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Inability of Ehrlich Ascites Tumor Cells to Volume Regulate Following a Hyperosmotic Challenge

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Summary. Ehrlich cells shrink when the osmolality of the suspending medium is increased and behave, at least initially, as osmometers. Subsequent behavior depends on the nature of the hyperosmotic solute but in no case did the cells exhibit regulatory volume increase. With hyperosmotic NaCl an osmometric response was found and the resultant volume maintained relatively constant. Continuous shrinkage was observed, however, with sucrose-induced hyperosmolality. In both cases increasing osmolality from 300 to 500 mOsM initiated significant changes in cellular electrolyte content, as well as intracellular pH. This was brought about by activation of the Na^+/H^+ exchanger, the Na/K pump, the $Na^+ + K^+ + 2Cl^-$ cotransporter and by loss of K^+ via a Ba-sensitive pathway. The cotransporter in response to elevated $[Cl^-]_i$ (~100 mM) and/or the increase in the outwardly directed gradient of chemical potential for Na^+, K^+ and Cl^-, mediated net loss of ions which accounted for cell shrinkage in the sucrose-containing medium. In hyperosmotic NaCl, however, the net Cl^- flux was almost zero suggesting minimal net cotransport activity.

We conclude that volume stability following cell shrinkage depends on the transmembrane gradient of chemical potential for $[Na^+ + K^+ + Cl^-]$, as well as the ratio of intra- to extracellular $[Cl^-]$. Both factors appear to influence the activity of the cotransport pathway.

Key Words Ehrlich tumor cells · volume regulation · hyperosmolality · ion transport

Introduction

Animal cells when suspended in hypo- or hyperosmotic media rapidly swell or shrink and in general behave according to the prediction of the Boyle-van't Hoff relationship. In many cell types, including the Ehrlich ascites tumor cell [20] suspension in hyposmotic media results initially in swelling, but this is followed almost immediately by a regulatory volume decrease (RVD), whereby the cells return to near normal volume within a few minutes. The decrease in cell volume is due to the loss of solute (K^+ and Cl^-) and water that is osmotically obligated to it. In Ehrlich cells the loss of these ions occurs as the result of a transient increase in the conductive K^+ and Cl^- permeabilities which returns to resting level at the termination of RVD [22].

The response to incubation in hyperosmotic media resulting in cell shrinkage is variable in that only a few cell types possess the ability to restore their volume. Furthermore, the experimental conditions necessary to trigger volume restoration (regulatory volume increase; RVI) under these conditions appears to differ among various cells. For example, duck and *Amphiuma* red cells exhibit RVI but do so by different transport mechanisms. In the *Amphiuma* red cell [7] an amiloride-sensitive Na^+/H^+ antiporter is activated upon shrinkage and this, along with the parallel exchange of intracellular HCO_3^- (or OH^-) for extracellular Cl^-, promotes the net uptake of solute and thereby an increase in cell volume. Avian red cells [33] on the other hand, utilize the Cl-dependent cation cotransport system. This transport pathway, in response to cell shrinkage, increases intracellular solute content by mediating the net uptake of the electrically neutral, quarternary complex, Na + K + 2Cl. Volume is restored because of the concomitant osmotic inflow of water. With the exception of avian and amphibian erythrocytes, most cells studied to date, including cultured ovary cells [32], HeLa [34], lymphocytes [12] and Ehrlich cells [17], are unable to restore their original volume when shrunk in hyperosmotic solution.

The present investigation was undertaken to characterize the behavior of the Ehrlich ascites tumor cell following shrinkage to determine why these cells fail to exhibit RVI. Since ion transport pathways (e.g., the Na/K pump, the Cl-dependent cation cotransport system and the Na/H exchanger) previously implicated in volume regulation are present, we were particularly interested in establishing the

response of these systems to the hyperosmotic challenge. Some of the present data have been presented in abstract form [28].

Materials and Methods

Cell Suspension

Ehrlich-Lettre' ascites tumor cells (hyperdiploid strain), grown in Ha/ICR white male mice, were harvested and washed as previously described [25]. The wash/incubation medium had the following composition (in mM): 140 NaCl, 5 KCl, 10 HEPES-NaOH buffered to pH 7.3. This solution was always filtered (Gelman Metricel; 0.45 μm) before use. Cells were washed twice and subsequently incubated at a density of 8 to 10 mg dry wt/ml for 30 min at 22°C.

Sampling

Following the preincubation period, the cell suspension was centrifuged, resuspended in fresh medium and incubated at 37°C under an atmosphere of 100% O_2. After 3–5 min, inhibitors and/or other agents were added. This was followed by the addition of a small volume of either 5 M NaCl, 1.5 M mannitol or 2.3 M sucrose which increased the osmolality of the medium from 295 to up to 600 mosmol/kg (mOsM). Sucrose and mannitol were dissolved in incubation medium so that the concentration of the extracellular electrolytes was not altered. Osmolalities were measured with an Osmette A freezing point depression osmometer.

Periodically during the next 10–15 min aliquots (1 ml) of cell suspension were removed and added to preweighed microcentrifuge tubes (1.5-ml capacity) containing 0.25 μCi (10 μl) of ^{3}H-mannitol. The samples were mixed, immediately centrifuged (15 sec at 15,000 × g), the supernatant collected and the tubes weighed before the addition of 1 ml of 1% (vol/vol) ice-cold perchloric acid (PCA).

