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Red blood cells of guinea pig were used to explore the effects of temperatures above 37°C on membrane permeability to $Na^+$ and $K^+$ as part of an effort to evaluate the Energy Depletion Hypothesis of heat stroke. Cell [$Na^+$] and [$K^+$] does not change at 41°C and 45°C for up to 2 hours of incubation in simple saline medium. Na-K pump activity measured as ouabain-sensitive $K^+$ influx increases with warming, as do $Na^+$ influx and $K^+$ influx. The rise in the pump activity matches the rise in $Na^+$ influx quantitatively, accounting for the lack of change in [$Na^+$] cell. The rise in $Na^+$ influx is in the face of a decrease in activity of Na-H exchange transport capacity. The rise in $K^+$ influx consists largely of activation of a single, carrier-mediated path, K-Cl cotransport and corresponds to an apparent activation of this path in the reverse direction ($K^+$ efflux), leading to dumping of $K^+$ at elevated temperature. Na-K-Cl, a third passive carrier pathway, shows a temperature optimum between 30°C and 37°C. A new hypothesis proposes that these diverse responses to temperature change contribute to the maintenance of ion balance.

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TABLE OF CONTENTS

# INTRODUCTION

Heat stress and heat stroke are ancient hazards of soldiers that persist in modern warfare. Collapse from working in a hot environment may involve not only failure of specific systems but also conflict among normally physiological responses. Both aspects are rooted in activities at the cellular level; important, but relatively unexplored, among these activities are those related to the maintenance of ion gradients across plasma membranes.

Maintenance of ion gradients governs cell volume, transport of nonelectrolytes, excitability of nerve and muscle, and the ionic composition of the extracellular fluid. The maintenance of ion gradients is one of the major components of basal energy expenditure (and therefore heat production) in the body.

Ion transport across the plasma membrane has been connected to the issue of heat stroke in various ways, but prominent among these has been the "Energy Depletion Model" (Hubbard, 1990). This hypothesis proposes that, with warming, a steep increase in passive permeability to $Na^+$ occurs in plasma membranes, that this increase leads to a rise in the intracellular $Na^+$ concentration and that this rise in turn increases the activation of the Na-K pump (Na-K ATPase), leading to excess ADP production, greater metabolic rate, and more heat production.

This project has sought to test the Energy Depletion Model by determining the validity of some of its key assumptions: Is there a steep rise in $Na^+$ permeability with heating? Does cell $[Na^+]$ rise with heating? Does Na-K pumping increase with heating at a greater rate than it would without increased $Na^+$ entry? Does the Na-K pump keep pace with $Na^+$ entry?

To answer these and ensuing questions a simple cell model was chosen, the mammalian erythrocyte. (In particular, guinea pig erythrocytes were chosen because of the similarity of their complement of transport pathways to that of human red cells and because of earlier work on the temperature

sensitivity of their pathways.) Even in such a simple model the issue of balance between pump and leak of $Na^+$ and $K^+$ ions has been a complex one because of the discovery of a plethora of passive carrier pathways for these ions. The pathways present in guinea pig red cells and of concern in this project are summarized in Fig. 1 and the legend thereof.

The research program has been, first, to address directly the questions listed above, and then to pursue the issue of which specific pathways may be altered in their activity at elevated temperatures. It was recognized from the outset that in the intact organism, increased $Na^+$ permeation might be caused by organismic factors such as the release of cytokines that would not be present under our constrained *in vitro* conditions and to which red cells might not be responsive in any case. The special value of the red cell/in vitro model, however, is that it provides a simple, quantifiable base for establishing the direct effects of temperature on these transport systems, before moving on to more complex situations.

## METHODS AND MATERIALS

*Collection and handling of blood.* Cells are drawn by heart puncture into a heparinized syringe from guinea pigs that have been anesthetized with xylazine and ketamine. The animal is then killed while it is still under deep anesthesia by cutting the spinal cord and severing the carotid arteries. The procedures used in this study were approved by the Animal Use Committee of the University of Georgia.

The blood is then diluted with simple incubation medium (150 mM-NaCl, 5 mM-KCl, 10 mM-glucose, 5 mM-adenosine, MOPS buffer, 10 mM, pH 7.4 and 0.1 mM EDTA), centrifuged and the 'buffy coat' of white cells removed. The red cells are then washed twice more in the same way, resuspended in the same medium to a 'hematocrit' of about 10 per cent and held in ice until use.

*Isotopic unidirectional influxes.* For determination of $Na^+$ influx $^{22}Na$ (in the presence of 150 mM $Na^+$, cold carrier) and for $K^+$ influx $^{86}Rb$ (in the presence of 5 mM $K^+$ cold carrier) are used. For $Na^+$ influx, triplicate samples are taken after 5 min incubation and after 25 min incubation and the computation of influx is based on linear uptake of isotope between these two times. For $K^+$ influx, only one sample time, 20 min, is necessary.

After incubation, cells are washed 3-4 times in ice-cold isotonic, buffered Na-free medium (107 mM $MgCl_2$, 10 mM Tris) using short (15 sec, 13,000 g) spins in a microfuge to remove excess isotope. Cells are lysed in trichloracetic acid and the precipate removed by centrifugation; the supernatant is placed in scintillation cocktail (for $Na^+$ fluxes) or water (for $K^+$ fluxes) and its radioactivity counted in a liquid scintillation counter. Influx is based on specific activity determined from a suitable standard made from stock isotope and on cell volume computed from absorption of hemoglobin at 540 nM of the suspension related to measured hematocrit.

*Ouabain-sensitive $K^+$ influx.* Na-K pump activity has been estimated by determining the difference in $K^+$ influx in the presence and absence of

ouabain (100 uM).

Effects of extracellular $K^+$ have been determined with a constant concentration of $^{86}Rb$ isotope in the medium over a range of $K^+$ concentrations in the medium (0.1-15 mM), although at the higher concentrations of $K^+$ it was necessary to use higher isotopic Rb concentrations to provide adequate final radioactivity to count. Since changing $K^+$ concentrations might have resulted in altered competition between $K^+$ and the Rb isotope, trial determinations of Km for the extracellular ion were carried out using cold-carrier $Rb^+$ and cold-carrier $K^+$. The results (Table 1) were the same for the two procedures, so that at least for the Na-K pump, $^{86}Rb$ appeared to be a satisfactory congener of $K^+$. The same concern might arise for other pathways, however, if detailed kinetics become an issue.

*Inhibitors of $K^+$ and $Na^+$ influx.* As indicated in Fig. 1 and in the Results, the inhibitors, bumetanide and amiloride, were used to differentiate fluxes through the Na-K-Cl cotransport system and through the Na-H pathway, respectively. Unless otherwise stated the concentrations used for these inhibitors were 0.01-0.2 mM for bumetanide (too low to interfere with K-Cl cotransport) and 1 mM for amiloride.

*$K^+$ efflux.* Efflux of $K^+$ was measured by preloading cells with isotope for 1-2 hr hours at room temperature, washing 3-4 times to remove excess, recombining with or without inhibitors in the medium, parceling to microfuge tubes, sampling at timed intervals, centrifuging and drawing off the supernatant for counting. Rate coefficient is calculated using an "infinity" value based on total suspension activity

$$kt = \ln(1-(a_t-a_o)/(a_{inf}-a_o)),$$

where k=rate coefficient of efflux (or fractional turnover rate), $a_t$ = activity at sample time, t, $a_{inf}$ = total activity, $a_o$ = activity at initial time point (3-6 min after placing loaded cells in nonradioactive medium).

*Steady-state experiments*. Cells were incubated at 3-5 per cent hematocrit at 37°C, 41°C, and 45°C for up to 3 hours in the simple experimental medium described above, unless otherwise specified. After incubation they were washed in Na-free medium with $MgCl_2$ or choline chloride replacing the NaCl osmotically. The cells were then lysed in 0.1 % triton and their content of Na and K was determined.

*Analytical methods*. Concentrations of $Na^+$ and $K^+$ are measured by flame emission photometry. Osmotic concentrations of media are verified by vapor pressure osmometry.

## RESULTS

*Steady state ion concentrations at elevated temperatures*. Cells were incubated for 1, 2 and 3 hours at 37°C, 41°C and 45°C and their cell [$Na^+$] and cell [$K^+$] were determined by flame emission photometry. The results (Table 2) showed no significant changes from initial values within two hours. By three hours there was a statistically significant decrease in cell [$K^+$] at 41°C and 45°C and a rise in cell [$Na^+$] at 45°C compared with initial values, but there was no statistically significant change from their time-matched controls at 37°C .

*Na-K pump activity at elevated temperatures*. Ouabain-sensitive $K^+$ influx (with $^{86}Rb$ being used as a substitute isotope for K, as described in Methods) was used measured as the criterion of Na-K pump activity (Table 3). Cells were incubated both with and without 2 mM $Mg^{2+}$ in the medium because of concern that long-term incubations in $Mg^{2+}$-free medium might deplete this important cofactor of the Na-K pump. However, no difference was observed in pump activity related to the presence of $Mg^{2+}$ (Table 3), and only results based on incubation without $Mg^{2+}$ in the medium are used hereafter for comparisons.

