood cells [25]. The sensitivity to inhibition of the different systems varies, however, with high affinity inhibition, in general, being limited to the cotransport system [34]. In the present experiments, furosemide effectively blocks net solute and water uptake (Fig. 4, Table 1) by inhibiting the cotransport system. The Na/K pump, however,

continues to operate and as a consequence reduces intracellular [Na^+] and increases [K^+] to levels that are typical of the physiological steady state. Our data suggest that the coupling of the Na/K pump is $1Na^+ : 1K^+$ and under the conditions of these experiments is electroneutral (Tables 1 and 2). This conclusion is supported by the observation that both the intracellular Cl^- and cell volume do not change (Fig. 4) in the presence of ouabain. If the pump were coupled $3Na^+ : 2K^+$, as has been suggested [11, 12], then in the presence of furosemide cell shrinkage, secondary to a net loss of Cl^- as NaCl, would be expected. This, however, was not observed. Net uptake of ($K^+ + Na^+ + Cl^-$) in the stoichiometric ratio of 1 : 1 : 2 (Fig. 3, Table 1) is readily demonstrated when the Na/K pump is inhibited. This is in agreement with findings in this as well as numerous other cell types [10, 14, 18] and appears to be a common feature of cation cotransport systems. The exchange of internal for external K^+ that occurs in parallel with the cotransport of ($Na^+ + K^+ + 2Cl^-$) may also be a commonly shared feature. Some years ago Schmidt and McManus [35] described in the duck red cell the 1 : 1 exchange of Rb^+ and K^+ that occurs in the absence of extracellular Na^+. More recently this process has been shown to be dependent on intracellular Na^+, is Cl dependent and diuretic sensitive [31]. These findings are consistent with the idea that the exchange represents a mode of operation of the cotransport system and in fact may be a partial reaction of the transport mechanism [30]. Our results are consistent with the idea that this exchange is mediated by the cotransport system in the Ehrlich cell also. Whether this represents a partial reaction of the cotransporter or is indicative of the backflux of the quartenary complex, ($Na^+ + K^+ + 2Cl^-$), remains to be determined.

Thus, as pointed out by Geck and Pfeiffer [12], the cotransport system and Na/K pump work in parallel to affect net K^+ uptake while at the same time operate in series to reduce internal Na^+. For each cycle the cotransporter mediates the uptake of 2 Cl^- for each ($K^+ + Na^+$), while the pump exchanges internal Na^+ for external K^+. The net result then is the uptake of four osmotically active particles (2KCl) followed by osmotically obligated water. It is important to emphasize that the Na/K pump plays a significant role in this overall process because it establishes a "potential" source of energy for the cotransport system, that is, the ionic gradients (*see below*).

The incremental change in cell volume is dependent on the net accumulation of ions and goes to zero as the net ion fluxes go to zero. It could be argued that the restoration of the normal cell volume or some derivative thereof inhibits or otherwise limits net movement through the cotransport system and thereby re-establishes the physiological steady state. While this is an attractive idea and may apply to other systems [1, 6, 9], it apparently is not the case in the Ehrlich cell. Rather, our results indicate that the final or resting cell volume is not dependent on the starting volume but is a function of KCl accumulation and Na^+ extrusion leading to the re-establishment of the steady-state ion gradients across the membrane.

In other systems it has been demonstrated that the net cotransport of ($Na^+ + K^+ + Cl^-$) depends on the magnitude and direction of the respective ionic gradients rather than on the direct utilization of metabolic energy [14, 21, 24]. Therefore, cotransport exhibits a cardinal feature of secondary active transport, namely, ion movement against an existing gradient that is driven by the energy contained within an oppositely directed ionic gradient. In an effort to determine whether cotransport in the Ehrlich cell is secondary active transport, Geck and coworkers [13] plotted the furosemide-sensitive uptake of water (as an index of cotransport activity) versus the sum of the individual chemical driving forces of K^+, Na^+ and Cl^-. At equilibrium, in the absence of net water flow, they found an inwardly directed driving force of about 1 kcal/mol. Although these results suggested primary active transport, with direct coupling of the cotransport system to a metabolic reaction, they were unable to demonstrate a linkage between ATP consumption and cotransport activity. In the present experiments we found that the chemical gradients of Na^+ and Cl^- were initially inwardly oriented and thereby favored net uptake. Potassium was rapidly taken into the cells even though its gradient was directed outwardly. Thus, it is possible, though certainly unproven, that the energy contained within the ($Na^+ + Cl^-$) chemical gradients drove K^+ uphill into the cells. However, in agreement with the findings of Geck [13], the net driving force does not disappear when the net fluxes of K^+, Na^+ and Cl^- are zero. This seems to be inconsistent with secondary active transport and raises the possibility for the involvement of other factors, including an alternate energy source. On the other hand, the persistence of an inward driving force for ($Na^+ + K^+ + 2Cl^-$) cotransport even in the steady state could serve to counterbalance net KCl efflux mediated by other pathways.

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