

Kinetics of the Sodium Pump in Red Cells of Different Temperature Sensitivity

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ABSTRACT Ouabain-sensitive K influx into ground squirrel and guinea pig red cells was measured at 5 and 37°C as a function of external K and internal Na. In both species the external K affinity increases on cooling, being three- and fivefold higher in guinea pig and ground squirrel, respectively, at 5 than at 37°C. Internal Na affinity also increased on cooling, by about the same extent. The effect of internal Na on ouabain-sensitive K influx in guinea pig cells fits a cubic Michaelis-Menten-type equation, but in ground squirrel cells this was true only at high $[Na]_i$. There was still significant ouabain-sensitive K influx at low $[Na]_i$. Ouabain-binding experiments indicated around 800 sites/cell for guinea pig and Columbian ground squirrel erythrocytes, and 280 sites/cell for thirteen-lined ground squirrel cells. There was no significant difference in ouabain bound per cell at 37 and 5°C. Calculated turnover numbers for Columbian and thirteen-lined ground squirrel and guinea pig red cell sodium pumps at 37°C were about equal, being 77–100 and 100–129 s^{-1} , respectively. At 5°C red cells from ground squirrels performed significantly better, the turnover numbers being 1.0–2.3 s^{-1} compared with 0.42–0.47 s^{-1} for erythrocytes of guinea pig. The results do not accord with a hypothesis that cold-sensitive Na pumps are blocked in one predominant form.

INTRODUCTION

Although the sodium pump has been the subject of extensive investigation, surprisingly little information is available on the effects of temperature on its kinetics. Most temperature studies have ignored the possibility of changes in kinetic parameters, and have concentrated on measuring total activity, presumably in the hope that activity is maximal, or that substrate affinities do not change with temperature. Evidence from other membrane transport systems (e.g., the glucose carrier: Sen and Widas, 1972; Levine and Stein, 1966; amino acid transport; Hoare, 1972), and membrane-bound (Silvius et al., 1978) and soluble enzymes (e.g., Hochachka and Somero, 1973) indicates that affinities can change markedly with temperature.

Recently we have reported (Willis et al., 1980) that the sodium pump in red cells from several species of hibernating mammals differs from that in several species of nonhibernator in showing a lower temperature sensitivity.

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Thus, if the sodium pump activity, measured as ouabain-sensitive K influx, at 5°C is compared with that at 37°C, most species' erythrocytes show a sodium pump activity ratio of 0.2–0.8%, whereas cells from hibernators show a ratio of 2–6%. This difference offers the possibility of examining the inhibition of overall pump activity at low temperature by contrasting the effects of cooling in the two types of cells. The greater relative decline in activity of ordinarily cold-sensitive mammalian cells may result from several factors. One possibility is that the kinetics of interaction of the pump with ligands (Na, K, or ATP) are altered such that substrate binding is diminished or else is increased less than in the cold-tolerant forms. A special case of this explanation would be a fundamental change in the overall pump reaction so that the actual translocation mechanism is affected. Alternatively, the number of pump molecules participating in transport could change with temperature, perhaps increasing with cooling in the hibernator, or falling in the nonhibernator. Finally, the simplest case would be a true difference in the enzymatic turnover of the pump at low temperature as could occur from a greater increase in constraint from the microenvironment in cold-sensitive cells.

In the present work, therefore, we have investigated the effects of temperature on several properties of the sodium pump by comparing red cells of high temperature sensitivity with those of lower sensitivity. The cells with high temperature sensitivity were those of guinea pig, a nonhibernating rodent, whereas less temperature-sensitive cells come from two species of ground squirrel that are capable of hibernation. The principal pump properties examined were internal Na affinity, external K affinity, and number of ouabain binding sites.

The results indicate that there are significant effects of temperature on both K_m and V_{max} for Na:K exchange through the sodium pump. Ouabain binding experiments confirmed that the number of binding sites did not change with temperature, which shows that there is a significant difference in pump turnover number between hibernator and nonhibernator species.

MATERIALS AND METHODS

Animals

Guinea pigs were purchased from animal suppliers; thirteen-lined ground squirrels were captured in Urbana, IL; Columbian ground squirrels were trapped near Edmonton, Alberta. Animals were maintained in the laboratory on rat chow for 3–30 wk before use.

Blood

Animals were bled by cardiac puncture after being anesthetized with nembutal or halothane. Samples were taken into sodium heparin, and the cells were washed three times by centrifugation in 30 vol of a solution containing: 145 mM NaCl, 5 mM KCl, 5 mM glucose, 15 mM imidazole, pH 7.4, at 25°C.

Fresh guinea pig and thirteen-lined ground squirrel red cells had internal Na concentrations of 10.2 ± 2.1 ($n = 6$) and 13.9 ± 2.8 ($n = 5$) mmol/liter cells, and K concentrations of 91 ± 6 ($n = 6$) and 87 ± 5 ($n = 5$) mmol/liter cells, respectively. In most experiments, internal Na and K concentrations were adjusted by nystatin treatment, so that Na levels were either saturating or constant (see below).