Measurement of Ion Fluxes

Net fluxes of Na^+, K^+ and Cl^- were estimated as the slopes {mEq/(kg dry wt) · min^{-1}} of the initial time-dependent change in the cellular electrolyte content. In this paper a net flux equal to 1 {mEq/(kg dry wt) · min^{-1}} is defined as "1 transport unit" and will be abbreviated as 1TU. The slopes were, in general, constant during the first 5–10 min of change. Net H^+ effluxes were measured with a Radiometer-Coopenhagen pH-stat as previously described [5]. H^- efflux was calculated from the rate of NaOH addition (mmol/min) necessary to maintain the extracellular pH constant at 7.30. This rate was normalized to the total dry wt of cells in the pH-stat chamber and is expressed as mEq. H^-/(kg dry wt) · min^{-1}. Errors are reported as ± SEM.

Measurement of Intracellular pH (pH_i)

Intracellular pH was estimated from the distribution (cell/medium) of dimethyloxazolidine-2,4-dione,5,5,-[2-^{14}C] (DMO). ^{14}C-DMO (0.025–0.05 μC_i/ml cell suspension) was added in a solution containing unlabeled DMO such that the final [DMO] was 10^{-5} M. In order to insure equilibration [5] DMO was added at least 3 min before the change in osmolality.

Analytical Methods

The cell pellets which were extracted with PCA were kept in an ice bath for 60 min. The samples were 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^+, K^+ and Cl^- as described previously [30]. Correction for Na^+, K^+ and Cl^- trapped within the extracellular space (ECS) was determined in each sample from the distribution of the ^{3}H-mannitol. Since mannitol is an impermeant solute [11; C. Levinson, *unpublished observations*], the radioactivity associated with the cell pellet served as a measure of the trapped fluid. Cell volume, which is reported in terms of kg cell water (corrected for ECS) per kg dry cell wt, was measured as described previously [4]. During the normal physiological steady state (290–300 mOsM; pH 7.3–7.4) cell volume is equivalent to 3.55 ± 0.35 kg water/kg dry wt [29].

Reagents

Oubain, sucrose, mannitol and amiloride were products of Sigma Chemical, St. Louis, MO, while ^{3}H-mannitol (19.1 C_i/mmol) and ^{14}C-DMO (47.2 mC_i/mmol) were purchased from New England Nuclear, Boston, MA. Bumetanide was kindly supplied by Hoffman-La Roche, Nutley, NJ. All other reagents were of the highest quality obtainable.

Results

Evidence that the Ehrlich tumor cell behaves like an osmometer and obeys the prediction of the Boyle-van't Hoff relationship is shown in Fig. 1. When the osmolality of the extracellular medium was increased from 286 to 590 mOsM by the addition of a hyperosmotic solution containing NaCl, sucrose or mannitol the cells shrunk (lost water) in proportion to the increase in osmolality. In these experiments cell water content was measured within 1 min of the change in osmolality. However, if this measurement was delayed a different pattern emerged. Figure 2 shows the results of six separate experiments in which the osmolality of the extracellular medium was increased by the addition of sucrose. Note that when the water content was measured within 1 min the relationship between cell water and osmolality was the same as that shown in Fig. 1. However, when measured 5–7 min after the change in osmolality the cells had shrunk by an additional increment. Although cells shrunk with the addition of NaCl, in contrast to sucrose, there was little additional change in water content even after 15 min (*data not shown*).

The time course of cell shrinkage during a repre-

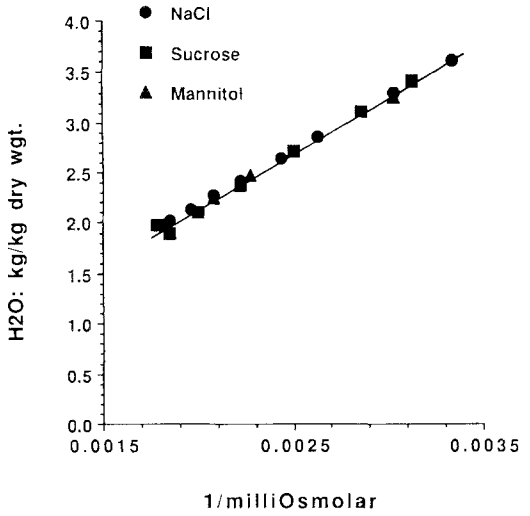

Fig. 1. Osmotic behavior of Ehrlich tumor cells. One-ml aliquots of cell suspension (300 mOsM) were added to preweighed tubes (1.5 ml) containing ^{3}H-mannitol + 5 M NaCl, 2.3 M sucrose or 1.5 M mannitol each dissolved in normal medium. Following 1-min incubation at 23°C the tubes were centrifuged and the water content measured. Osmolalities ranged from 286 to 590 mOsM. Results are plotted according to the Boyle-van't Hoff relation: water content = $(1/\text{mOsM}_{\text{medium}}) \cdot$ water content steady-state cells

