# Hypo-osmotic Stimulation of Active Na⁺ Transport in Frog Muscle: Apparent Upregulation of Na⁺ Pumps

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**Summary.** The purpose of this work was to determine if hypotonicity, in addition to the stimulation of active $Na^+$ transport (Venosa, R.A., 1978, *Biochim. Biophys. Acta* **510:**378–383), promoted changes in (*i*) active $K^+$ influx, (*ii*) passive $Na^-$ and $K^+$ fluxes, and (*iii*) the number of $^3H$-ouabain binding sites.

The results indicate that a reduction of external osmotic pressure ($\pi$) to one-half of its normal value ($\pi = 0.5$) produced the following effects: (*i*) an increase in active $K^+$ influx on the order of 160%, (*ii*) a 20% reduction in $Na^+$ influx and $K^+$ permeability ($P_K$), and (*iii*) a 40% increase in the apparent density of ouabain binding sites. These data suggest that the hypotonic stimulation of the $Na^+$ pump is not caused by an increased leak of either $Na^+$ (inward) or $K^+$ (outward). It is unlikely that the stimulation of active $Na^+$ extrusion and the rise in the apparent number of pump sites produced by hypotonicity were due to a reduction of the intracellular ionic strength. It appears that, at least in part, the stimulation of active $Na^+$ transport takes place whenever muscles are transferred from one medium to another of lower tonicity even if neither one was hypotonic (for instance $\pi = 2$ to $\pi = 1$ transfer). Comparison of the present results with those previously reported indicate that in addition to the number of pump sites, the cycling rate of the pump is increased by hypotonicity. Active $Na^+$ and $K^+$ fluxes were not significantly altered by hypertonicity ($\pi = 2$).

**Key Words**     sodium pump · density · hypotonicity · frog muscle

## Introduction

Most animal cells respond to osmotic challenges with changes in volume by swelling (hypotonic solutions) or shrinking (hypertonic solutions). For a reasonably wide range of osmotic pressure ($\pi$), frog muscle fibers behave as quasi-perfect osmometers showing a linear relationship between their volume at equilibrium and $1/\pi$. Osmolarity values between one-half and twofold that of the normal Ringer are well within the linear range (Dydynska & Wilkie, 1963; Reuben et al., 1963; Parisi, Montoreano & Lew, 1965; Blinks, 1965).

In some cell types volume changes in anisotonic media are transient. These so-called osmoconformers exhibit volume regulation; in an anisotonic medium after the initial change, this volume tends to return to that in the isotonic media. This phenomenon is apparently due to changes in ionic permeabilities.

Active transport systems do not seem to play any significant role in either the regulatory volume decrease or increase (*see* reviews by Hoffman, 1977; Macknight & Leaf, 1985; Siebens, 1985).

When frog muscle fibers, which do not behave as osmoconformers, are exposed to hypotonic media their ionic transport properties are altered in a manner very different from osmoconformers. In addition to swelling, they undergo, quite uniquely, a marked increase in active $Na^+$ extrusion (Venosa, 1978).

This is a puzzling finding because, as is generally accepted, in frog muscle fibers as well as in most cells, active $Na^+$ transport increases with $[Na^+]_i$. Therefore, it would be expected that a reduction in the osmolarity of the external medium and the subsequent fall of $[Na^+]_i$ due to the osmotic water inflow should produce, if anything, a decrease in the active $Na^+$ extrusion. As just mentioned, and at variance with this expectation, the opposite effect, i.e., increased $Na^+$ pump activity, is observed.

The intent of this work was to determine if the stimulation of the active $Na^+$ transport mechanism produced by hypotonic media in frog muscle fibers was associated with an increase in the number of pumps. The results indicate that, indeed, swelling is accompanied by an apparent upregulation of pump sites measured as an increase in $^3H$-ouabain binding. This increase, however, is smaller than that previously observed for active $Na^+$ transport (Venosa, 1978).

## Materials and Methods

Small isolated sartorius muscles from *Leptodactyllus ocellatus* (Argentine frog) were used. After dissection they were fastened to light stainless steel holders by means of thin surgical threads attached to the tendons.

### Solutions

The normal Ringer solution had the following composition (in mM): NaCl 115; KCl 2.5; CaCl$_2$ 1.8; Na$_2$HPO$_4$ 2.15; and NaH$_2$PO$_4$ 0.85 (pH = 7.2).