Nystatin Loading

Preliminary experiments established the nystatin concentration necessary for loading the cells with Na plus K plus choline (or tetramethylammonium [TMA]) mixtures. The optimal concentration varied between 20 and 50 $\mu\text{g/ml}$ nystatin, depending on the species and the batch of nystatin used. A concentrated (5 mg/ml) solution of nystatin in methanol was diluted into the loading solution immediately before use. Packed, choline-washed red cells were added to 40 vol of loading solution and incubated at 20°C for 60 min. The loading solution usually had the composition (Na plus K plus choline or TMA) 150 mM Cl, 0.5 mM K_2PO_4 , 0.4 mM inosine, 0.4 mM adenosine, 5 mM glucose, 10 mM imidazole or Hepes, pH 7.4, at 20°C, with sucrose from 28 to 40 mM, depending on the species. After loading, the cells were washed five times by centrifugation (2,800 g, 5 min) in the loading medium and then twice in the choline medium.

Measurement of Influx

K influx as a function of external K was measured at 2% hematocrit in a medium consisting of 145 mM NaCl, 0.01–10 mM KCl (containing ^{42}K), 5 mM glucose, 10 mM imidazole, adjusted to pH 7.4 at each incubation temperature. Ouabain was present in some tubes at a final concentration of 0.1 mM. Cells to be incubated at low temperatures were pre-exposed to ouabain at 37°C for 15 min.

At time zero and at various times from 10 to 300 min the tubes were centrifuged in an Eppendorf 320B microfuge (Eppendorf Gerätebau Netheler & Hinz GmbH, Hamburg, Federal Republic of Germany) (15 s) and aliquots of supernatant were taken to measure the external K concentration. The cells were washed four times in a medium containing 107 mM MgCl_2 , 10 mM Tris, pH 7.5, at 4°C. The final pellet was lysed with 1 ml of Triton X-100 (0.1%) and a 0.1-ml aliquot diluted for hemoglobin OD_{540} measurement. The remaining 0.9 ml was deproteinized with trichloroacetic acid and processed for Cerenkov counting in a β -scintillation spectrometer. 1 wk later the samples were diluted with 2 vol of concentrated LiNO_3 medium (1,000 ppm) and analyzed for Na and K by flame photometry. In some experiments aliquots were taken immediately after deproteinization and analyzed for phosphate by the method of Weil-Malherbe and Green (1951).

When K influx was measured as a function of $[\text{Na}]_i$, the cells were “buffered” against changes in $[\text{Na}]_i$ by varying the composition of the external solution between 5 and 40 mM Na, with choline replacement. The external K was constant at 7.5 mM, ^{42}K being 1–2 $\mu\text{Ci/ml}$.

K Efflux

Cells were loaded with ^{42}K by incubation for 2–6 h at 37°C, sometimes after nystatin loading. Six rapid washes in choline medium using the microcentrifuge were carried out in 10 min, and the cells were resuspended in the appropriate medium at 1% hematocrit. After the appropriate additions (ouabain, K), the samples were incubated at the reaction temperature, when 1-ml aliquots were taken at 5-min intervals and centrifuged, and the supernatant was assayed for ^{42}K by Cerenkov counting. Samples for cell Na and K and hemoglobin were taken at the end of the experiment. Data were expressed as rate constant from the ratio of the fractional loss of isotope with time to the initial intracellular isotope concentration.

Ouabain Binding

Ouabain binding was determined by one of two methods. In the first case the procedure was to expose the cells to 10^{-8} M ouabain (containing ^3H -ouabain) for

varying periods (10–360 min), followed by rapid washing and determination of bound ^3H and of fractional pump inhibition by subsequently measuring ^{42}K influx by re-incubating the washed cells in ^{42}K media. This technique (“washing method”) was difficult to apply to guinea pig erythrocytes because ouabain binding is very reversible at every temperature. In some experiments, therefore, cells were spun through a dibutylphthalate (DBT) layer instead of being repeatedly washed (e.g. see Ferreira and Lew, 1977). The DBT method yielded the same number of ouabain binding sites as the washing method. With the washing method it was impossible subsequently to measure fractional inhibition of K influx, because the ouabain binding reversed during the second incubation. We therefore determined ouabain binding and ^{42}K influx simultaneously over several time intervals (0, 1, 2, and 3 h at 5°C ; 15, 30, and 45 min at 37°C) using high ^3H -ouabain concentrations (0.1–6 μM) and K concentrations (0.1–0.5 mM at 5°C ; 1–6 mM at 37°C) to give significant displacement, thereby establishing equilibrium binding levels in the range 15–75% inhibition. After lysis and trichloroacetic acid precipitation of protein, ^{42}K was counted by Cerenkov radiation in a 1-ml aqueous sample. 1 wk later the samples were transferred to counting vials and assayed for ^3H by β -scintillation spectrometry after the addition of 15 ml of a toluene/Triton X-100-based scintillation cocktail.