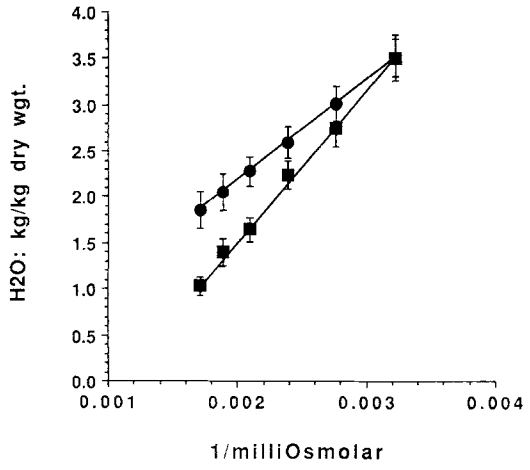

Fig. 2. Response of Ehrlich tumor cells to hyperosmolality induced by sucrose. One-ml aliquots of cell suspension (300 mOsM) were added to tubes containing ^{3}H-mannitol + 2.3 M sucrose dissolved in normal medium. After 1-min (●) or 5–7 min (■) inubation at 23°C the tubes were centrifuged and the water content measured. Osmolalities ranged from 313 to 590 mOsM. Results are plotted according to the Boyle-van't Hoff relation

sentative experiment is shown in Fig. 3. The cell water content measured in normal medium (297 mOsM) was 3.7 kg water/kg dry wt but decreased within about 30 sec to 2.11 when the osmolality was increased to 520 mOsM by the addition of either

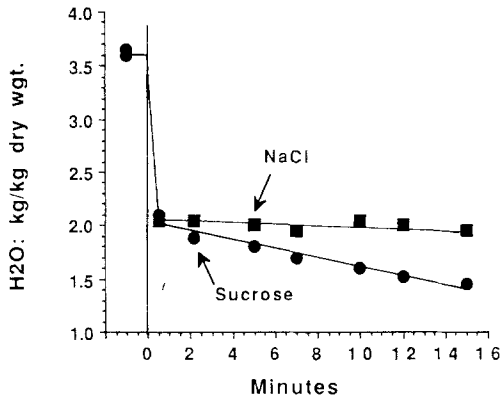

Fig. 3. Time course of the change in water content induced by hyperosmolality (520 ± 11 mOsM). In a representative experiment of this series (n = 6), 2.3 M sucrose medium or 5 M NaCl was added to cell suspension maintained at 37°C. Periodically during the next 15 min samples were removed for the measurement of cell water content. After the initial shrinkage the net efflux of water was 0.051 (sucrose) and 0.003 (NaCl) kg/(kg dry wt) · min^{-1}, respectively

sucrose or NaCl. This change is consistent with the prediction of the Boyle-van't Hoff relationship (final water content = {297/520 mOsM} · 3.7) and represents a 43% decrease in cell volume. In the sucrose-containing medium the cells continued to shrink and by 15 min had shrunk an additional 33% (2.11 to 1.4 kg water/kg dry wt) while over the same time period those in NaCl-containing medium shrunk less than 4%.

Since the membrane of the Ehrlich cell is highly permeable to water [17], the initial shrinkage represents the loss of water in response to the imposed osmotic gradient. Therefore, it seemed reasonable to assume that after dissipation of the osmotic gradient continued shrinkage must represent the loss of solute with water obligated to it. To test this idea a series of experiments was undertaken in which the cellular electrolytes (K^+, Na^+, Cl^-) were measured following the abrupt increase in osmolality. The data presented in Fig. 4 show that when the osmolality was increased from 295 to 502 mOsM by the addition of sucrose there was a prompt loss of K^+ and Cl^-. Intracellular Na^+, however, remained almost constant. In this experiment which is representative of 18 separate measurements (Table 1), the initial net efflux of K^+ and Cl^- was 10.6 and 13.1 TU, respectively, while water loss (Fig. 4*B*) amounted to 0.049 kg/(kg dry wt) · min^{-1}. Thus, the osmolality of the solution leaving the cells was calculated to be 484 mOsmol/kg water. If NaCl rather than sucrose was added the pattern of electrolyte change was different. As illustrated in Fig. 5 the addition of sufficient 5 M NaCl solution to raise the osmolality to 500

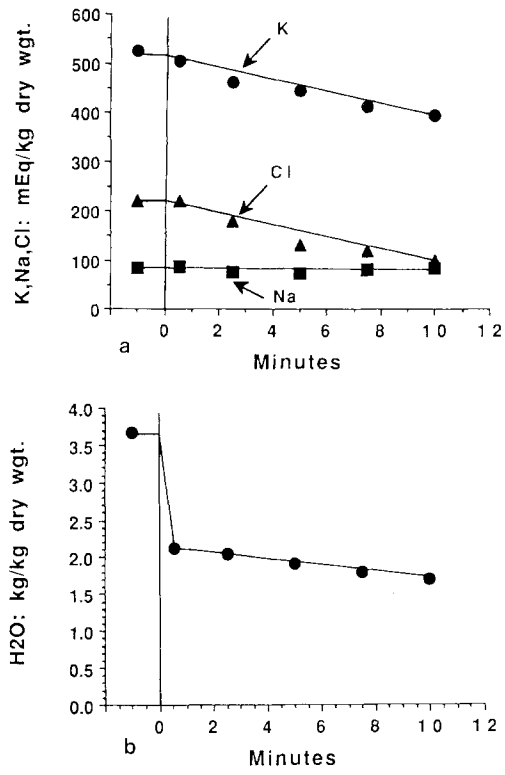

Fig. 4. Effect of a hyperosmotic medium (sucrose) on the time-dependent changes in cellular electrolytes and water. (*A*) Na^+, K^+ and Cl^- content expressed as mEq/kg dry cell wt. (*B*) Cell water content in kg/kg dry cel wt. At time zero 1 ml of 2.3 M sucrose medium was added to 14 ml cell suspension maintained at 37°C/O_2. While the electrolyte composition was unchanged the osmolality increased from 295 to 502 mOsM