In cells in which influx was determined directly after their introduction to high temperature, ouabain-sensitive $K^+$ influx rose 19 per

cent between 37°C and 41°C ($P<0.01$), but the further 4 per cent rise at 45°C was not statistically significant. In order to determine whether a greater increase related to temperature might occur after cells had been incubated at high temperature, cells were preincubated for 1 hour before the introduction of isotope to determine $K^+$ influx. This procedure had the overall effect of reducing $K^+$ influx at all temperatures. The rise between 37°C and 41°C was proportionately similar to that in nonpreincubated cells (19 per cent), but there was also a possibly statistically significant ($P<0.05$) further rise of 15 per cent at 45°C (Table 3).

A secondary objective was to determine whether any changes occurred in the affinity of the Na-K pump for its transported ligands in the temperature range of interest. To determine the affinity of the pump for extracellular $K^+$, ouabain-sensitive influx was measured over a range of concentrations, and a typical Michaelis-Menten relationship was observed (Fig. 2). The average value of Km determined in this manner among 4 similar experiments was $0.50 \pm 0.03$ mM at 37°C, $0.67 \pm 0.12$ at 41°C and $0.54 \pm 0.06$ at 45°C. None of the differences among the three temperatures investigated was statistically significant.

## *$Na^+$ influx*

*$Na^+$ influx vs. T in cells incubated without inhibitors.* Change in overall $Na^+$ permeability with temperature has been estimated in two ways, by determination of unidirectional $Na^+$ influx with isotopic $^{22}Na$ and by net change in cell $[Na^+]$ in the presence of ouabain to block Na-K pump activity.

When cells were incubated in the presence of 100 uM ouabain for 1 to 3 hours at 37°C, 41°C and 45°C, cell $[Na^+]$ rose progressively at all temperatures, but the rate was clearly greater at 45°C than at 37°C (Fig. 3). The uptake of $Na^+$ at 41°C was intermediate between that at 37°C and 45°C and appeared to be steepest during the third hour.

Na influx measured as unidirectional $Na^+$ influx with isotopic $Na^+$ revealed a moderately steep temperature sensitivity that became steeper at higher temperatures (Fig. 4).

*Amiloride-sensitive $Na^+$ influx*. There are several components of $Na^+$ influx as indicated in Fig. 1. Prominent among these is the entry through the Na-H exchange pathway. Previous studies had indicated that this pathway is either absent or represents a small fraction of influx at 37°C in cells that are not challenged by cytoplasmic acidity or cell shrinkage. (At temperatures below 37°C, however, it increases dramatically in untreated cells.) Accordingly, in order to observe effects of elevated temperature on this pathway, it was necessary to activate it by cell shrinkage (i.e., hypertonic incubation). An example of such an experiment in which sucrose in the medium was employed to increase the osmotic concentration is shown in Fig. 5 A. In this experiment amiloride-inhibited, hypertonically activated $Na^+$ influx declined with warming and virtually disappeared at 45°C.

Results with sucrose as the hypertonic agent, however, proved to be quite variable, and it was realized that sucrose probably had a secondary, countervailing effect of making cells more permeable to $Na^+$. Accordingly, we repeated the experiments using increasing concentrations of NaCl to create hypertonicity. There was also a problem with this approach, however, in that amiloride is a competitive inhibitor of $Na^+$, and increasing $Na^+$ concentration might diminish the relative amiloride inhibition. Therefore, for these experiments cells incubated in hypotonic medium with amiloride were used as the "baseline" control. The results, shown in Fig. 5 B and Table 4, indicate that the very large effect of hypertonicity on $Na^+$ entry is greatly inhibited by warming. Thus, the $Q_{10}$ of 2.2 for the minimal influx in 200 mOsM medium with amiloride is a normally high value, whereas in hyperosmotic medium the $Q_{10}$ was fractional (i.e., negative temperature effect) (Table 4).

(It should be noted that the 23 *fold* increase in $Na^+$ influx between 200 mOsM [about 100 mM $Na^+$] and 500 mOsM [about 250 mM $Na^+$] is far greater than could be accounted for by any merely kinetic activation caused by the 2.5 fold increase in $Na^+$ concentration. In fact this carrier pathway is known to be saturated at about 150 mM $Na^+$ [Zhao and Willis, 1963].)

### *$K^+$ influx*

*"Total passive" (ouabain-insensitive) $K^+$ influx.* When cells were incubated in the presence of 100 uM ouabain, to prevent $K^+$ influx through the Na-K pump, the remaining $K^+$ influx, representing all "passive" routes (channels and carriers) exhibited a steep dependence on temperature (Fig. 6). $K^+$ influx nearly doubled between 37°C and 45°C.

*Electrodiffusive ("Residual") $K^+$ influx.* Several attempts were made to estimate the dependence of fundamental permeability of the guinea pig red cell membranes on temperature (see Fig. 1). Since this truly "leak" pathway cannot be blocked by inhibitors, the chief criteria for determining it are (1) the minimal flux remaining (hence, "residual") when other known pathways have been blocked and (2) linear dependence on extracellular $K^+$ at concentrations above about 15 mM (at which level carrier mediated paths are saturated). Accordingly, our initial attempts to determine this path were based on influx in the presence of ouabain and bumetanide (a standard approach in earlier studies of our own and others) and on slope of the rising limb of the the curve of $K^+$ influx *vs.* $[K^+]_o$. These results were duly reported in the quarterly reports, but are not reported here, because in pursuing the further analysis of other pathways of $K^+$ influx, it was found that, contrary to our expectation, K-Cl cotransport is not "silent" in cells in isotonic medium even at 37°C, and that at higher temperatures this component rises steeply (see below). K-Cl cotransport is not blocked by bumetanide at sub-millimolar concentrations, so that its contribution had not been excluded in the earlier measurements.

Since K-Cl cotransport is activated by swelling and inhibited by shrinkage it was necessary to resort to the combination of hypertonic incubation with ouabain and bumetanide present to obtain a better measure of (hopefully) "truly" residual, electrodiffusive influx.

Measured in this way, the residual influx showed a very low temperature dependence (Fig. 7), barely doubling over the thirty degree range between 15°C and 45°C.

*Bumetanide-sensitive $K^+$ influx*. Na-K-Cl cotransport can reliably be estimated by the difference in $K^+$ influx in the presence and absence of a low concentration (e.g., 10-200 uM) bumetanide. Initial results over a narrow range of elevated temperatures suggested that this activity either remained the same or decreased with warming. When a larger series was carried out over a wider temperature range (Figs. 7 and 8) it appeared that there was a decrease in this activity above 37°C and below 28°C.

In other cell systems Na-K-Cl has been shown to be activated by cell shrinkage and to be involved in reswelling shrunken cells by downhill influx of $Na^+$ and coupled $Cl^-$ and $K^+$ entry. To determine whether the pathways is osmotically reactive in guinea pig red cells, two trial experiments were carried out to measure bumetanide-sensitive $K^+$ influx in these cells. As seen in Fig. 9, bumetanide-sensitive $K^+$ influx more than doubled in hypertonic media at 37°C, but it was unresponsive to shrinkage at 45°C and 20°C. When cells were incubated in hypertonic medium (450mOsM) over a range of temperature to maximize their Na-K-Cl cotransport, bumetanide-sensitive influx exhibited a virtual plateau between 37°C and 30°C and declined both at higher and at lower temperatures (Fig. 8).

*K-Cl cotransport*. As described above, an important involvement of temperature in regulation of K-Cl cotransport (and, therefore, potentially, in control of ion regulation and cell volume) was recognized when we attempted to estimate the component of $K^+$ influx through this pathway at elevated temperatures. By comparing influx in hypertonic, hypotonic and

isotonic media at 37°C, 41°C and 45°C (Fig. 10 A) we found a large increase in influx in isotonic medium with warming, but little or no increase in hypertonic medium (see also Fig. 7). As expected, $K^+$ influx in hypotonic medium was elevated at all temperatures but increased only relatively slightly with warming.

The K-Cl pathway is selectively and strongly activated by incubation with n-ethyl maleimide (NEM) at 37°C (Lauf *et al.*, 199), much more so than by hypotonic incubation. When we treated cells with NEM at higher temperatures, this maximal activity declined with warming. Thus, while the gap between maximal ouabain-insensitive $K^+$ influx (in NEM) and minimal ouabain-insensitive $K^+$ influx (in hypertonic medium with bumetanide) declined between 37°C and 45°C (Table 5), the influx in ouabain-insensitive influx in isotonic medium approached the maximum possible with full K-Cl activation in NEM (Table 5).

In order to establish whether the large increase in $K^+$ influx with warming in isotonic medium was entirely through K-Cl cotransport, it was necessary to determine whether the increase was dependent upon the presence of chloride as a cotransported ion. This was done by dividing cells into two suspensions, one with chloride ion and the other in which chloride was replaced with nitrate ion. Cells were preincubated in these media for 20 - 30 minutes and washed in the same medium before $K^+$ influx was measured in the corresponding media, with ouabain and bumetanide present as before.