Materials

All reagents were purchased from the Sigma Chemical Company, St. Louis, MO, except ATP, which was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Choline chloride and tetramethylammonium chloride were recrystallized from hot ethanol. ^{24}Na and ^{42}K were made in the University of Illinois reactor; ^3H -ouabain was purchased from Amersham Searle Corp., Arlington Heights, IL.

Abbreviations and Terms

The terms app K_m and app V_{\max} refer to usual Michaelis-Menten-type kinetic constants. $[\text{K}]_o$, $[\text{K}]_i$, $[\text{Na}]_o$, and $[\text{Na}]_i$ mean external and internal concentrations per liter cell water.

RESULTS

External K Affinity

With red cells loaded to a constant high internal Na (nominally 100 mmol/liter cell water), the external K affinity in high Na medium was determined by measuring ouabain-sensitive K influx in guinea pig and ground squirrel red cells at various temperatures. Figs. 1A and B give the ouabain-sensitive K influx in one experiment on ground squirrel red cells at 5, 15, 30, and 37°C . The data follow sigmoid curves, and are fitted well by a modified Hill expression of the form

$$V = \frac{V_{\max}}{\left(1 + \frac{K_m}{[\text{K}]}\right)^2},$$

consistent with the existence of two equivalent external K sites (Sachs and Welt, 1967). As the temperature decreases from 37 to 5°C there is an *increase* in K affinity and a decrease in V_{\max} . Data with fitted curves for guinea pig erythrocytes are illustrated in Figs. 1C and D, where similar effects are seen.

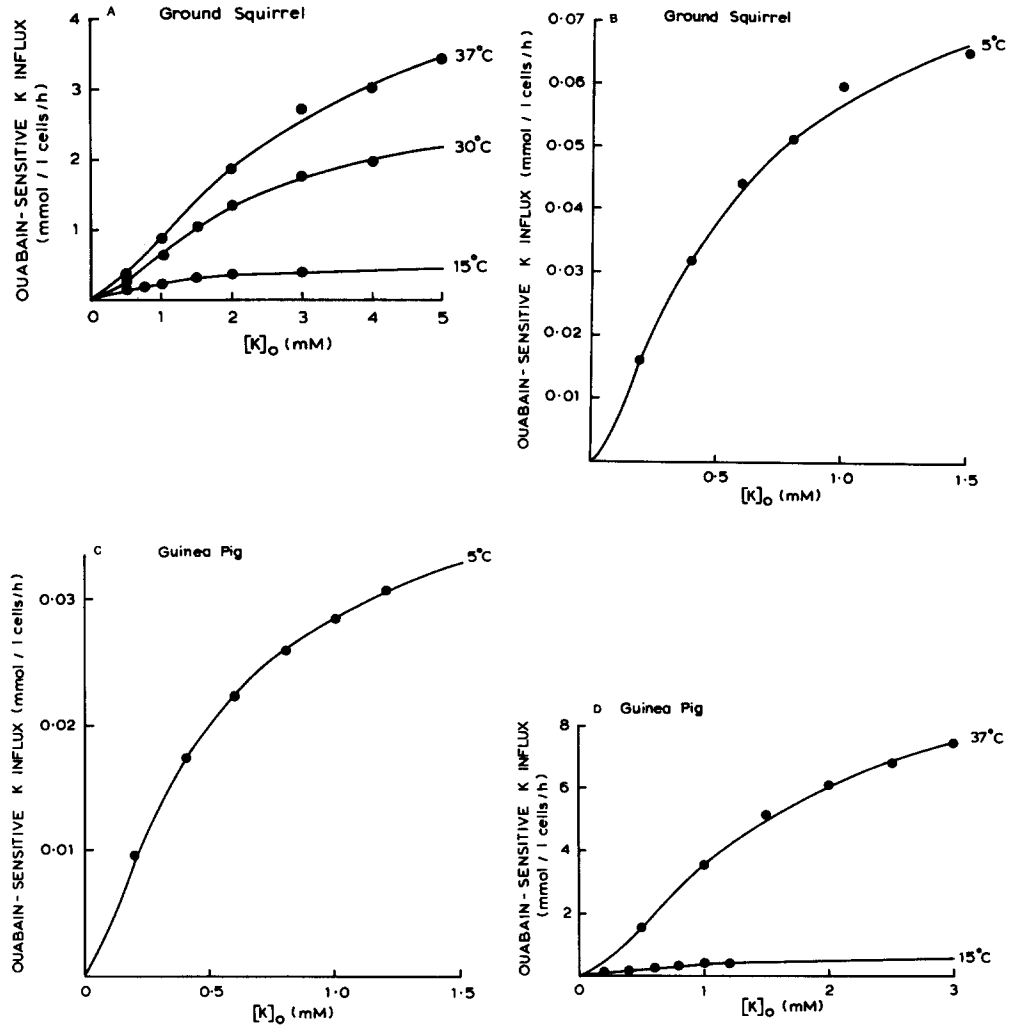


FIGURE 1. Temperature dependence of the external K affinity of the sodium pump in thirteen-lined ground squirrels (A and B) and guinea pig (C and D) erythrocytes.