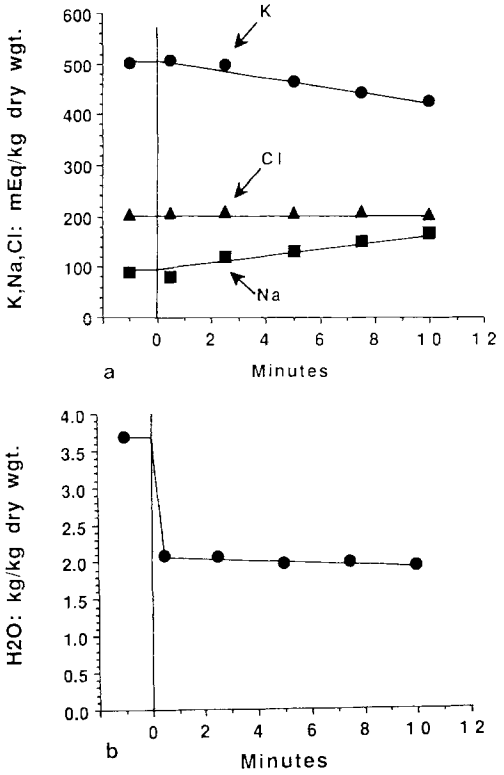

Fig. 5. Effect of a hyperosmotic medium (NaCl) on the time-dependent changes in cellular electrolytes and water. (*A*) Na^+, K^+ and Cl^- content expressed as mEq/kg dry cell wt. (*B*) Cell water content in kg/kg dry cell wt. At time zero 0.3 ml of 5 M NaCl was added to 14 ml cell suspension maintained at 37°C/O_2. The osmolality increased from 295 to 500 mOsM

Table 1. Effect of hyperosmotic stress on net ion and water fluxes[a]

	K^+	Na^+ (mEq/kg) dry wt $(min)^{-1}$	Cl^-	Water (kg/kg dry wt $(min)^{-1}$)
SUCROSE: 511 ± 5 mOsM				
Control (18)	−10.2 ± 1	0.7 ± 0.5	−13.1 ± 1.4	−0.054 ± 0.004
Ouabain (13)	−16.1 ± 1.1	4.5 ± 0.8	−12.5 ± 0.8	−0.052 ± 0.007
Bumetanide (4)	−4.5 ± 0.5	4.5 ± 0.8	−1.9 ± 1	−0.015 ± 0.006
Ouab. + Bumet. (19)	−12.3 ± 1	9.4 ± 0.9	−2.1 ± 0.5	−0.015 ± 0.003
Ouab. + Bumet. + Ba^{2+} (3)	0.7 ± 1	8.5 ± 3	0.7 ± 1	−0.010 ± 0.02
NaCl: 507 ± mOsM				
Control (7)	−9.8 ± 1.3	10.7 ± 0.9	0.8 ± 0.8	−0.01 ± 0.005
Ouabain (5)	−22.8 ± 1.5	22.3 ± 1.6	0	−0.005 ± 0.005
Ouab. + Bumet. (3)	−21.9 ± 2	21.1 ± 1.3	0	−0.002 ± 0.004
Ouab. + Bumet. + Ba^{2+} (3)	−2.5 ± 1.2	8.1 ± 2	0	0

[Ouabain] = 1 mM; [Bumetanide] = 25–50 μM; [Ba^{2+}] = 3–5 mM.

[a] Direction of net fluxes: (+) refers to uptake and (−) to extrusion.

Errors are expressed as ± SE with the number of measurements shown in parenthesis.

mOsM resulted in the loss of K^+ (9.5 TU) but with an equivalent gain in Na^+ (10 TU), while Cl remained almost constant. The change in cell water content was less than 0.01 kg/(kg dry wt) · min^{-1}.

In both instances the osmotic withdrawal of water results not only in concentrating the cellular electrolytes in proportion to the degree of shrinkage, but in addition triggers a sequence of events leading to changes in the electrolytes. In the case of sucrose the net loss KCl is responsible for the additional loss of cell volume while with the addition of NaCl there is almost no net change in osmolyte content and consequently little additional change in cell volume. Thus, not only is the Ehrlich cell unable to restore its volume following hyperosmotic challenge, but under some circumstances the cells continue to shrink.

In an effort to investigate this phenomenon we examined the effect of hyperosmolality on the activity of the principal ion transport pathways. The data shown in Table 1 summarizes experiments in which the effects of ouabain (Na/K pump inhibitor), bumetanide (Cl-dependent K^+, Na^+ cotransport inhibitor) and barium (K channel inhibitor, [15]) were studied on the net transport of K^+, Na^+, Cl^- and water following osmotic shrinkage induced by either sucrose of NaCl. Since Ba^{+2} may be less specific than other types of transport inhibitors, its effect on net Na^+ and K^+ transport is defined only in the presence of ouabain and bumetanide so as to minimize nonspecific effects.