The results (Fig. 10 B) showed that with no chloride in the medium, $K^+$ influx in hypotonic, hypertonic and isotonic media were very little different from each other and corresponded closely with the minimal influx in ordinary hypertonic medium containing chloride, bumetanide and ouabain. In short, not only is hypotonic activation of K-Cl cotransport blocked in chloride-free medium but thermal activation is blocked as well.

### *$K^+$ efflux*

The results above, showing apparently large activation of K-Cl cotransport with warming based on unidirectional $K^+$ influx raised the possibility for increased $K^+$ dumping at high temperatures, because the net balance of the K-Cl cotransport pathway is usually outwards under physiological circumstances.

As a first step toward investigating this possibility it was necessary to determine whether the increase seen in $K^+$ influx through this pathway is matched by a comparable change in $K^+$ efflux. Accordingly, cells were loaded with $^{86}Rb$ by preincubation with the isotope for one hour. Excess isotope was removed by rapid centrifugations and resuspension in initially isotope-free medium and then the efflux estimated by the appearance of radioactivity in the supernatant as described in Methods and in the legend of Fig. 11.

The results of one such experiment are shown in Fig. 11. Cells were incubated in hypotonic medium to activate K-Cl cotransport maximally and in hypertonic medium with bumetanide to achieve minimal $K^+$ permeability. The efflux in isotonic medium at 37°C corresponded to that in hypertonic medium (i.e., very slow); at 45°C the efflux in isotonic medium corresponded to the efflux in hypotonic medium (fast) and it was intermediate at 41°C. Replications of this experiment to obtain statistical comparisons are in progress.

## DISCUSSION

*Overview*. The results presented above and in the appendix confirm some expectations and contain some surprises. As anticipated in the proposal, $Na^+$ permeability, as expressed both by unidirectional $Na^+$ influx and by net gain in $Na^+$ in ouabain-inhibited cells, does increase steeply with warming. However, countervailing this increase, the tendency or availability of the Na-H exchange pathway to admit $Na^+$ appears to be stringently reduced by warming and $Na^+$ transport through the Na-K-Cl cotransport (inferred from $K^+$

bumetanide-sensitive $K^+$ influx) may be reduced as well.

Similarly, overall $K^+$ permeation also appears to be steeply temperature-dependent above 37°C, but, when analyzed, this increase appears *not* to be due to generalized leakiness, but rather to the turning on of a specific, regulated, carrier pathway. True "leakiness", if we may so describe the residual, non-inhabitable influx of $K^+$ (in hypertonic medium with ouabain and bumetanide) seems to have a remarkably *low* temperature dependence, contrary to our former conclusions (Hall and Willis, 1986). In short, for $K^+$ permeation with warming, one pathway increases only slightly ("residual, leak", pathway 2 in Fig. 1), one pathway declines (Na-K-Cl cotransport, pathway 5 in Fig. 1), and one pathway, K-Cl cotransport, increases spectacularly (pathway 4 in Fig. 1).

Notwithstanding the dramatic and diverse changes in passive permeation of $Na^+$ and $K^+$ with warming, the steady-state cell concentrations of $Na^+$ and $K^+$ are well maintained in this cell model at 41°C and 45°C for at least two hours under the minimal conditions of incubation provided to them. This observation returns us to the issue of the Energy Depletion Hypothesis, and it also raises a new question: whether the temperature-specific alteration in activity of pathways is contributing to the maintenance of ion balance and cell volume or is endangering them. These issues are discussed below.

*Whither the Energy Depletion Hypothesis?* Although the rise in Na-K pump activity with warming (Table 3) was quantitatively less than anticipated, the maintenance of $Na^+$ and $K^+$ gradients in cells incubated for long intervals suggests that the pump was keeping pace with the elevated $Na^+$ entry.

This point can be estimated quantitatively over short time intervals by comparing unidirectional $Na^+$ influx with pump-mediated $Na^+$ efflux. We have not yet measured the latter directly, but it can be inferred from ouabain-sensitive $K^+$ influx, if one assumes the pump maintains its standard

stoichiometry of 3 $Na^+$:2 $K^+$:1 ATP. The relevant data are summarized in Table 6, and they suggest that the increased pump efflux of $Na^+$ does match the increased $Na^+$ influx up to at least 41°C.

Thus, it appears that increased $Na^+$ entry is a component in driving the pump faster rate at 41°C than it would experience without the increased $Na^+$ entry, and, to this extent, the prediction of the Energy Depletion Hypothesis is borne out.

The question then arises how the pump manages to keep pace without a measurable rise in cytoplasmic [$Na^+$]. Conceivably, change to a new steady state may be too slow or too small to have been detected in these experiments. An alternative possibility is that the affinity of the pump is increased with warming, permitting an "anticipatory" increase in pump activity without elevated cytoplasmic [$Na^+$]. Clarification of this point is one of the objectives of the second half of the contracted funding period, along with checking of Na-K pump stoichiometry and dependence of ouabain-insensitive efflux on extracellular $Na^+$ (as a measure of exchange diffusion).

Finally, the small fractional rise in Na-K pump activity observed need not be regarded as a hindrance to the Energy Depletion Hypothesis, because this study was intended from the outset to establish baseline values and responses in a standard cell without complicating factors such as increased levels of cytokines, catechol amines and lactate. Such factors might be expected to play an exacerbating role in the intact exercising subject.

Of these factors, lactate may be the only one to which red cells might be assumed to be sensitive, and pursuit of the issue of the Energy Depletion Hypothesis with the confounding variables of humoral factors may require an alternative cell model with an appropriate complement of receptors (e.g., endothelial cells).

*Differential effects of temperature on passive permeation pathways.* The unexpected differential effects of temperature on three pathways of $K^+$

entry and three pathways of $Na^+$ entry, described in the Results and outlined above in the first paragraph of the Discussion, raise questions both as to mechanism and as to physiological relevance.

*Mechanism*. With regard to mechanism, it should be pointed out that three of the pathways involved, are known to be activated in other, analogous, systems by phosphorylation (Na-H exchange carrier, Na-K-Cl cotransporter) or dephosphorylation (K-Cl cotransport). It has been proposed that Na-H exchange and K-Cl cotransport, which are oppositely affected by cell shrinkage and swelling and by phosphorylation and dephosphorylation, are complementarily regulated through a single protein kinase substrate (Parker *et al.*, 1991). The possibly complementary effects of warming on these two pathways raise the same possibility for response to temperature as for response to anisosmotic challenge.

There can be little doubt that the steep rise in ouabain-insensitive $K^+$ permeation with warming observed in this study (Fig. 6) was due almost entirely to activation of K-Cl cotransport: (1) it was largely abolished by hypertonic incubation (with bumetanide present to prevent activation of the Na-K-Cl cotransporter); (2) it was dependent upon the presence of chloride in the medium (Fig. 10); and (3) there was a corresponding increase in hypertonically suppressed $K^+$ efflux (Fig. 11), though the last point requires further substantiation. Jennings and Al-Rohil (1990) proposed that the steep temperature dependence between 37°C and 25°C of this pathway in rabbit red cells was due to the change in the equilibrium for phosphorylation with temperature. They substantiated this point by showing that when the pathway was fully activated with NEM there was very little temperature sensitivity. We have extended this observation to higher temperatures and have shown that the putatively maximal activity induced by NEM actually *decreases* with warming (Table 5), so that in untreated cells flux through this pathway approaches the maximum attainable at 45°C.

If, as proposed by Parker *et al.* (1991), it is not the carrier itself, but a regulatory intermediate that is being phosphorylated, then it is possible to conceive of the concerted changes in Na-H exchange *capacity* (Fig. 5, Table 4) and K-Cl cotransport as arising from the temperature dependence of the equilibrium constant of one particular reaction. Insofar as Na-K-Cl cotransport appears to behave by yet another pattern (Fig. 8), it would not fit into such a simple scheme.

*Physiological relevance*. With regard to physiological significance, one immediate conclusion is that the ability of cells regulate cell pH would be impaired at higher temperatures if they relied primarily on the Na-H exchange mechanism. This possibility has already been raised for cells in culture (Kiang *et al.*, 1990). Since increased acidification is one of the complications of cells being stressed by working in a hot environment and since cell acidification has been raised as a possible contributing factor in cell leakiness (Gaffin and Hubbard, 1995), this is a potentially crucial observation.

Another issue is that increased K-Cl cotransport raises the possibility of increased dumping of $K^+$, leading to hyperkalemia. Here, the question is whether the increased $K^+$ efflux merely balances an increased $K^+$ influx through the activated Na-K pump at high temperature or whether it is in excess of that increase. This is a point that we intend to explore at an early date. In either case the outcome is important: If K-Cl cotransport is in excess of pump influx, then we may have isolated an important source of hyperkalemia, a potentially important cause of collapse in the heat. If the efflux balances the Na-K pump influx of $K^+$, then we may have identified an important cellular regulation for compensating for temperature change. The latter possibility is outlined below.

*Temperature compensation of ion regulation in mammalian cells: a new hypothesis*. In the view of most investigators the stoichiometry of the Na-K pump of 3 $Na^+$:2 $K^+$:1 ATP is invariant under normal physiological

conditions. If this is correct, then it follows that unless temperature change affects the permeability of $Na^+$ and $K^+$ to the same extent, the Na-K pump can only maintain a given steady state at a single temperature.