Curves fitted as follows. Ground squirrel 5, 15, 30, and 37°C:

$$v = \frac{0.0939}{\left(1 + \frac{0.283}{K}\right)^2} + \frac{0.534}{\left(1 + \frac{0.508}{K}\right)^2} + \frac{3.42}{\left(1 + \frac{1.26}{K}\right)^2} + \frac{5.82}{\left(1 + \frac{1.53}{K}\right)^2}$$

Guinea pig at 5, 15, and 37°C:

$$v = \frac{0.0441}{\left(1 + \frac{0.241}{K}\right)^2} + \frac{0.667}{\left(1 + \frac{0.41}{K}\right)^2} + \frac{12.42}{\left(1 + \frac{0.88}{K}\right)^3}$$

Table I summarizes the app K_m and V_{max} data for several experiments on the external K affinity. In both species the app $K_m[K]_0$ at each of two sites is 0.3 mM at 5°C (corresponding to a $K_{1/2}$ of 0.75 mM). Increasing the temperature reduced the apparent affinity until at 37°C it was fivefold lower in the thirteen-lined ground squirrel, and 3.5-fold lower in the guinea pig. At the plasma K level of 6–10 mM in the ground squirrel (Pengelly and Kelly, 1967) and 6–7 mM in the guinea pig (Senturia et al., 1972), the pump would be 60–80% saturated by external K at 37°C and 90–96% saturated at 5°C.

Effects of Internal Na

To measure Na pump activity at constant, low intracellular Na concentrations, it was necessary to adjust Na influx to compensate Na loss during the incubation period. The most effective way to do this was an empirical adjustment of external Na (from 5 to 40 mM) so that the inward Na leak about balanced the outward sodium movement. Internal Na concentration ($[Na]_i$) was measured for every flux condition before and after the incubation period, with the value for $[Na]_i$ used in plotting the data representing the

TABLE I
 K_m AND V_{max} VALUES FOR K INFLUX IN GROUND SQUIRREL
AND GUINEA PIG RED CELLS AT VARIOUS TEMPERATURES

Temperature	Ground squirrel		Guinea pig	
	K_m	V_{max}	K_m	V_{max}
°C				
37	1.59±0.03	2.74±1.0	0.93±0.02	11.3±0.5
30	1.46±0.10	2.54±0.5	—	—
15	0.59±0.06	0.40±0.07	0.38±0.06	0.52±0.17
5	0.31±0.01	0.067±0.011	0.29±0.03	0.040±0.004

Data are fitted as $v = \frac{V_{max}}{\left(1 + \frac{K_m}{K}\right)^2}$. Results are for triplicate experiments on four animals.

mean and range (i.e., initial to final $[Na]_i$) measured for those cells. Intracellular Na in ouabain-treated cells tended to be higher than in the controls, but no significant effect of intracellular Na on ouabain-insensitive Na fluxes was found over the range of $[Na]_i$ in these experiments.

The effect of lowering the incubation temperature on the activation of ouabain-sensitive K influx by intracellular Na in guinea red cells is demonstrated by comparing Figs. 2A and B. As was the case with the external K affinity, there is an increased affinity for $[Na]_i$ at lower temperatures. The data were fit by a cubic Michaelis-Menten expression

$$V = \frac{V_{max}}{\left(1 + \frac{K_m}{[Na]}\right)^3}$$

as previously described for human erythrocytes (Garay and Garrahan, 1973).