The standard isotonic medium was similar to the normal Ringer except that 62 mM NaCl, i.e., one-half of the total osmolarity, was replaced by an osmotically equivalent concentration of sucrose (1 M NaCl = 1.6 M sucrose as determined with a Wescor 5100 osmometer). The relative osmotic pressure, $\pi$, was changed between one-half ($\pi = 0.5$) and twice its normal value ($\pi = 2$) by changing [sucrose] between 0 ($\pi = 0.5$) and 293 mM ($\pi = 2$). By these means all the experimental solutions had the same ionic strength.

### Na$^+$ and K$^+$ Fluxes

The unidirectional fluxes of Na$^+$ ($^{24}$Na$^+$) and K$^+$ ($^{42}$K$^+$) were measured as previously described (Venosa & Horowicz, 1973; Venosa, 1974; Kotsias & Venosa, 1987). The $^{24}$Na$^+$ uptake periods ranged from 15 to 20 min. The uptake values were corrected for backflow as previously described (Venosa, 1974). The $^{42}$K$^+$ uptake was a linear function of time for at least 40 min and therefore it was not corrected for backflow. Active K$^+$ influx was taken as the difference between the influx in muscles equilibrated in a given $\pi$ in the presence of 50 $\mu$M ouabain (passive influx) and that in their paired companions under identical conditions but in the absence of ouabain.

$^{24}$Na$^+$ and $^{42}$K$^+$ were purchased from Comision Nacional de Energia Atomica, Argentina.

### Electrical Measurements

Resting membrane potential was measured by means of conventional electrophysiological techniques using glass microelectrodes filled with 3 M KCl (5–20 M$\Omega$ resistance). Action potentials were elicited by stimulating small bundles of fibers with square pulses lasting 1 msec and delivered through a pair of thin platinum electrodes. The action potentials were displayed and saved in the memory of a digital oscilloscope (Tektronix 5223) and recorded by a Gould 2400 pen recorder.

### $^3$H-Ouabain Binding

$^3$H-ouabain binding was measured using the technique of Venosa and Horowicz (1981) which is based on the very slow release of the specifically bound drug and on the kinetics of the binding at different ouabain concentrations ([OUA]). Briefly, paired muscles, after osmotic equilibration (one in $\pi = $ x and the other in $\pi = 1$ for 90 min), were exposed to the same media containing a given concentration of labeled ($^3$H) ouabain (New England Nuclear) for different periods of time. After the exposure to $^3$H-ouabain they were washed from 8 to 10 hr in a series of tubes

containing 3 ml of the same solutions without ouabain. At the end of the run the preparations were dissolved overnight in 1 ml of Protosol (New England Nuclear). After neutralization with 1 ml of 1 M malic acid and the addition of 15 ml scintillation cocktail (POPOP 250 mg; PPO 5 g; ethanol 500 ml; toluene 2 liter; Triton X-100 1.25 liter) the samples were counted in a beta counter (Tracor Analitic 1191). Internal standards were used to correct for quenching.

Typically, washout curves show an initial rapid loss due to the release of the drug from the extracellular space and unspecific binding sites. This is followed by a slow single exponential component with a time constant of several hours (*see* Fig. 2), consistent with the virtually irreversible block of active Na$^+$ efflux produced by the drug in this preparation. The extrapolated value of this component to time zero of the washout represents the binding to specific sites (*b*) at the end of the exposure period to the glycoside.

As previously shown (Venosa & Horowicz, 1981), in frog skeletal muscle, specific ouabain binding follows a time course that is reasonably well fitted by Eq. (1)

$$b = B(1 - \exp(-t/\tau)) \tag{1}$$

where $b$ and $B$ are the amounts of ouabain (in pmol $\cdot$ g$^{-1}$ or molec $\cdot$ $\mu$m$^{-2}$ of sarcolemma, *see below*) bound at time $= t$ and time $= \infty$ (i.e., at equilibrium), respectively, and $\tau$ is the time constant of the uptake.

The value of $B$ at a given [OUA] was determined by measuring $b$ at different times of exposure ($t$) using several preparations and fitting Eq. (1) to the data (*see* Fig. 3). The determination of $B$ at a fixed $t$ is unreliable unless $\tau$ is known and $t$ is considerably greater than $\tau$.

To compare the data from these experiments with those obtained in other species, the surface membrane area (T-tubules excluded) per gram of muscle was estimated. As done before with muscles from *R. pipiens* (Venosa, 1974) the value was calculated from the extracellular space (Na$^+$ space $= 29.5 \pm 1.0$ (SEM) ml/ 100 g), muscle length, weight and number of fibers per muscle from 14 representative muscles. Fibers were counted in histological cross sections using light microscopy. The mean number of fibers per muscle was $910 \pm 20$, the calculated mean fiber diameter $62.0 \pm 1.3$ $\mu$m and the mean surface area (T-tubules excluded) $430 \pm 9$ cm$^2$ $\cdot$ g$^{-1}$ of muscle.