We have observed, however, that the steady-state concentrations of $Na^+$ and $K^+$ do not appear to change markedly as guinea pig red cells are warmed up. Furthermore we have found that the *fundamental* permeability for $Na^+$ and $K^+$ (i.e., that through pathway 2 in Fig. 1) show a very different dependence on temperature, that for $K^+$ has a very low dependence on temperature and that for $Na^+$ is steeply dependent on temperature (Figs. 3, 4 and 7).

Therefore, the changes in activity of regulated carrier pathways with warming and cooling may be compensatory and may have the result of keeping total passive fluxes of $Na^+$ and $K^+$ in balance with the Na-K pump and with each other. *With warming above 37°C*, as increased $Na^+$ leak activates the pump, active uptake of $K^+$ would exceed the passive loss of $K^+$; increased K-Cl cotransport prevents this imbalance. *With cooling below 37°C*, reduced Na-K pumping would fall behind passive loss of $K^+$; decreasing K-Cl cotransport (down to about 20°C) and increasing influx of $Na^+$ through the Na-H exchange pathway and activation of the pump, prevent this imbalance.

Different kinds of cells have different complements of carrier and channel pathways for $Na^+$ and $K^+$ that are regulated by phosphorylation or other cytoplasmic factors. If temperature compensation of ion balance over a range occurs, and if it is generalizable to other cells, the effective pathways of compensation may vary, but it may be the regulatory sensing and coupling mechanisms (such as the phosphorylatable regulatory intermediate, postulated by Parker *et al.* [1991] and mentioned above) that comprise the common theme.

## CONCLUSIONS

1. Guinea pig red cells maintain ion balance and, specifically, avoid increased $[Na^+]_{cell}$ for up to two hours at 41°C and 45°C.

2. This stability of ion gradients is in the face of steep rise in both passive $Na^+$ influx and passive $K^+$ influx.

3. Increased Na-K pump activity balances the rise in $Na^+$ influx.

4. Increased $Na^+$ influx with warming is probably through basic, non-carrier mediated "leak" (pathway 2, Fig. 1), because both the Na-H exchange pathway and Na-K-Cl cotransport appear to be inhibited at temperatures above 37°C.

5. The increased $K^+$ influx at elevated temperatures is largely through K-Cl cotransport - it is virtually abolished by hypertonicity (leading to cell shrinkage) and by removal of chloride from the medium.

6. Unresolved questions are:

   A. Does increased pump activity rely in part on increased affinity for cytoplasmic $[Na^+]$? (And is the 3 $Na^+$:2 $K^+$ stoichiometry of the pump the same at elevated temperature as at 37°C?)

   B. Is increased $K^+$ efflux at elevated temperature balanced by the Na-K pump uptake of $K^+$?

   C. Does diminished Na-H exchange capacity at higher temperatures lead to greater cytoplasmic acidity and does this contribute to leakiness for $Na^+$?

   D. Do the converse effects of warming on Na-H exchange and K-Cl cotransport represent coordinate control, and, if so, is it mediated by phosphorylation/dephosphorylation of the carriers or of an intermediate?

E. Does Na-K-Cl cotransport play a role in modulating ion balance/cell volume with changing temperature?

F. Does the opening of the Na-H pathway at reduced temperature elevate Na-K pump activity?

G. Are the complex of thermal responses observed in this study generalizable to other cell types?

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Table 1. Comparison of kinetics of $^{86}Rb$ influx with $K^+$ and $Rb^+$ as cold carrier.

| Cold carrier: | (N) | $K^+$ | $Rb^+$ |
|---|---|---|---|
| Vmax (mmol/l cells/h) | 5 | 4.7 ± 1.2 | 4.2 ± 1.3 |
| $K_{1/2}$ (mM K *or* RB) | 5 | 2.2 ± 0.4 | 1.8 ± 0.2 |

Cells were incubated 20 min in medium containing concentrations of K or Rb ranging from 0.5 mM to 20 mM, and $^{86}Rb$ uptake was determined. Kinetic constants were computed from Lineweaver-Burk analysis.

Table 2. Effect of high temperature on cell $[K^+]$ and cell $[Na^+]$ of guinea pig red blood cells.

| | | Cell $[K^+]$ (mM) | | |
|---|---|---|---|---|
| Time (hr) | (N) | 37°C | 41°C | 45°C |
| 0 | 12 | 83.7 ± 2.6 | -- | -- |
| 1 | 10 | 83.9 ± 4.1 | 81.1 ± 3.6 | 83.1 ± 2.6 |
| 2 | 11 | 80.6 ± 3.4 | 80.1 ± 3.5 | 77.7 ± 2.6 |
| 3 | 9 | 76.9 ± 4.9 | 79.7 ± 3.2* | 75.7 ± 2.6* |
| | | Cell $[Na^+]$ (mM) | | |
| Time (hr) | | | | |
| 0 | 11 | 6.5 ± 1.3 | | |
| 1 | 9-11 | 6.1 ± 0.3 | 6.2 ± 0.3 | 7.2 ± 6.0 |
| 2 | 11 | 6.6 ± 1.0 | 6.8 ± 1.0 | 7.0 ± 0.9 |
| 3 | 7-10 | 7.0 ± 1.4 | 7.3 ± 1.1 | 8.2 ± 1.5+ |

*Change from initial, $P<0.01$ by paired t-test

+Change from initial, $P<0.05$ by paired t-test

Table 3. Effect of mild elevation of temperature on Na-K pump.

| | Temperature (°C) | $K^+$ Influx (mmole/l/h) | |
|---|---|---|---|
| $[Mg^{2+}]$ in Medium (mM): | | 0 | 2 |
| A. No preincubation | 37 | 2.52 ± 0.32 | 2.58 ± 0.32 |
| | 41 | 2.89 ± 0.36* | 3.00 ± 0.35* |
| | 45 | 3.02 ± 0.37 | 2.94 ± 0.40 |
| B. One hour preincubation at each temperature | | | |
| | 37 | 1.56 ± 0.21 | 1.70 ± 0.21 |
| | 41 | 1.86 ± 0.20+ | 1.87 ± 0.25 |
| | 45 | 2.14 ± 0.27+ | 2.40 ± 0.24* |

Numbers represent means ± S.E. of 6 cases. *$P<0.01$ for comparison with next lower temperature; +$P<0.05$ for comparison with next lower temperature (paired t-test in both cases). "Na-K pump" = ouabain-sensitive $K^+$ influx (i.e., difference between total influx and flux remaining in presence of 0.1 mM ouabain).

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Table 4. Effect of elevated temperature on $Na^+$ influx in hypotonic, isotonic and hypertonic media.

| | $Na^+$ influx (mmoles/l cells/h) | | | | |
|---|---|---|---|---|---|
| Temp. | +200 mOsM | 300 mOsM | 400 mOsM | 450 mOsM | 500 mOsM |
| 37°C | 1.4 ± 0.2 | 2.3 ± 0.2 | 7.8 ± 1.0 | 17.7 ± 1.9 | 31.8 ± 2.7 |
| 41°C | 1.7 ± 0.1 | 2.4 ± 0.1 | 5.3 ± 0.6 | 11.3 ± 1.3 | 19.3 ± 0.8 |
| 45°C | 2.6 ± 0.4 | 3.3 ± 0.5 | 4.8 ± 0.6 | 6.4 ± 0.5 | 9.5 ± 0.6 |
| *$Q_{10}$: | 2.2 | 1.6 | 0.5 | 0.3 | 0.2 |

Same data as Fig. 5, but including results in 500 mOsM medium and showing $Q_{10}$.

*$Q_{10}$ values are based on comparison with influx at 37°C.

+Hypotonic medium also contained 2 mM amiloride to block residual Na-H exchange and any Na-Mg exchange.

Table 5. Relative activation of K-Cl cotransport by warming in isotonically incubated guinea pig red blood cells.

| T (°C) | $K^+$ Influx (mmoles/l cells/h) | | | |
|---|---|---|---|---|
| | A Hypertonic | B Isotonic+ NEM | C Isotonic | Relative Activation (C-A)/(B-A)x100 |
| 37°C | 0.16 ± 0.01 | 1.53 ± 0.09 | 0.20 ± 0.01 | **2.9** |
| 41°C | 0.15 ± 0.03 | 1.36 ± 0.04 | 0.36 ± 0.06 | **17.4** |
| 45°C | 0.20 ± 0.04 | 0.95 ± 0.04 | 0.66 ± 0.09 | **61.3** |
| 48°C | 0.24 ± 0.06 | 0.65 ± 0.04 | 0.56 ± 0.10 | **78.0** |

Minimum $K^+$ influx was obtained in hypertonic medium; K-Cl cotransport was maximally activated by preincubation with n-ethyl maleimide (NEM) at 37°C. All cells were incubated with 0.05 mM bumetanide and 0.1 mM ouabain. Cells treated with NEM were suspended with 5 mM NEM at 37°C for 15 min and were then centrifuged and resuspended in NEM-free medium and incubated for flux in parallel with untreated cells.

Means ± S.E. of 3 experiments are shown.