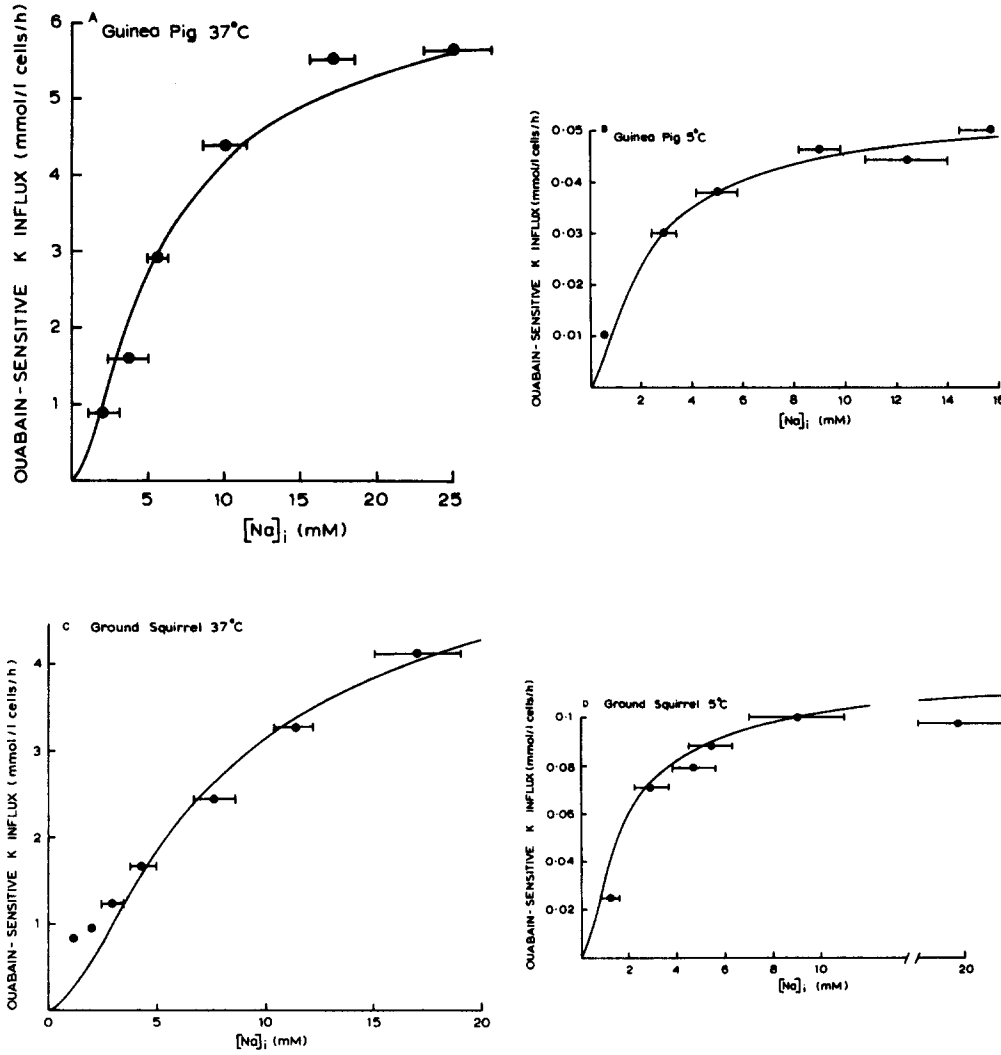


FIGURE 2. Temperature dependence of the internal Na affinity of the sodium pump in guinea pig (A and B) and thirteen-lined ground squirrels (C and D). Fitted curves at 5 and 37°C for guinea pig are

$$v = \frac{0.055}{\left(1 + \frac{0.65}{Na}\right)^3}; \frac{6.9}{\left(1 + \frac{1.82}{Na}\right)^3}$$

At 5 and 37°C for ground squirrel they are

$$\frac{0.117}{\left(1 + \frac{0.5}{Na}\right)^3}; \frac{6.0}{\left(1 + \frac{2.4}{Na}\right)^3}$$

In all these experiments $[K]_i$ is fixed at ~ 90 mmol/liter cells (range 84–96), with choline or TMA substitution making up the difference. Uptake was measured over three time intervals at each temperature, the fluxes remaining linear provided $[Na]_i$ remained about constant over the incubation period. Lowering the temperature produced an increased affinity for Na at the inside site, as shown by the data for four experiments summarized in Table II.

Similar experiments were performed at 5 and 37°C in thirteen-lined ground squirrel cells. The results obtained were similar to those for guinea pig red cells; the data conformed to the same Michaelis-Menten cubic expression and yielded comparable app K_m values. Table II includes results for four experiments at high $[Na]_i$ for ground squirrels, giving values for app K_m and V_{max} . However, at low $[Na]_i$ the fluxes measured at 37°C in ground squirrel cells are higher than those predicted by the fitted curve (e.g., see the low $[Na]_i$

TABLE II
APPARENT K_m AND V_{max} VALUES FOR Na AT THE INTERNAL SITES IN GROUND SQUIRREL AND GUINEA PIG ERYTHROCYTES AT 5 AND 37°C

Temperature °C	Experiment	Ground squirrel		Guinea pig	
		K_m	V_{max}	K_m	V_{max}
37	1	2.4	6.0	1.8	6.9
	2	2.1	2.7	2.9	10.4
	3	1.6	4.4	2.3	9.6
	4	1.4	3.7	3.7	12.4
5	1	0.50	0.117	0.65	0.055
	2	0.70	0.059	0.78	0.040
	3	0.35	0.092	0.91	0.037
	4	0.43	0.067	0.89	0.065

Data fitted as $v = \frac{V}{\left(1 + \frac{K_m}{Na}\right)^3}$ from $(S/v^{-1/3})$ vs. S plots for six data points for $[Na]_i$

values from 5 to 25 mmol/liter cells (ground squirrels) and 2–30 mmol/liter cells (guinea pigs). Values for cell K were between 92 and 106 mmol/liter cells (ground squirrels and 84–96 mmol/liter cells (guinea pigs).

points; Fig. 2C). This was a consistent finding, another example of which is illustrated in Fig. 3, and it was from experiments of this kind that we obtained previously high affinity app K_m values for $[Na]_i$ in ground squirrel cells (Ellory and Willis, 1978).