All the experiments were performed at room temperature (20–22°C).

### Statistics and Curve Fitting

Student's $t$ test was used to estimate the statistical significance of differences. If not otherwise stated, values are expressed as means $\pm$ 1 SEM.

Curve fitting was done by nonlinear regression (Graph PAD, ISI® software).

## Results

### Na$^+$ and K$^+$ Influx Under Anisotonic Conditions

With regard to the mechanism by which hypotonicity stimulates the Na$^+$ pump in frog muscle fibers, two possibilities should initially be considered. One

**Table.** Effect of tonicity on K$^+$ permeability ($P_K$)

| $\pi$ | K$^-$ influx (pmol · cm$^{-2}$ · sec$^{-1}$) | $V_m$ (mV) | $P_K$ (nm · sec$^{-1}$) |
|---|---|---|---|
| 1 | 2.77 ± 0.15 (n = 11) | −89.7 ± 0.7 (n = 108f/6m) | 3.00 ± 0.16 |
| 0.5 | 1.90 ± 0.09 (n = 9) | −76.9 ± 1.2 (n = 35f/4m) | 2.36 ± 0.11[a] |
| 2 | 2.95 ± 0.20 (n = 10) | −89.5 ± 0.7 (n = 27f/4m) | 3.20 ± 0.23 |

$\pi$: tonicity relative to normal Ringer; $V_m$: resting membrane potential; f/m: fibers/muscles.

[a] $P < 0.01$ with respect to $\pi = 1$. Means ± 1 SEM. Preparations were in the presence of 50 $\mu$M ouabain.



**Fig. 1.** Active K$^+$ influx (ouabain sensitive) as a function of relative osmotic pressure ($\pi$). The means ± 1 SEM. are: 0.40 ± 0.10 pmol · cm$^{-2}$ · sec$^{-1}$ ($n = 4$) at $\pi = 1$, 1.05 ± 0.13 pmol · cm$^{-2}$ · sec$^{-1}$ ($n = 6$) at $\pi = 0.5$ and 0.36 ± 0.09 pmol · cm$^{-2}$ · sec$^{-1}$ at $\pi = 2$. The values at $\pi = 1$ and $\pi = 0.5$ are statistically different ($P < 0.05$)

possibility, although unlikely, would be that pump stimulation is due to an increase in the inward leak of Na$^+$ large enough to raise [Na$^+$]$_i$ in a region close to the high affinity site for Na$^+$ of the Na$^+$/K$^+$-ATPase in spite of the increased cellular water content. This mechanism can readily be discarded because: (i) the hypotonic effect on active Na$^+$ transport is not noticeably altered in a Na$^+$-free medium (Venosa, 1978), (ii) the overshoot of the action potential increased from 19.1 ± 0.7 mV ($n = 94$) in $\pi = 1$ to 27.8 ± 1.0 mV ($n = 35$) in $\pi = 0.5$ suggesting that the intracellular Na$^+$ activity was significantly reduced in the hypotonic medium, and (iii) hypotonicity actually produced a barely significant reduction in Na$^+$ influx of about 20%. Thus, in four muscles equilibrated in $\pi = 0.5$ the mean Na$^+$ influx was 2.9 ± 0.5 pmol · cm$^{-2}$ · sec$^{-1}$ while in their paired companions equilibrated in $\pi = 1$ it was 3.7 ± 0.7 pmol · cm$^{-2}$ · sec$^{-1}$ ($P < 0.06$).

The second mechanism that deserves to be considered is that concerned with the possibility that hypotonicity might increase K$^+$ permeability ($P_K$) and thereby the leakage of K$^+$ from the cells which by raising [K$^+$]$_o$ in proximity to the high affinity site for K$^+$ would, in turn, stimulate the pump. This can also be ruled out because $P_K$ falls by about 20% in $\pi = 0.5$ while it was not changed by $\pi = 2$. $P_K$ was calculated from the passive K$^+$ influx (ouabain insensitive) and the mean resting membrane potential under the same experimental conditions using the constant field flux equation (Hodgkin & Katz, 1949). The data and calculated $P_K$'s are summarized in the Table.