When sucrose was used as the hyperosmotic agent, net K^+ efflux (16.1 TU) was maximal when the Na/K pump was inhibited by ouabain. Under these conditions 24% (3.8 TU) of K^+ loss was mediated by the bumetanide-sensitive pathway while 76% (12.3 TU) was lost via the Ba-inhibitable K^+ pathway. Chloride transport was unaffected by ouabain but was strongly inhibited by bumetanide. In fact, over 83% of Cl^- efflux (10.4 TU) appears to be transported by the bumetanide-sensitive pathway. In the presence of ouabain plus bumetanide there was a net Na^+ influx (9.4 TU) which is attributed to the activity of the Na/H exchanger (*see below*) and was only marginally affected by Ba^{+2} (8.5 TU). Influx, however, was almost quantitatively balanced by efflux mediated by the bumetanide-sensitive pathway (4.9 TU) plus the Na/K pump (4.5 TU). Therefore, under control conditions (absence of inhibitors) the net Na^+ flux was essentially zero. Results of these studies indicate that continued loss of cell volume (water) after the initial shrinkage is, in large part, due to transport of K^+, Na^+ and Cl^- out of the cell facilitated by the bumetanide-sensitive or cotransport pathway. This is demonstrated most clearly in Fig. 6 which shows the effect of bumetanide on the response of Ehrlich cells to hyperosmolality. Figure 6*A* demonstrates that after the initial osmotic response bumetanide almost completely blocked the subsequent loss of water. Figure 6*B* displays the time course of the change in the intracellular [Cl^-]. With the osmotic withdrawal of water, the [Cl^-] rose to approximately 100 mEq/kg water {62 mEq/kg water · (512/307) mOsM}; but then paralleled the decrease in water and by 25 min had returned to the normal physiological steady-state level of 62 mEq/kg water. Since bumetanide blocked the loss of Cl^-, its concentration remained elevated.

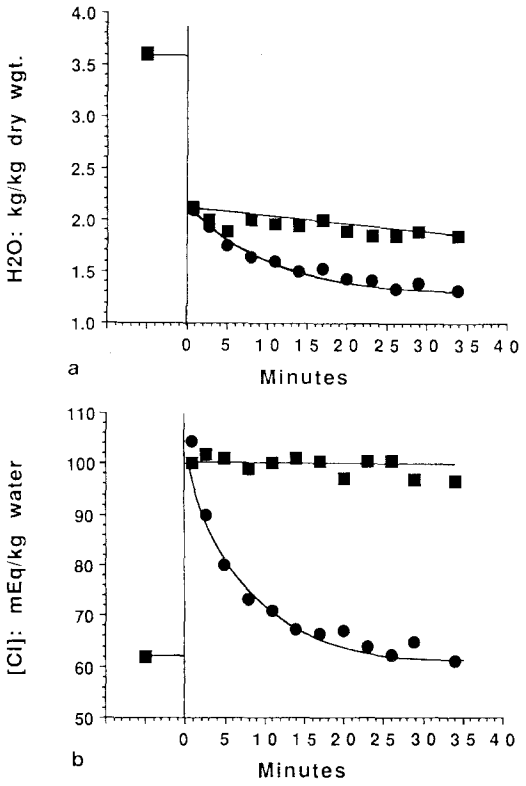

Fig. 6. Effect of a hyperosmotic medium (sucrose) on the time-dependent changes in cellular [Cl^-] and water and inhibition by bumetanide. (*A*) Cell water content in kg/kg dry cell wt. (*B*) Cl^- concentration expressed as mEq/kg cell water. Control ●, 50 μM bumetanide ■. At time zero 1 ml of 2.3 M sucrose medium was added to 14 ml cell suspension ± bumetanide maintained at 37°C/O_2. The osmolality increased from 307 to 512 mOsM. Representative experiment displayed; three others gave similar results.

The results are quite different when NaCl is used to increase the osmolality (Table 1). Although the maximum loss of K^+ also occurs when the Na/K pump is inhibited (22.8 TU), bumetanide had no effect on K^+ efflux. Ba^{2+}, however, blocked 88% (19.4 TU) of net K^+ loss. Sodium gain which paralleled K^+ loss, was also unaffected by bumetanide. Ba^{2+}, on the other hand, inhibited 62% of Na^+ entry. There is good reason to believe that the residual Na^+

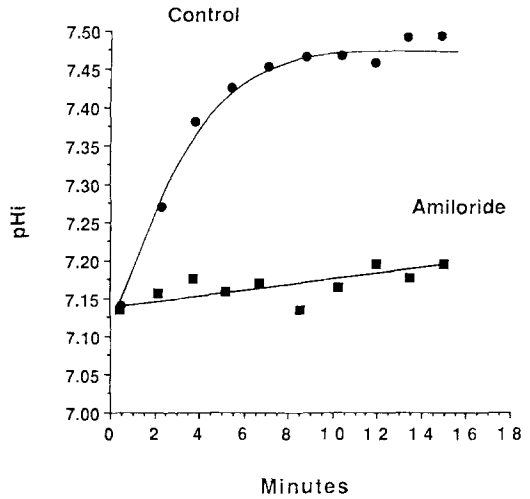

Fig. 7. Intracellular pH and the effect amiloride. At t_0 minus 20 sec amiloride (1 mM) was added to 14 ml cell suspension equilibrated with ^{14}C-DMO and maintained at 37°C/O_2. At time zero 1 ml of 2.3 M sucrose medium was added to increase the osmolality from 301 to 505 mOsM. Extracellular pH = 7.35 ± 0.5. Representative experiment displayed; four others gave similar results