The most likely explanation of these results would be the presence of an additional high Na affinity or Na-independent component of ouabain-sensitive K influx in ground squirrel cells at 37°C. One possibility would be an increased K:K exchange (Simons, 1974), although this seems unlikely because intracellular phosphate was measured as <0.6 mM in these cells. Ouabain-sensitive K efflux from cells with low $[Na]_i$ was therefore measured in five experiments. There is a variable amount of K:K exchange; in two experiments, no ouabain-sensitive K efflux was detectable, whereas a significant flux

amounting to 0.4–0.5 mmol/liter cells was measured in two other experiments (Table III, Fig. 3). There was no explanation for this variability. In one experiment (Fig. 3) we measured K influx and efflux simultaneously using parallel batches of cells with low $[Na]_i$. The results indicated a significant K:K exchange. This could not, however, completely account for the high ouabain-sensitive K influx seen at low $[Na]_i$, because subtracting the K:K exchange component from the total ouabain-sensitive K influx still left a residual flux that was too high to conform with the simple cubic fitted curve obtained at high $[Na]_i$ (Fig. 2C).

Ouabain Binding

Because the foregoing experiments showed no obvious difference between ground squirrel and guinea pig sodium pumps in terms of temperature effects on internal and external cation affinities, it was necessary to investigate pump turnover directly via ouabain binding studies.

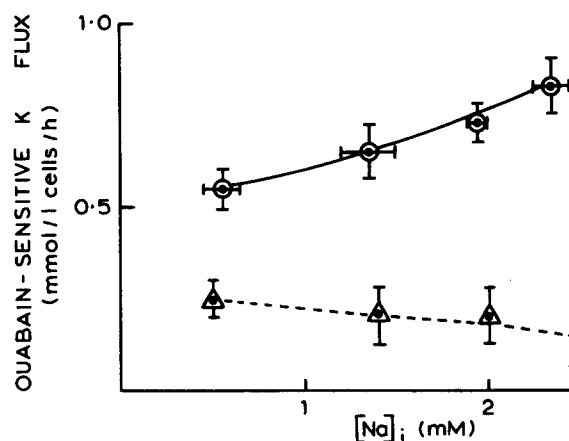


FIGURE 3. Simultaneous measurements of K influx and efflux in thirteen-lined ground squirrel erythrocytes at 37°C. Measurements made on parallel batches of cells; efflux determinations were made using cells incubated in unlabeled K-choline medium, but processed identically; O, influx; Δ , efflux.

Preliminary experiments with ground squirrel erythrocytes established that they behaved like human red blood cells in having high affinity binding and virtually no reversal after 1 h of subsequent incubation in ouabain-free medium. We were therefore able to use the simple technique of pre-exposure to 3H -ouabain at either 5 or 37°C, followed by washing and a rapid ^{42}K influx determination to establish fractional pump inhibition. Some experiments were also carried out on thirteen-lined ground squirrel red cells using the K displacement technique developed for guinea pig erythrocytes (see below). Data for an experiment where both experimental approaches were carried out on Columbian ground squirrel erythrocytes at 5 and 37°C are presented in Fig. 4, where the function molecules bound per cell is plotted as a function of fractional pump inhibition. The relationship is linear and passes through the

origin. Extrapolation to 100% inhibition allows the determination of the total number of pump sites binding ouabain with ~800 sites/cell for guinea pig and Columbian ground squirrel red cells, and 280 sites/cell for thirteen-lined ground squirrel cells. Control samples, run in parallel in the presence of excess (10^{-3} M) cold ouabain, gave 6–12 sites per cell. Data for further experiments on Columbian and thirteen-lined ground squirrels are presented in Table IV.

TABLE III
K EFFLUX MEASURED IN NYSTATIN-TREATED GROUND
SQUIRREL ERYTHROCYTES AT 37°C

Experiment	Cell cations		Efflux rate constant (k_0^K)		
	Na	K	Control	Ouabain	k_0^K pump difference
	<i>mmol/liter cells</i>				
1	6.3	42	0.034±0.002	0.031±0.002	0.003±0.003
2	2.4	31	0.072±0.006	0.059±0.003	0.013±0.007
3	1.8	34	0.048±0.004	0.046±0.002	0.002±0.005
4	4.1	20	0.051±0.003	0.026±0.001	0.025±0.003

External K concentration = 6 mM.