Stimulation of the pump by hypotonic solutions should also be expressed as an increase in the active inward movement of K$^+$. This is illustrated in Fig. 1 where it can be seen that in muscles equilibrated in $\pi = 0.5$ there was an increase in active (ouabain sensitive) K$^+$ influx on the order of 160% with respect to that found in $\pi = 1$ (which is very close to
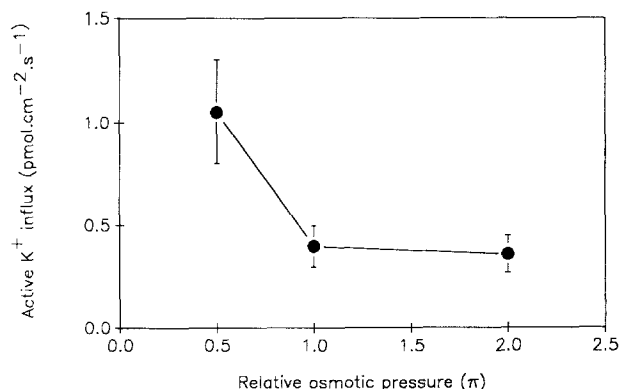
the 170% increase in active Na$^+$ efflux rate coefficient estimated from previous results (Venosa, 1978). On the other hand, the mean active K$^+$ influx in muscles equilibrated in $\pi = 2$ was not significantly different from that in $\pi = 1$. The lack of effect of $\pi = 2$ on the active K$^+$ transport will be further discussed below in connection with the experiment shown in Fig. 6. It should be pointed out that ouabain (50 $\mu$M) significantly reduced K$^+$ influx in all muscles regardless of the osmotic pressure in which they had been equilibrated.

### OUABAIN BINDING IN $\pi = 0.5$

Figure 2 shows the release of ouabain, into a ouabain-free medium, from a pair of muscles which had previously been equilibrated (one in $\pi = 0.5$ and the other in $\pi = 1$) and subsequently exposed for 30 min to the same solutions containing 20 $\mu$M labeled ($^3$H) ouabain. Specific ouabain binding is given by extrapolating the monoexponential component (dashed lines) to time = 0. In this particular experiment binding amounted to 97 pmol · g$^{-1}$ in $\pi = 1$ and 298 pmol · g$^{-1}$ in $\pi = 0.5$. Binding at different times of exposure and inhibitor concentrations was always higher in preparations equilibrated at $\pi = 0.5$ than in those kept in $\pi = 1$.

Figure 3 illustrates the time course of ouabain binding in paired sartorii, one member of each pair equilibrated in $\pi = 0.5$ and the other in $\pi = 1$. It can be seen that at all times of exposure to 40 $\mu$M ouabain (a concentration at which cardiac steroids maximally inhibit active Na$^+$ transport in this preparation; Horowicz, Taylor & Waggoner, 1970) bind-
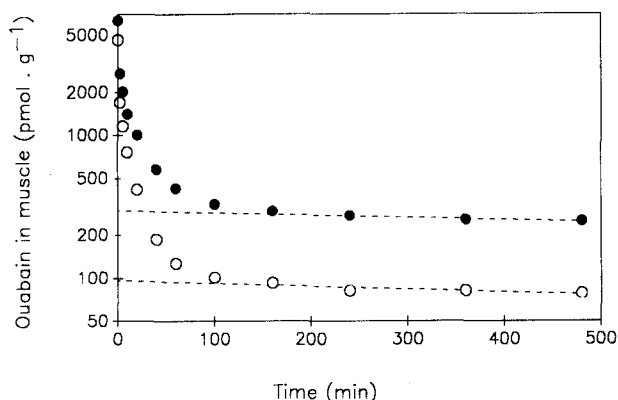
**Fig. 2.** Semilog plot of the washout of ouabain from a pair of muscles which had been equilibrated in $\pi = 1$ (O) and $\pi = 0.5$ (●), and exposed to 20 $\mu$M labeled $^3$H-ouabain for 30 min in their respective media. Dashed lines: Slow single exponential component (time constants: 43 and 36 hr for the upper and lower curve, respectively) corresponding to the release of the inhibitor from specific binding sites. The extrapolation to time zero represents the specific binding (pmol/g) at the end of the exposure period (*see* text)



**Fig. 4.** Density of ouabain binding sites in paired sartorii equilibrated in $\pi = 0.5$ (●) and $\pi = 1$ (O) as a function of ouabain concentration. Each point represents the mean maximum binding ($B$ in Eq. (1)) $\pm$ 1 SEM (absent when smaller than the symbol) obtained from experiments similar to that illustrated in Fig. 3. The curves represent the fit of Eq. (2) to the experimental points. The values of the calculated parameters are: for $\pi = 1$—$B_m = 3500 \pm 310$ molec $\cdot \mu$m$^{-2}$ and $K = 5.4 \pm 1.8$ $\mu$M; for $\pi = 0.5$—$B_m = 4860 \pm 160$ molec $\cdot \mu$m$^{-2}$ and $K = 1.1 \pm 0.2$ $\mu$M