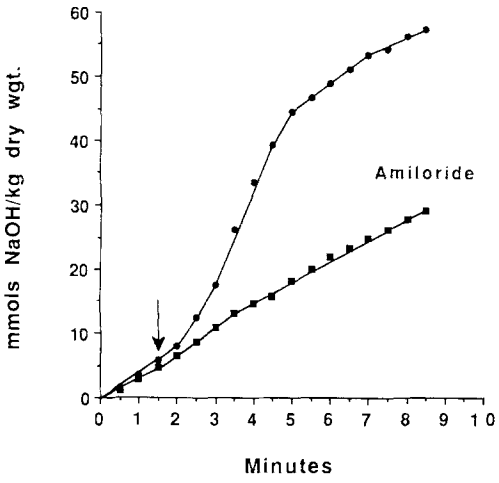

Fig. 8. NaOH delivered by the pH-stat in response to hyperosmolality. Ehrlich cells (6 mg dry wt/ml) were incubated in weakly buffered medium (pH_o 7.35, 298 mOsM) ± 1 mM amiloride. After a steady-state rate was established the osmolaity of the medium was rapidly increased (at the arrow) to 505 mOsM by the addition of either 5 M NaCl or 2.3 M sucrose medium. pH_o was maintained constant by the addition of microliter quantities of 5 mM NaOH dissolved in weakly buffered medium. A single representative experiment is shown; 10 others gave similar results

entry (8.2 TU) is mediated by the Na/H exchanger (*see below*). Intracellular Cl^- content was unaffected; in fact, we could find no evidence that shrinkage resulted in the net transport of Cl . Therefore, the lack of effect of bumetanide on Cl content or on the cation fluxes is consistent with the idea that net cotransport activity is not activated when cells are shrunk in hyperosmotic NaCl. Because of this cell volume remains stable following shrinkage.

As noted above (Table 1), a component of net Na^+ influx was revealed when the Na/K pump and cotransport system were inhibited. Since it is known in other systems that shrinkage activates the Na/H exchanger [14], we set out to determine whether this pathway was activated in the Ehrlich cell and thereby contributed to net Na^+ uptake. Two types of experiments were performed. In the first, Ehrlich cells which were equilibrated with ^{14}C-DMO were shrunk by the addition of hyperosmotic NaCl or sucrose. Figure 7 shows that intracellular pH (pH_i) rapidly increased from a resting value of about 7.15 to 7.47 within 7 min. Alkalinization, however, was almost completely blocked by amiloride. In the second type of experiment, net H^+ efflux was measured following an osmotic shrinkage. The data in Fig. 8 show that after a delay of about 1 min there is a rapid increase in H^+ efflux which persists for about 5 min. The increase in H^+ efflux, as well as a small fraction (about 20%) of the steady-state rate {4.3 ± 0.25 mEq/(kg dry wt) · min^{-1}}, is inhibited by amiloride. Additional support for the idea that osmotic shrinkage activates the Na/H exchanger comes from the observation that both intracellular alkalinization and extracellular acidification are completely blocked in the absence of extracellular Na^+ (*data not shown*).

Although osmotic activation of the Na/H exchanger appears to be independent of the nature of the hyperosmotic agent, net outward ion transport, mediated by the bumetanide-sensitive pathway (cotransporter), occurs only when osmolality is increased without altering the electrolyte composition of the medium. Since the effect of any nonpenetrating osmotic agent on cell composition should be the same, differences in the cellular responses elicited by sucrose when compared to NaCl must be related to differences in the transmembrane ion gradients that are generated.

Table 2 shows the electrolyte composition of cells and media during the normal physiological steady state and how this changes when media are made hyperosmotic (~500 mOsM) by the addition of either 200 mmol sucrose/liter or 100 mmol NaCl/liter. Note that in both media, with the osmotic withdrawal of water, intracellular [Na^+] increases from 36 to about 60 mEq/kg water while [K^+] increases from 163 to about 250 mEq/kg water. Although the [Cl^-] increases from 64 to over 100 mEq/kg water, in sucrose-containing medium it subsequently decreases with time to about 62 mEq/kg water. This re-

Table 2. Electrolyte composition of Ehrlich cells following incubation in standard medium and media made hyperosmotic by the addition of sucrose of NaCl

	K^+	Na^+ (mEq/kg water)	Cl^-	r_{Cl} (cell/medium)
Isosmotic: Cells	163 ± 3	36 ± 3	64 ± 0.7	0.43 ± 0.01
(302 ± 1) Medium	5.7 ± 0.2	133 ± 0.7	149 ± 0.6	
Sucrose: [a]Cells (initial)	253 ± 9.9	60 ± 4	112 ± 2.2	0.73 ± 0.01
[b]Cells (30 min)	265 ± 11	72 ± 8	62 ± 5	0.43 ± 0.04
(511 ± 5) Medium	4.9 ± 0.2	129 ± 1.3	143 ± 1.3	
NaCl: [a]Cells	238 ± 15	54 ± 5.7	103 ± 5	0.40 ± 0.03
(507 ± 4) Medium	4.6 ± 0.3	226 ± 4.5	257 ± 4.7	