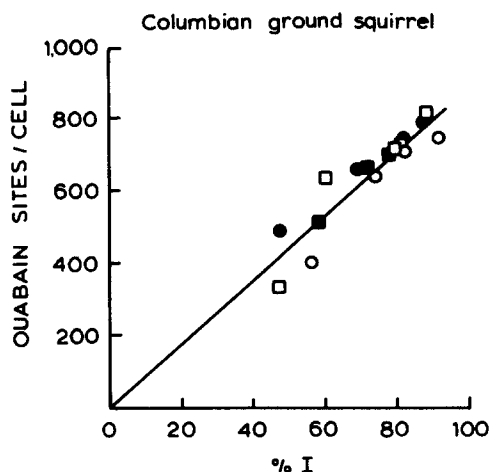


FIGURE 4. ^3H -ouabain binding and sodium pump inhibition in Columbian ground squirrel erythrocytes at 5 and 37°C. Data presented for both time course and K equilibrium experiments. ○, time, 37°C; □, time, 5°C; ●, K equilibrium, 37°C; ■, K equilibrium, 5°C. Line, drawn by eye, corresponds to 880 sites/cell at 100% inhibition.

Although the 37°C values were numerically lower than the 5°C values the difference was not statistically significant ($P < 0.1$). The constancy of the number of sites operative at both temperatures and the linearity of the relationship of bound ouabain to percent inhibition indicate that the sodium pumps are behaving as the same simple population at both 5 and 37°C in ground squirrels. From the flux and ouabain-binding data we can calculate a

turnover number for the pump in these cells of 80–110 s^{-1} at 37°C, falling to 1.5–2.2 s^{-1} at 5°C.

Ouabain-binding experiments proved more difficult in the guinea pig because glycoside binding is very reversible in this species (Willis and Ellory, 1982). We therefore adopted a K-displacement binding method, which allowed bound ouabain and pump inhibition to be followed over time (Fig. 5). The DBT method results in very rapid cell sedimentation, which demonstrated that reversal did not occur during rapid washing and confirmed the maximum number of measured pump sites. Data derived from experiments of the kind shown in Fig. 5 were used to produce plots of molecules bound vs. pump inhibition, and extrapolated to 100% inhibition. Results of two experiments

TABLE IV
NUMBER OF OUABAIN BINDING SITES AND PUMP TURNOVER IN
ERYTHROCYTES FROM GUINEA PIGS AND COLUMBIAN AND
THIRTEEN-LINED GROUND SQUIRRELS

Species	Temperature °C	Method*	Experiment 1		Experiment 2	
			Sites/cell	Turn- over number s^{-1}	Sites/cell	Turn- over number s^{-1}
Guinea pig	37	[K] ₀	768±81	129	783±66	106
	5	[K] ₀	934±70	0.47	863±93	0.42
Thirteen-lined ground squirrel	37	Time course	266±30	101	289±11	97
	5	Time course	280±17	1.42	348±28	1.01
Thirteen-lined ground squirrel	37	[K] ₀	359±31	87	454±53	88
	5	[K] ₀	387±23	1.27	511±46	1.22
Columbian ground squirrel	37	Time course	812±31	81	766±54	77
	5	Time course	751±47	2.30	640±36	2.19

* Ouabain binding was either measured by washing the cells after exposure to ouabain for variable periods (time course) or by using differing K levels to vary the percent inhibition with simultaneous measurements of ⁴²K influx ([K]₀).

are given in Table IV. Guinea pig erythrocytes have more sites per cell than thirteen-lined ground squirrels, but the calculated pump turnover number for these cells at 37°C is comparable (~100 s^{-1}). Again the total number of sites measured at 5°C was slightly greater than at 37°C, but the difference was not significant ($P < 0.05$). The principal effect at 5°C was decreased pump turnover number (0.4–0.5 s^{-1}) compared with the hibernator cells.

DISCUSSION

Overview

The fact that mammalian tissues gain Na and lose K on cooling demonstrates that the sodium pump usually is more sensitive to low temperature than passive membrane permeability to these ions. Exceptions to this rule, usually,

but not always, involving greater pump activity at low temperature (Willis et al., 1980, 1981), are found among tissues of mammals capable of deep hibernation. A comparison of the effects of cooling between cells of greater and lesser temperature sensitivity offers the possibility of better understanding of the details of pump inhibition at low temperature. The present work differs from most efforts in this subject in two respects: its use of intact cells instead of isolated Na-K-ATPase, and its emphasis on kinetics rather than membrane composition (see Ellory and Willis, 1981; Willis et al., 1981).

Regardless of the membrane compositional and structural constraints that lead to arrest of the pump on cooling, it would be useful to know the