**Fig. 3.** Time course of ouabain binding in muscles osmotically equilibrated at $\pi = 0.5$ (●) and their paired controls at $\pi = 1$ (O) both exposed to 40 $\mu$M ouabain. The curves represent the nonlinear exponential fit (Eq. (1)) to the data. Each circle is the mean of five to eight muscles and the triangles the mean from four pairs of cut ($\triangle$) and intact muscles (▲) both equilibrated at $\pi = 0.5$. Error bars: $\pm$ 1 SEM (absent when smaller than the symbol)

ing was higher in $\pi = 0.5$ than in $\pi = 1$. The exponential fit of the data (Eq. (1)) yielded: $B = 4550 \pm 180$ molec $\cdot \mu$m$^{-2}$; $\tau = 9.5 \pm 1.2$ min in $\pi = 0.5$, and $B = 2980 \pm 170$ molec $\cdot \mu$m$^{-2}$; $\tau = 16 \pm 2$ min in $\pi = 1$.

The possibility that the binding increase in $\pi = 0.5$ was due to the reduction of either [K$^+$]$_i$ or the internal ionic strength upon swelling of the fibers was precluded by the following experiment. In four
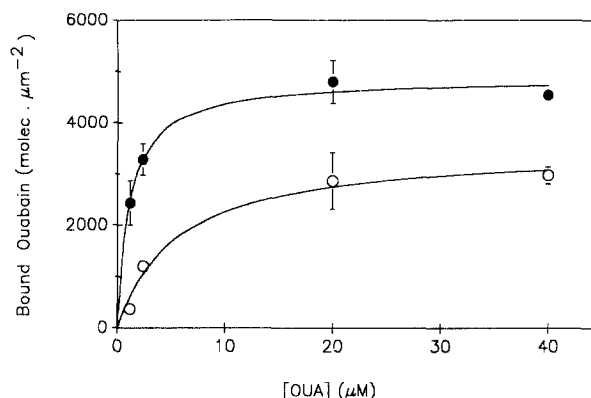
muscles the sarcolemma was disrupted by cutting it in 16 places (eight from each edge alternately). The length of each cut was about 3/4 of the muscle width. The cut muscles and their intact paired companions were equilibrated in $\pi = 0.5$. A subsequent 30-min exposure to 40 $\mu$M $^3$H-ouabain showed that while in the intact muscles the binding was similar to that measured in other intact muscles equilibrated in $\pi = 0.5$ (filled triangles in Fig. 3), in the cut muscles it was significantly lower and close to that observed in intact muscles equilibrated in $\pi = 1$ (open triangles in Fig. 3). This indicates that neither low [K$^+$]$_i$ nor the fall of the internal ionic strength by themselves caused the binding increase in $\pi = 0.5$. In addition we have previously shown that cutting, *per se*, does not affect ouabain binding in normal Ringer (Venosa & Horowicz, 1981).

The relationship between the magnitude of ouabain binding, $B$, and glycoside concentration, [OUA], was reasonably well fitted by the following hyperbolic function:

$$B = \frac{B_m[\text{OUA}]}{K + [\text{OUA}]} \tag{2}$$

where $B_m$ is the maximum binding and $K$ represents the [OUA] at which $B = B_m/2$.