Table 6. Balance between Na-K pump and $Na^+$ influx.

| | Ion fluxes (mmole/l cells/h) | | |
|---|---|---|---|
| | 37°C | 41°C | 45°C |
| Measured ouabain-sensitive $K^+$ influx | 2.5 ± 0.3 | 2.9 ± 0.4 | 3.0 ± 0.4 |
| Computed Na-K pump efflux of $Na^+$ | 3.8 | 4.4 | 4.5 |
| Measured $Na^+$ influx | 3.6 ± 0.4 | 4.1 ± 0.3 | 4.9 ± 0.2 |

Data for $K^+$ influx and $Na^+$ influx are from separate experiments, represented in Table 3 and Fig. 4, respectively. Mean values for observations are based upon results from 6 or more individual guinea pigs. Computed value for pump efflux of $Na^+$ as based on assumed stoichiometry of 3 $Na^+$:2 $K^+$.

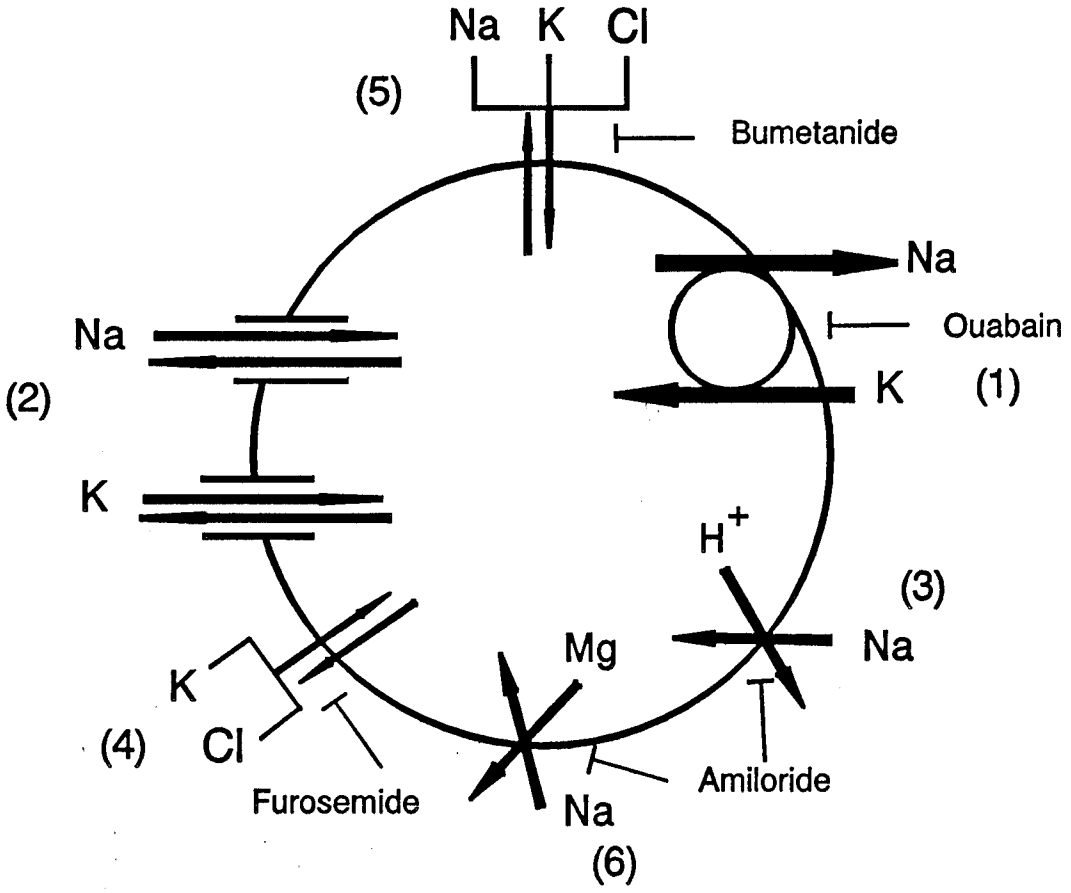



Fig. 1. Pathways for $Na^+$ and $K^+$ permeation under consideration in this study. Under normal physiological conditions the ouabain-insensitive Na-K pump (1) carries $Na^+$ out and $K^+$ into the cell. Each of the other pathways can transport $Na^+$ and/or $K^+$ in either direction, but diffusive leak for $Na^+$ (2) is predominantly inward; for $K^+$ (2) it is outward. The Na-H exchanger (3) primarily moves $Na^+$ into the cell in exchange for $H^+$ at 37°C. It is activated by shrinkage and cytoplasmic acidification. It is inhibited by amiloride. The K-Cl cotransporter (4) is activated by swelling and by oxidation; it is inhibited by 1 mM loop diuretic (furosemide, bumetanide). The Na-K-Cl cotransporter (5) is activated by cell shrinkage and inhibited by 0.01 mM bumetanide.

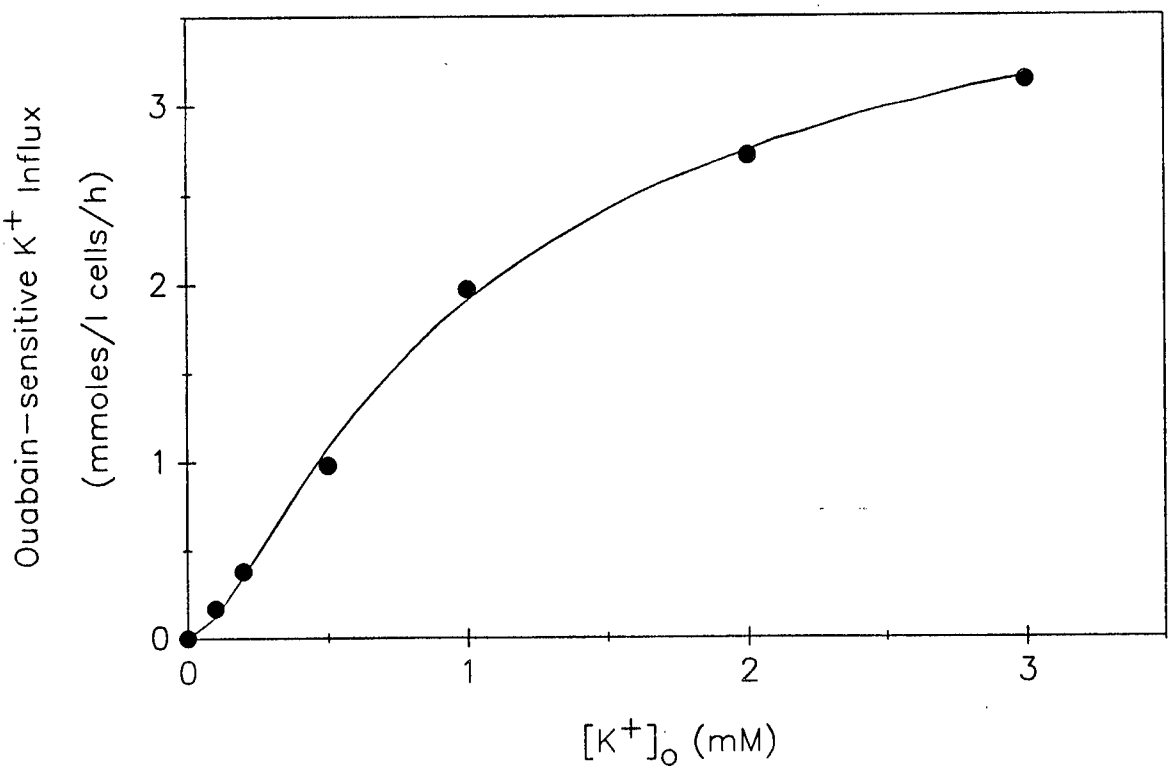



Fig. 2. Fine-scale kinetics of ouabain-sensitive $K^+$ influx. The points represent the difference between "total" and "ouabain" values shown in A. The line drawn is computed from the equation,

$$\text{ouabain-sensitive } K^+ \text{ influx} = Vmax/(1 + Km/[K^+]o)^2,$$

where Km represents the affinity constant of the Na-K pump for $K^+$ and Vmax represents the maximum velocity with saturating $[K]_o$ and the prevailing conditions in the red cell (i.e. for ATP and intracellular Na). (See Ellory and Willis, 1982). Results shown are for a single experiment, typical of several. Group results are presented in Table 2.