[a] Measurements made within 1 min after the osmolality was changed.
[b] Measurements made 30 min after the change in osmolality.
Errors are expressed as SE and the number of determinations: isosmotic = 10, sucrose = 18, and NaCl = 9.

establishes the normal steady-state Cl^- distribution ratio (cell/environment), and it is during this period that additional cellular water is lost (Fig. 6). In addition to Cl^- there is also a net loss of K^+ (Fig. 4), yet its concentration does not change. This suggests that the composition of the solution leaving the cells is approximately 250 mEq KCl/kg water. In contrast to this, although hyperosmolality induced by the addition of NaCl also causes the cellular $[Cl^-]$ to rise, its distribution ratio does not change. Rather it remains similar to that of cells incubated in normal physiological media.

Discussion

Ehrlich cells incubated under physiological conditions of ionic composition and osmolality maintain a constant volume by regulation of their total solute content. However, with an increase in osmolality the cells rapidly shrink indicating a relatively high permeability to water [17, 19]. Quantitative measurements (Figs. 1 and 2) indicate that in a hyperosmotic environment the cells behave, at least initially, as osmometers following the prediction of the Boyle-van't Hoff relationship (linearity of cell water content *vs.* 1/extracellular osmolality). Subsequent behavior of the Ehrlich cell, however, depends on the nature of the hyperosmotic solute. For example, with the addition of NaCl to isotonic medium, cell volume decreases as predicted and the resultant volume is maintained relatively constant for as long as measurements were made. The inability of cells to recover, that is, exhibit RVI (Fig. 3) under these conditions was first demonstrated in the Ehrlich cell by Hempling [17] and more recently in lymphocytes [18], MDCK cells [31] and Chinese hamster ovary cells [32]. Similarly, HeLa cells [34], C6 glioma [9] and lymphocytes [12] fail to show RVI but do maintain volume stability when challenged hyperosmotically by the addition of mannitol or sucrose to the medium. This is in contrast to our finding that exposure of Ehrlich cells to hyperosmotic media containing sucrose results in a continuous decrease in cell volume which finally stabilizes in about 25 min (Figs. 3 and 6). A similar observation was made by Hendil and Hoffmann [19] who also found a slow decrease in Ehrlich cell volume in media made hyperosmotic (400 mOsM) by sucrose.

Two interrelated questions arise. First, why is the response of the Ehrlich cell dependent on the nature of agent used to induce the osmotic stress? Second, why can't these cells recover volume following a hyperosmotic challenge? It is important to recognize that when Ehrlich cells shrink by the addition of a nonpenetrating, nonelectrolyte such as sucrose the $[K^+]_o$, $[Na^+]_o$ and $[Cl^-]_o$ remain constant. This is in contrast to the effect of adding hyperosmotic NaCl which, of course, raises the extracellular $[Na^+]$ and $[Cl^-]$ while maintaining $[K^+]$ constant. In both cases the internal ion concentrations will be increased by a factor that is directly proportional to the change in osmolality (Table 2). The gradients of ionic chemical activity, however, will be quite different.

Shrinkage by the addition of sucrose results in what appears to be the simple net loss of KCl (Fig. 4, Table 1). However, when the effects of transport inhibitors, which are assumed to be specific, are considered it is apparent that net solute efflux and the resultant loss of cell water is dependent on the activity of the bumetanide-sensitive or cotransport pathway. Ionic movements through other pathways, such as the Na/K pump, Na/H exchanger and Ba-inhibitable K^+ loss, appear to balance so that they do not contribute to the net change in solute content (Table 1). However, inspection of Table 1 reveals a discrepancy between net ion movement and cell

volume response in cells shrunken in hyperosmotic NaCl. In the presence of ouabain, bumetanide and Ba^{2+} there is an apparent solute gain (Na^+) with no concomitant change in cell volume. Since the Na/H exchanger is considered to be osmotically active [7, 8], this finding suggests that other osmolytes must leave the cell (e.g., amino acids).