Figure 4 shows the binding in muscles equilibrated in $\pi = 1$ and their paired companions in $\pi = 0.5$ at [OUA]'s ranging from 1.2 to 40 $\mu$M. While $B_m$ was increased from 3500 $\pm$ 310 molec $\cdot \mu$m$^{-2}$ in $\pi = 1$ to 4860 $\pm$ 160 molec $\cdot \mu$m$^{-2}$ in $\pi = 0.5$, $K$ was
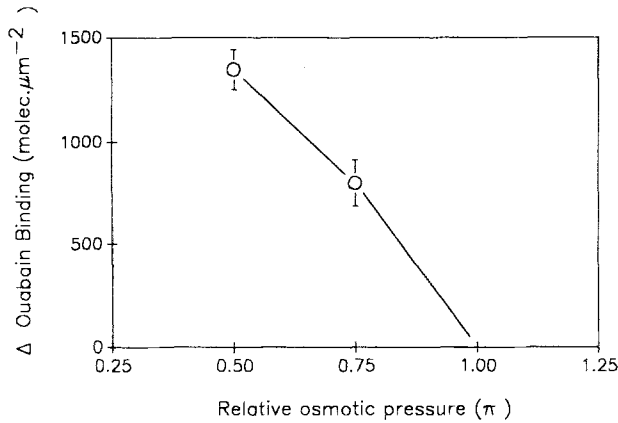
**Fig. 5.** Increase in the density of ouabain binding sites over that found in $\pi = 1$ in muscles equilibrated in $\pi = 0.75$ ($n = 7$) and $\pi = 0.5$ ($n = 32$) and exposed to 40 $\mu$M ouabain for 40 min. Means $\pm$ 1 SEM: 1350 $\pm$ 95 ($n = 32$) molec $\cdot$ $\mu$m$^{-2}$ in $\pi = 0.5$ and 800 $\pm$ 110 ($n = 7$) molec $\cdot$ $\mu$m$^{-2}$ in $\pi = 0.75$



**Fig. 6.** Effect of $\pi = 2$ and of the return from $\pi = 2$ to $\pi = 1$ on the fractional loss of $^{24}$Na$^+$ from eight muscles in the absence (●) and their paired companions in the presence (▲) of 30 $\mu$M ouabain. The solid line without symbols represents the difference between the two experimental curves, i.e., the ouabain-sensitive component of the Na$^+$ efflux. The ouabain-insensitive component of the efflux (▲) increased significantly at $\pi = 2$ ($P < 0.01$ for the 50–80 min period) with respect to its last value of the initial period at $\pi = 1$

reduced from 5.4 $\pm$ 1.8 $\mu$M in $\pi = 1$ to 1.1 $\pm$ 0.2 $\mu$M in $\pi = 0.5$.

It is clear from Figs. 3 and 4 that after a 40-min exposure to 40 $\mu$M [OUA] the binding sites are virtually all occupied, i.e., the amount of bound ouabain (molec $\cdot$ $\mu$m$^{-2}$) under these conditions represents a reliable estimate of the binding capacity ($B_m$) in this preparation. Figure 5 illustrates the increase in $B_m$ ($\Delta B_m$) promoted by $\pi = 0.75$ and $\pi = 0.5$ estimated by 40-min exposure to 40 $\mu$M [OUA]. The increase in $B_m$ as $\pi$ decreases is consistent with the increase of the active Na$^+$ efflux observed under similar experimental conditions (Venosa, 1978).

It is not unreasonable to postulate that the hypotonic effect on the Na$^+$ active transport system might be triggered by the increase in cell volume which would be, somehow, sensed by membrane deformation. It is known that passive stretch increases active Na$^+$ transport in frog sartorius muscle (Harris, 1954; Rapoport & Bidinger, 1974). However, this effect did not occur as a result of an increase in the number of binding sites. Control experiments showed that stretch, if anything, reduces ouabain binding. In seven muscles stretched by 25%, relative to their *in situ* length, and exposed to 40 $\mu$M $^3$H-ouabain for 30 min the binding was 2680 $\pm$ 190 sites $\cdot$ $\mu$m$^{-2}$ as compared with 3280 $\pm$ 220 sites $\cdot$ $\mu$m$^{-2}$ in their paired companions kept at body length ($P < 0.02$).

The stimulation of the Na$^+$ pump by swelling not only takes place with $\pi < 1$, but as is shown in Fig. 6, in muscles exposed to $\pi = 2$ for 1 hr and then returned to $\pi = 1$. A marked and transient increase of the active Na$^+$ extrusion occurred. This
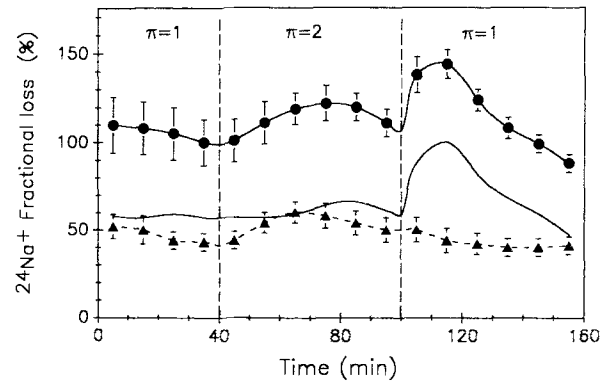
observation suggests that, at least in part, pump stimulation is produced whenever the cell volume increases regardless of whether it occurs below or above $\pi = 1$.