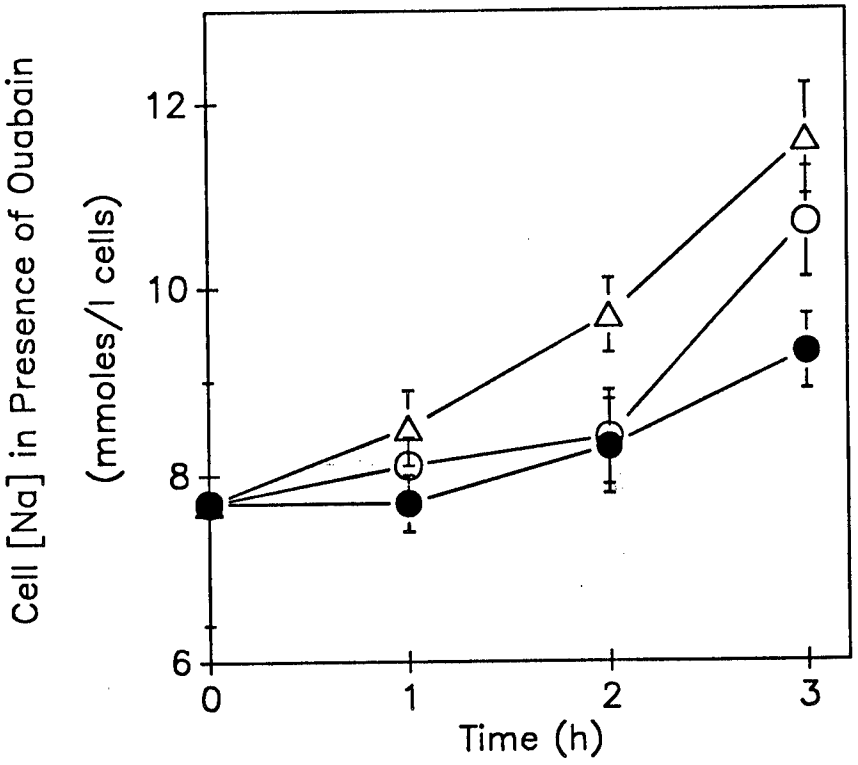



Fig. 3. Increase in cell [$Na^+$] of guinea pig red cells incubated with ouabain. Solid circles, 37°C, open circles, 41°C, triangles, 45°C. Means ± S.E. of 8-10 cases are shown.

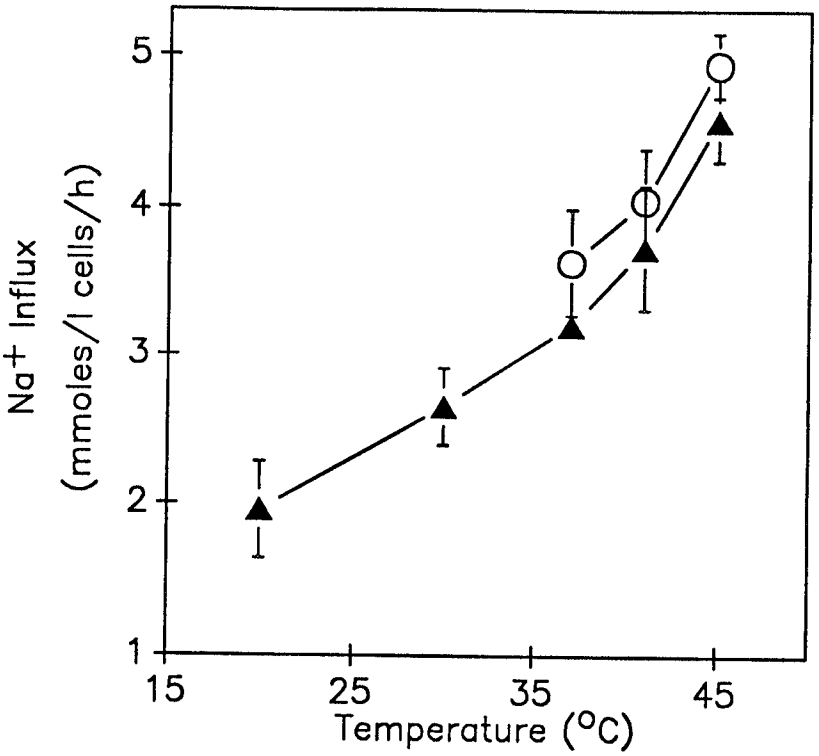



Fig. 4. Effect of temperature on $Na^+$ influx in guinea pig cells. Triangles, means ± S. E. of 3 experiments carried out over 20°C - 45°C. Open circles, means ± S.E. of 7 experiments carried out at 37°C, 41°C and 45°C (includes values of experiments represented as triangles).

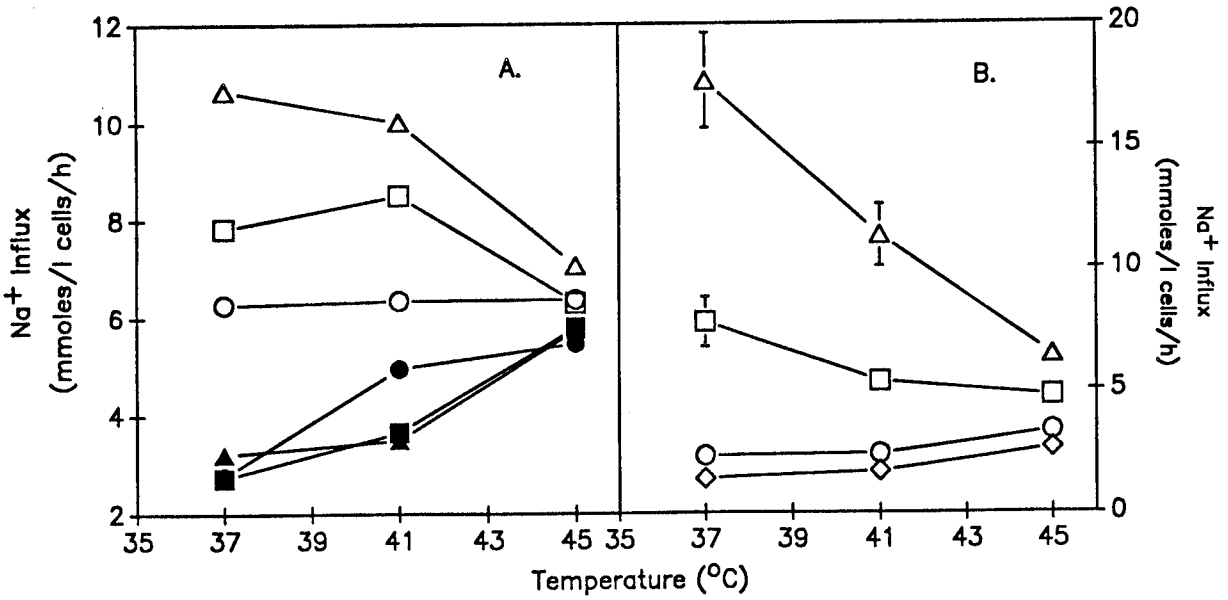



Fig. 5. Effect of higher temperature on shrinkage-activated $Na^+$ influx in guinea pig red cells.

A. Guinea pig red cells were incubated in media made hypertonic by the addition of sucrose: 410 mOsM, circles, 450 mOsM, triangles, and 510 mOsM, squares. Cells were also incubated with (closed symbols) or without (open symbols) 1 mM amiloride. Tonicity was increased by adding sucrose to isotonic medium. The value of 410 mOsM was chosen based on preliminary experiments that showed that this was the lowest osmolality at which an easily observable increase in $Na^+$ influx could be observed. Amiloride-insensitive $Na^+$ influx (closed symbols) increases with warming, but shrinkage-activated and amiloride-sensitive $Na^+$ influx (difference between control and amiloride-exposed cells) decreases with warming. Results represent means of triplicate determinations on red cells of a single animal and are representative of two similar experiments.

B. Guinea pig red cells were incubated in media made hypertonic with NaCl As a baseline control (i.e., Na-H exchange blocked) cells were incubated in 200 mOsM medium containing 2 mM amiloride (diamonds), as well as in isotonic medium (circles). Squares, 400 mOsM; triangles, 450 mOsM. Means $\pm$ S.E. of 3 experiments on separate individual guinea pigs are shown and are the same data as presented in Table 4. It should be noted that $Na^+$ influx at 37°C in 500 mOsM was too high to show on same scale, but values are given in Table 4.