Based on the initial bumetanide-sensitive fluxes we estimate the stoichiometry of the cotransport pathway to be: Cl^- (10.4 TU) : K^+ (3.8 TU) : Na^+ (4.9 TU) or approximately $2Cl^- : K^+ : Na^+$. There is evidence suggesting that the magnitude and direction of ion movement through this pathway is strongly influenced by the sum of the transmembrane chemical potential gradients for Na^-, K^+ and Cl^- [16, 24]. Calculation of this gradient ($\Delta\mu_{net} = RT \ln \{[K]_o \cdot [Na]_o \cdot [Cl]_o^2\}/\{[K]_i \cdot [Na]_i \cdot [Cl]_i^2\}$) using the data shown in Table 2 indicates that after the initial osmotic response, the driving force acting on Na^+, K^+ and Cl^- is outwardly directed and equal to 1.63 ± 0.08 kcal/mol. If the activity of this pathway were governed exclusively by $\Delta\mu_{net}$ then we would expect the gradient to fall to zero as the net flux approached zero. This, however, is not the case. Rather, 30 min after the hyperosmotic challenge when cell volume has stabilized, $\Delta\mu_{net}$ remained outwardly directed and equal to 1.07 ± 0.06 kcal/mol. Clearly, something other than the transmembrane chemical potential gradient is responsible for limiting the loss of Na^+, K^+, Cl^- and water. The finding that the loss of ions and water correlates with the change in $[Cl^-]_i$ (Fig. 6) and that equilibrium is established when the Cl^- distribution ratio (r_{Cl}) returns to its normal physiological level (0.43; Table 2), suggests that the Cl^- gradient may serve as an important regulatory determinant. For example, after the initial osmotic response r_{Cl} increases to 0.73 (Table 2) which is considerably higher than that measured under physiological conditions of ionic composition and osmolality. Since during the normal physiological steady state the cotransporter does not catalyze net ion transport [3, 26], it is tempting to speculate that with the rise in $[Cl^-]_i$ the symmetry of the transporter is altered such that it now mediates net efflux of the quarternary complex. This continues until the Cl^- gradient re-establishes its normal equilibrium value. That the activity of the cotransporter is somehow influenced by the relationship between intra- and extracellular $[Cl^-]$ and not exclusively by the gradient of chemical potential of Na^+, K^+ and Cl^- has been proposed previously [27, 29, 34]. This view is further supported by the observation that even under isosmotic steady-state conditions (Table 2) there exists a small outwardly directed gradient ($\Delta\mu_{net}$ = 0.22 ± 0.03 kcal/mol) which favors net salt loss and thereby cell shrinkage. This, however, does not occur and raises the possibility that net cotransport activity in the physiological steady state is regulated by the Cl^- distribution ratio. In fact, Cl has recently been implicated as playing a key and perhaps regulatory role in controlling the response of the cotransorter of the squid axon to hyperosmolality [6].

The postulation that the Cl^- gradient is important in the regulation of cotransporter activity can be used to explain volume stability following NaCl-induced hyperosmolality. Since $[Cl]_i$ increases in direct proportion to the amount of NaCl added, r_{Cl} and thereby, the Cl^- gradient would not change significantly (Table 2). Under these conditions we would anticipate minimal net cotransport activity, even though bumetanide-sensitive unidirectional fluxes of Cl^- can readily be measured (C. Levinson, *unpublished observations*). Therefore, even in the presence of an outwardly directed passive driving force ($\Delta\mu_{net}$) of 0.39 ± 0.01 kcal/mol there is no net cotransport activity.

Our first indication that Na/H exchange is stimulated by cell shrinkage was the finding that a component of Na^+ uptake was insensitive to inhibition by either ouabain or bumetanide (Table 1). In contrast to cytoplasmic acidification which activates the Na/H exchanger by increasing the $[H^+]_i$ [5, 10], shrinkage *per se* has no effect on pH_i (Fig. 7). Therefore, a possible mechanism underlying the activation of the exchanger by shrinkage in Ehrlich, as in other cell types [14], is an alkaline shift in the pH_i dependence of a so called "modifier" site located on or near the antiporter. According to this model [1, 2] the set point of the modifier site which normally promotes only limited Na/H exchange at about pH_i 7.1, is adjusted upward. Consequently, when the exchanger is activated by shrinkage pH_i increases to a stable value of about 7.5. This presumably represents the value of the new set point. The mechanism by which osmotic shrinkage increases pH_i sensitivity and thereby activates the Na/H exchanger is unknown. Several authors have speculated that protein kinase C mediated phosphorylation of the antiporter itself or an associated protein is responsible [14]. While there are no definitive data, control through a biochemical cascade involving phosphorylation is an attractive hypothesis. The observation that after cell shrinkage there is a delay of about 1 min (Fig. 8) before maximal activation of Na/H exchange is consistent with that idea. A similar delay in activation has been reported in the lymphocyte [13].

An important consequence of Na^+ uptake by the Na/H exchanger is an osmotic gain which should be associated with an increase in cell volume. Since Na/H exchange operates with a 1 : 1 stoichiometry [2], the extrusion of $1H^+$ which is replaced by cellu-

lar buffers, results in the net gain of $1Na^+$. As pointed out by Cala [7, 8], this could be an important mechanism for volume restoration in osmotically shrunken cells. However, no evidence for an increase in volume as a consequence of activation of the Na/H antiporter could be found. A likely explanation is that the gain in Na^+ is off-set by the loss of K^+ through the Ba-inhibitable pathway (Table 1). Thus, the two types of ionic mechanisms, Na/H exchange and K^+, Na^+ and Cl^- cotransport, that could facilitate volume restoration after osmotic shrinkage appear to be ineffective. This does not imply that under all conditions the Ehrlich cell is unable to restore volume in response to hyperosmolality. It is well established that when Ehrlich cells are subjected to a cycle of RVD and subsequent re-exposure to isosmotic (~300 mOsm) medium, the shrinking phase is followed by reaccumulation of ions and water (RVI) and ultimately swell to the normal physiological volume [23]. The role of the RVD cycle may be necessary to deplete cells of intracellular ions, particularly Cl^- and thereby establish a gradient that favors net uptake [21]. This possibility is currently under investigation.

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