Figure 6 also shows that hypertonicity ($\pi = 2$), produced a modest, if any, increase of the ouabain-sensitive component of Na$^+$ fractional loss. This is consistent with the lack of the effect of $\pi = 2$ on active K$^+$ transport (Fig. 1). A result at variance with the augmented pump activity that might be expected as a consequence of the increase in [Na$^+$]$_i$.

An increase in passive Na$^+$ efflux is not a likely explanation for the increase in Na$^+$ efflux in the presence of ouabain since, based on independence of unidirectional fluxes (Ussing, 1949), it represents a negligible fraction (<1%) of the total efflux. Most of the increase in Na$^+$ efflux observed when $\pi = 2$ is probably the result of an increase in the ouabain-insensitive Na$^+$ exchange diffusion component. In normal Ringer ([Na$^+$]$_o$ = 120 mM) this component amounts to about 38% of the Na$^+$ unidirectional fluxes, and its absolute value is not altered by isotonic reduction of [Na$^+$]$_o$ to 60 mM (Horowicz et al., 1970; Venosa & Horowicz, 1973; Venosa, 1974), that is, under conditions similar to $\pi = 1$. The fact that in four muscles equilibrated in $\pi = 2$ the mean Na$^+$ influx was 3.92 $\pm$ 0.44 pmol $\cdot$ cm$^{-2}$ $\cdot$ sec$^{-1}$ while in their paired controls, kept in $\pi = 1$, it was 2.35 $\pm$ 0.18 pmol $\cdot$ cm$^{-2}$ $\cdot$ sec$^{-1}$ ($P = 0.06$) favors the notion that the increase in Na$^+$ efflux produced by $\pi = 2$ mainly represents an increase in the Na$^+$ exchange diffusion component.

## Discussion

The aim of this work was to find out whether the stimulation of active Na$^+$ transport produced by hypotonicity in frog skeletal muscle (Venosa, 1978) was also accompanied by an increase in the number of Na$^+$ pumps measured as an increase in ouabain binding.

The results show that hypotonicity produces an apparent upregulation of pump sites—a new finding. The stimulation of the ouabain-sensitive K$^+$ influx by $\pi = 0.5$ provides further evidence of sarcolemmal Na$^+$/K$^+$-ATPase involvement in the effect of hypotonicity on Na$^+$ efflux. The apparent reduction of $P_{Na}$ and $P_K$ in muscles equilibrated in $\pi = 0.5$ precludes the possibility that the stimulation of the Na$^+$/K$^+$ active transport was due to leakage of Na$^+$ (inward) and/or K$^+$ (outward) which might produce local increases of [Na$^+$]$_i$ and [K$^+$]$_o$ close to their high affinity pump sites. The active component of K$^+$ influx was clearly detectable under all osmotic conditions. In $\pi = 1$, it was on the order of 13% of the total influx. Previous reports are contradictory. Keynes (1965), working with sartorii from *R. temporaria,* and Sjodin (1965), using the same preparation from *R. pipiens,* were unable to detect any active component K$^+$ influx. On the other hand, in sartorii from *R. esculenta* O'Donnell, Kovacs and Szabo (1975) found that about 27% of the inward movement of K$^+$ could be blocked by ouabain.

In $\pi = 1$ the passive (ouabain-insensitive) K$^+$ influx was 2.8 pmol · cm$^{-2}$ · sec$^{-1}$ while the active K$^+$ influx (ouabain sensitive, Fig. 2) was 0.4 pmol · cm$^{-2}$ · sec$^{-1}$. The total influx in isotonic medium (3.2 pmol · cm$^{-2}$ · sec$^{-1}$) is similar, although somewhat lower than the values found earlier in toe muscle from *R. temporaria* (4.1 pmol · cm$^{-2}$ · sec$^{-1}$) (Keynes, 1954) and in single fibers from the same species (5.4 pmol · cm$^{-2}$ · sec$^{-1}$) (Hodgkin & Horowicz 1959a). The calculated $P_K$ here in $\pi = 1$ was 3.0 nm · sec$^{-1}$ (Table), a value identical to that reported by Sjodin (1965) in sartorius from *R. pipiens,* but that appears to be significantly lower than the mean value (9 nm · sec$^{-1}$) obtained by Hodgkin and Horowicz (1959b) in single fibers from *R. temporaria* under experimental conditions close to those used here.