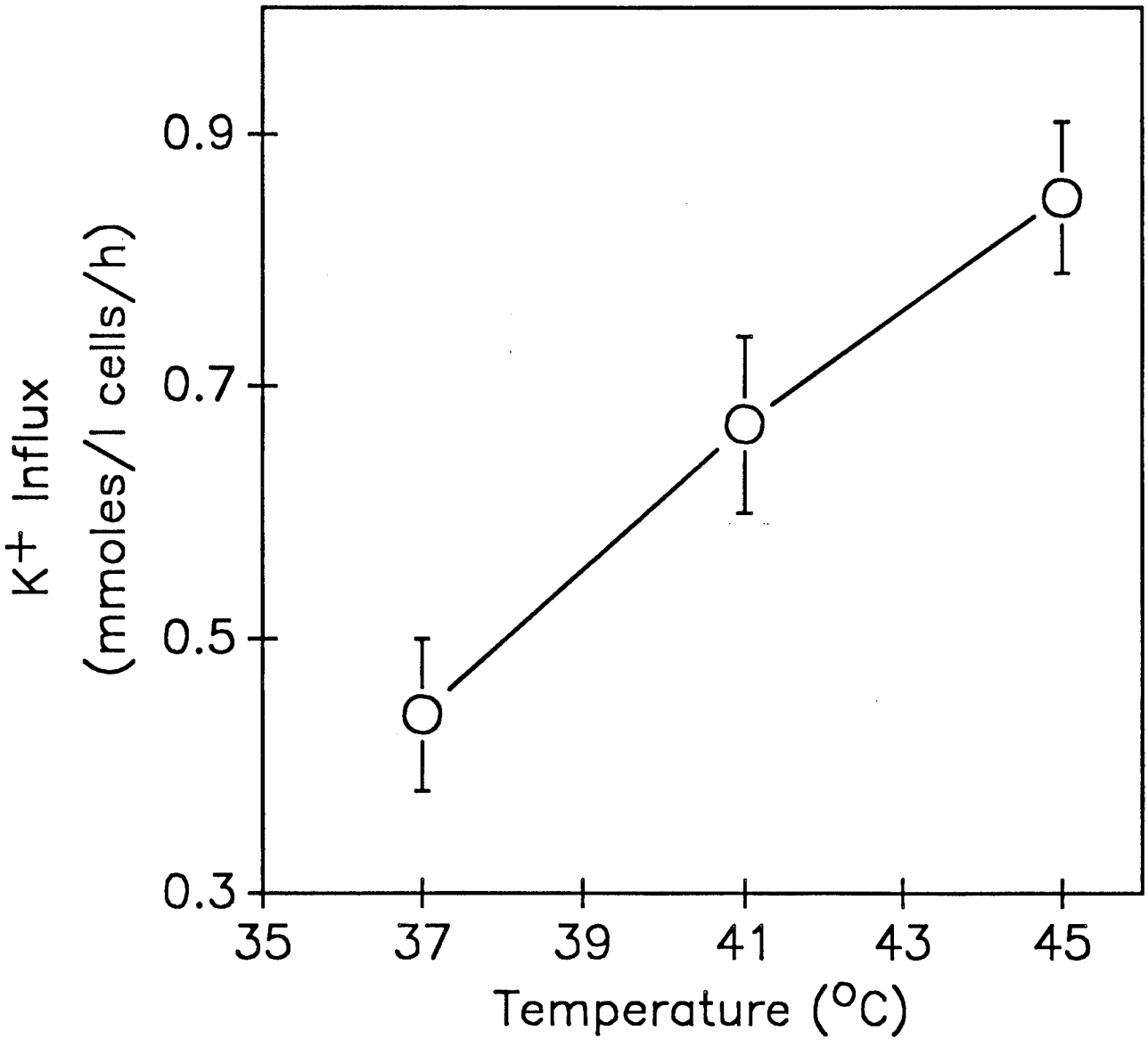



Fig. 6. Effect of temperature on ouabain-insensitive $K^+$ influx in guinea pig red cells. Means ± S.E. of 6 cases shown.

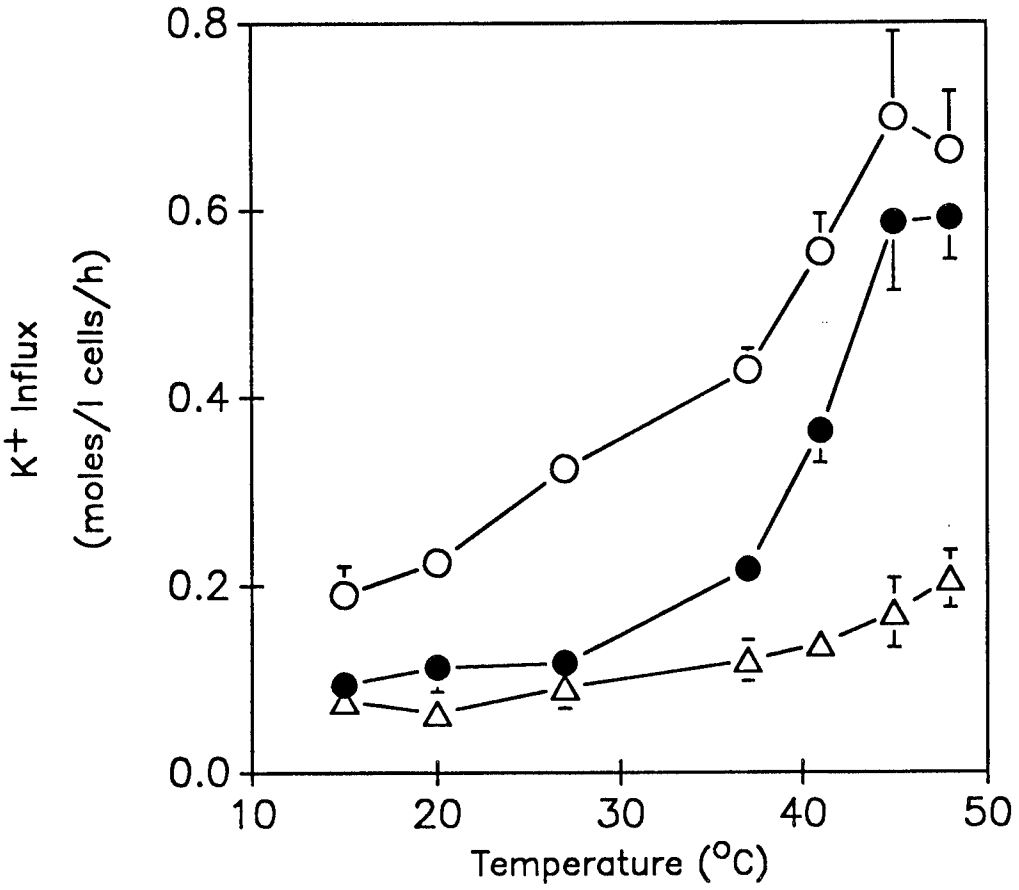



Fig. 7. Effect of temperature on three components of ouabain-insensitive $K^+$ influx in guinea pig red cells. Guinea pig red cells were incubated at various temperatures between 15°C and 48°C with ouabain (0.1 mM) and with or without bumetanide (0.05 mM) and in isotonic (300 mOsM) or hypertonic (470 mOsM) medium. (Medium was made hypertonic by addition of sucrose.) $K^+$ influx was measured using $^{86}Rb$ in the presence of 5 mM $K^+$. Means ± S.E. of 6 or more cases are shown. Triangles, $K^+$ influx in cells incubated in hypertonic medium in the presence of bumetanide and ouabain; this component represents the newly defined minimal, "residual" influx. Solid circles, $K^+$ influx in cells incubated in isotonic medium with ouabain and bumetanide. The difference between this curve and that of cells incubated in hypertonic medium (triangles) presumably represents K-Cl cotransport (see Fig. 10). Open circles, $K^+$ influx in cells incubated in isotonic medium with ouabain alone. This curve represents "total passive $K^+$ influx" (see also Fig. 6); the difference between this curve and that with ouabain and bumetanide (solid circles), presumably represents Na-K-Cl cotransport (see Fig. 8).

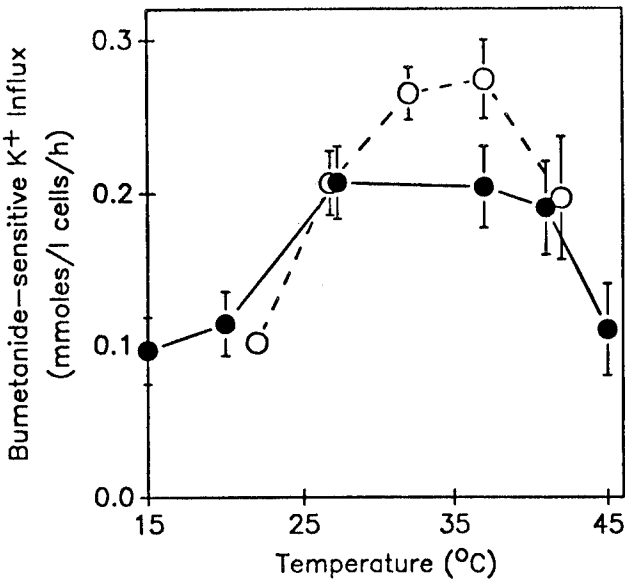



Fig. 8. Effect of temperature on Na-K-Cl cotransport in guinea pig red cells. Solid circles, difference between $K^+$ influx in cells incubated in isotonic medium with ouabain and with or without bumetanide (i.e., data computed from same results as in Fig. 7, difference between open circles and closed circles). Point represent means ± S.E. of 6 or more experiments. Open circles: cells were incubated in hypertonic medium (450 mOsM), in order to fully activate Na-K-Cl cotransport (see Fig. 9). The points represent means ± S.E. where larger than symbols) of four experiments.

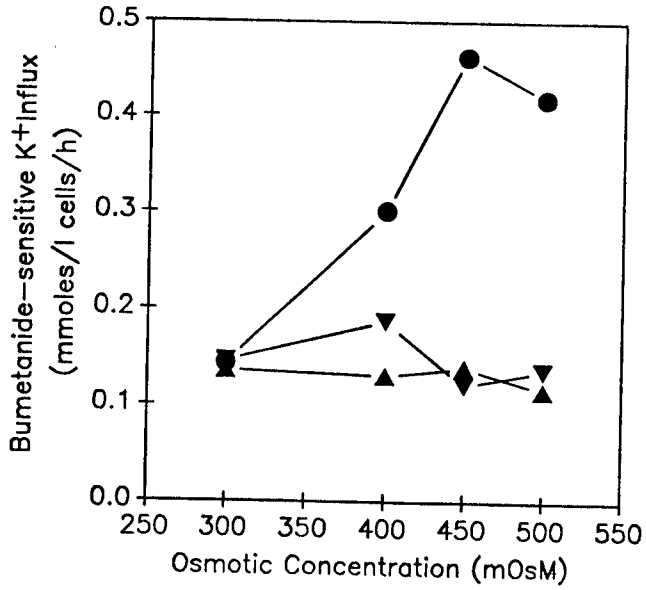



Fig. 9. Activation of Na-K-Cl cotransport in guinea pig red cells by hypertonicity. Points represent the results of a single experiment, representative of two. Circles, 37°C; triangles, 20°C; inverted triangles, 45°C. Cells were incubated with ouabain and with or without bumetanide. The difference is plotted as bumetanide-sensitive $K^+$ influx (ice, Na-K-Cl cotransport).