In skeletal muscle, depending on the experimental conditions and preparation, stimulation of the Na$^+$ pump may or may not be associated with an increase in the number of pump sites. For example, stimulation of the active Na$^+$ transport by insulin and adrenaline increases the rate of ouabain binding in mammalian skeletal muscle without changing the apparent number of pump sites (Clausen & Hansen, 1977). On the other hand, Erlij and Grinstein (1976)

suggested that the stimulation of the Na$^+$ pump by insulin in frog muscle takes place through an upregulation of the number of pump sites.

Hypotonic stimulation of the pump in frog muscles equilibrated in $\pi < 1$ increases both the rate constant of ouabain binding (1/$\tau$) and the apparent number of pumps (Figs. 3 and 4).

The increase in active Na$^+$ and K$^+$ flux and the increase in the apparent number of pumps under hypotonic conditions do not seem to be linearly related. In $\pi = 0.5$, for instance, the increase in ouabain binding was 40% of that in $\pi = 1$ (*see* Fig. 4), while the increase in active Na$^+$ efflux estimated from previous data (Venosa, 1978) was on the order of 170%. These results are in good agreement with the increase in active K$^+$ influx (160%) obtained in these experiments. In $\pi = 0.75$, on the other hand, active Na$^+$ efflux increased by about 50% (Venosa, 1978) and ouabain binding about 26%. It would appear, therefore, that as $\pi$ decreases the cycling rate, i.e., the number of Na$^+$ ions transported per pump site and unit time, increases. The possibility that the magnitude of bound ouabain in $\pi = 0.5$ represents two populations of sites (that present in $\pi = 1$ and an additional one made available to the inhibitor by equilibration at $\pi = 0.5$) could not be confirmed. Thus, the data at $\pi = 0.5$ are unsuitable for the fit to a sum of two hyperbolic functions of [OUA] (one for the binding values at $\pi = 1$ and the other for the difference between paired values at $\pi = 0.5$ and at $\pi = 1$).

The mechanism of the increase in the apparent number of pump sites produced by hypotonic solutions is by no means clear. The fact that in cut muscles equilibrated in $\pi = 0.5$ the magnitude of the ouabain binding is similar to that found in intact muscles equilibrated in $\pi = 1$ makes it unlikely that the effect is the result of either a decrease in the concentration of some intracellular solute or a fall in internal ionic strength. On the other hand, a possibility worth considering is that the hypotonic effect might be mediated by the deformation of the membrane by swelling. It is not clear, however, how that deformation could be transduced into both the increase in the active Na$^+$ transport reported earlier (Venosa, 1978) and in the apparent upregulation of pump sites described here. Some circumstantial evidence makes the stretch hypothesis plausible. Passive stretch stimulates active Na$^+$ transport (Harris, 1954; Rapoport & Bidinger, 1974) and produced a reduction of about 18% rather than an increase in ouabain binding (*see* Results). Vanderburgh and Kaufman (1981) suggested that longitudinal stretch activates pump sites already present in the membrane; therefore, if in the present experiments the stretch of the sarcolemma plays a role in the increase

of the apparent number of pump sites this might be due to the membrane strain in the circumferential direction. The sarcolemma stretches anisotropically; it is more compliant in the circumferential than in the longitudinal direction (Fields, 1970; Rapoport, 1973). Nevertheless, the hypothesis remains to be tested directly.

The experiment shown in Fig. 6 shows that active Na$^+$ transport, or at least a component of it, is sensitive to volume increases regardless of whether they occur above or below the normal fiber volume.

A previous estimate of the distribution of Na$^+$ pumps between surface and tubular membrane (Venosa & Horowicz, 1981) based on fiber detubulation by osmotic shock with glycerol (Howell & Jenden, 1967) indicated that about one-fifth of them would be in the T-tubules. These results may have been affected by the increase in ouabain binding due to swelling. Detubulated fibers remain somewhat swollen after glycerol treatment (about 30% volume increase at the time of binding measurements). A re-estimation of the distribution of ouabain binding sites in the sarcolemma taking into account the swelling effect suggests that about one-half rather than one-fifth of the pumps may be located in the tubules (Venosa, 1990).

It is reasonably well established that stretch activates ionic channels. Stretch-activated channels first described in muscle fibers (Guharay & Sachs, 1984) increase their conductance, mainly to cations, in a variety of cells. It also has been proposed that hypotonicity increases Ca$^{2+}$ conductance of stretch-activated channels in cells of a choroid plexus epithelium as a result of stretch (Christensen, 1987).

It is, therefore, not unreasonable to postulate that in frog muscle fibers osmotic swelling is transduced, through membrane deformation, into both an increase in active Na$^+$ extrusion and an upregulation of the apparent number of pump sites. The quantitative relationship and mechanistic connection between these changes, however, remains to be shown.

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