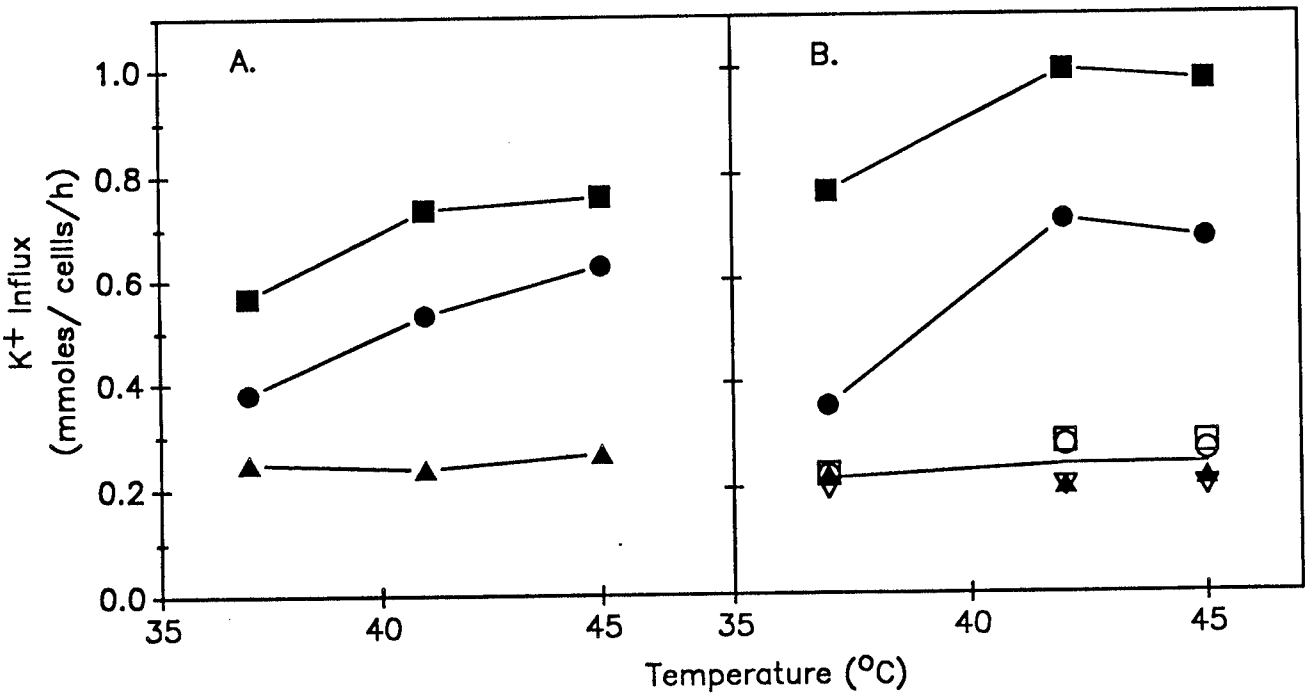


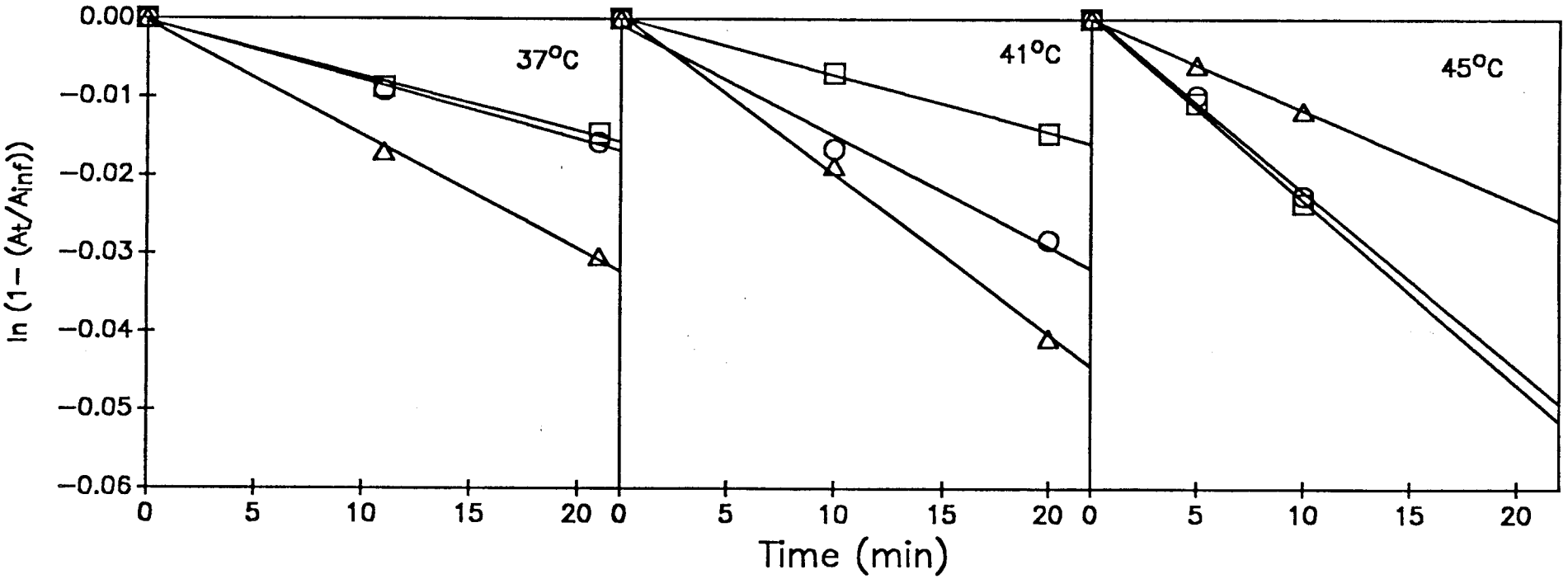

Fig. 10. Effect of warming and anisosmotic incubation on ouabain-and-bumetanide insensitive $K^+$ influx.

A. Cells were incubated at three temperatures in media with three widely different osmotic concentrations. Circles, hypo-osmotic medium (200 mOsM); triangles, isotonic medium (300 mOsM); squares, hypertonic medium (510 mOsM). Hypotonicity was achieved by mixing isotonic medium with NaCl-free medium. Hypertonicty was achieved by adding sucrose to isotonic medium. All media contained 0.1 mM ouabain and 0.2 mM bumetanide.

B. $K^+$ influx in guinea pig red cells in the presence and absence of chloride. Results are for triplicate samples for a single experiment representative of four similar experiments. All cells were incubated with 100 uM ouabain and 10 uM bumetanide. Squares, hypotonic medium (220 mOsM); circles, isotonic medium (310 mOsM); triangles, medium made hypertonic (470 mOsM) by the addition of sucrose; closed symbols, medium with chloride; open symbols, media with nitrate replacing chloride.

Fig. 11. $K^+$ efflux in guinea pig red cells: effect of tonicity and temperature. Results are for a single experiment (duplicate samples for each point) representative of three similar experiments. Symbols: squares, hypotonic medium; circles, isotonic medium; triangles, hypertonic medium containing 10 uM bumetanide. Cells were loaded for 1 h in medium containing $^{86}Rb$ as three separate suspensions; half-way through this period water or sucrose was added to two of the suspensions to make them hypertonic or isotonic. The suspensions were centrifuged and washed three times in non-radioactive media of the corresponding osmolarity. They were then incubated in the same three media and the supernatants were sampled at the indicated intervals.

The term, $(1-(A_t/A_{inf})$, on the ordinate represents the fractional loss of isotope ($A_t$= activity in suspension at time, t; $A_{inf}$=total radioactivity in system). The slope of the regression lines shown (i.e., $\ln(1-(A_t/A_{inf}))$ *vs.* time) is "k", the rate coefficient of efflux. It represents the fraction of turnover per unit time and if multiplied by the $K^+$ concentration of the cell would give *unidirectional* $K^+$ efflux. The three panels show that in this experiment $K^+$ efflux in isotopic medium at $37^oC$ is close to the minimal rate in hypertonic cells with Na-K-Cl cotransport blocked by bumetanide, whereas $K^+$ efflux from isotonic cells at $45^oC$ approaches the maximum in hypotonic cells with activated K-Cl cotransport.


0.00
−0.01
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ln (1− (At/Ainf))
37°C
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5
10
15
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Time (min)