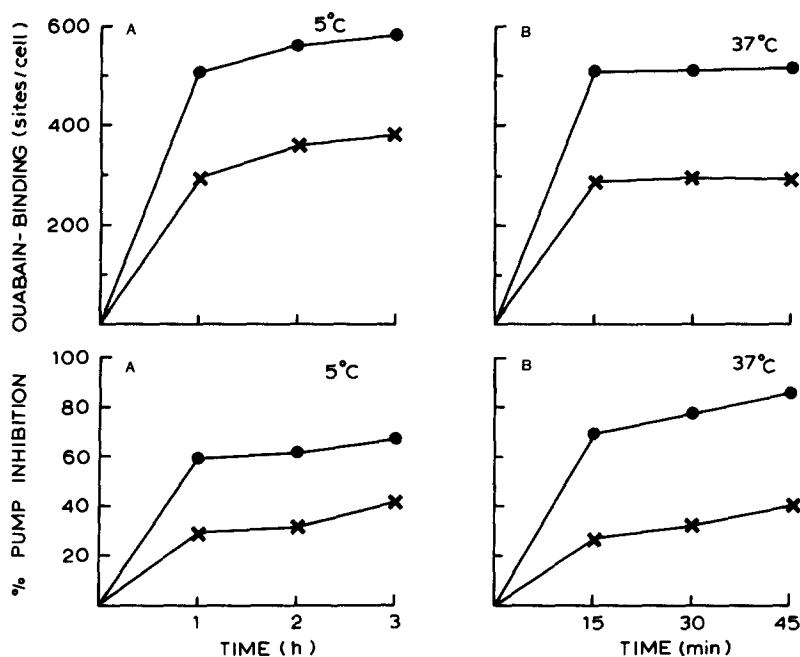


FIGURE 5. ³H-ouabain binding to guinea pig erythrocytes at 5 and 37°C measured by the K equilibrium method. (A) 5°C; (B) 37°C; ●, K 1 mM (37°C), 0.1 mM (5°C); X, 5 mM K (37°C), 0.5 mM (5°C). The concentration of ouabain was 0.39 μM in each case.

conformational state of the mechanism in which it stops. To this end no one has systematically investigated ion affinities or ouabain binding as a function of temperature in either intact or isolated preparations, even though there has been some interest in the possibility of maintained "partial" reactions of isolated Na-K-ATPase at low temperature in the face of negligible overall ATP hydrolysis (Skou, 1965; Grisham and Barnett, 1973; Swann and Albers, 1981). The use of intact cells for these purposes is especially desirable because the effects of temperature on isolated Na-K-ATPase and cell sodium pump function do not coincide (Ellory and Willis, 1978; Willis et al., 1978).

Kinetics of Na and K Activation of Pump

Our first aim was to characterize temperature-induced changes in Na and K affinities. The present results demonstrate that both the external and internal ionic affinities of the sodium pump for substrate (e.g., $[K]_o$ and $[Na]_i$) increase with lowered temperature. Both the app $K_m[K]_o$ and app $K_m[Na]_i$ change by about the same fractional amount, which relates to plasma levels of K and to intracellular Na such that the pump would be ~50% saturated at 37°C in vivo, but close to 100% saturated at 5°C. With regard to the external affinity, there were no obvious differences between cold-resistant and cold-sensitive erythrocytes.

For the internal sites the results are more complicated. In guinea pig red cells, the results resemble the simple cubic fit for Na activation at the internal site found in human red cells (Garay and Garrahan, 1973); the presently measured app K_m of 1.8 mM at three sites with a high $[K]_i$ agrees well with their results. Lowering the temperature to 5°C increased the affinity roughly threefold, but the kinetics remained simple. For the ground squirrel the results show a pronounced deviation from a simple cubic fit in the low range of $[Na]_i$ values. This deviation cannot be accounted for completely by K:K exchange because this flux is small and variable in these cells. We have also found similar effects in red cells of another species (hamster; unpublished results).

It is by no means obligatory that increased Na and K affinities at low temperature will lead to an increase in pump turnover and might in fact militate for the opposite. Thus, the present finding of an increased app K affinity could reflect an increase in $E_2(K)$, the occluded K-binding form of the enzyme, at low temperatures (see, for example, Robinson and Flashner, 1979, for a review and nomenclature). Similarly, the increased Na affinity could result from an E_1 stabilization. Conversely, increased affinity could be a factor causing the stabilization.

Number of Pump Sites

The other major effort of this study was to estimate changes in steady state ouabain binding with decreased temperature. One possible explanation of the differential pump rates in the two types of animals at low temperature is a change in the number of operational sodium pumps. Combinations that would explain the result include the "freezing out" of a fraction of pumps in temperature-sensitive cells or augmentation of pumps at low temperatures in ground squirrels. In fact, the results suggest that the ouabain binding characteristics of both guinea pigs and ground squirrels at high and low temperature are rather straightforward because the number of pump sites did not change significantly with temperature. At the higher temperature the turnover number of the pump was the same for both species, $\sim 100 \text{ s}^{-1}$. This number compares well with the values for human red cells (Joiner and Lauf, 1978). On cooling, the turnover number for guinea pig red cells (the temperature-sensitive species) falls much more than for ground squirrel.

The present finding of a simple linear function even at low temperature

leads us to conclude that the effect of cold on the sodium pump is absolute in the sense that no separation of partially active components (i.e., capable of binding ouabain though not transporting) was apparent, contrary to what would be the expectation from a hypothesis based on blockage of $E_2 \rightarrow E_1$.

Conclusions

The two main conclusions of this study seem unremarkable. There is little in the changes of affinities to cations to explain cold sensitivity in guinea pig red cells. The results with ouabain binding suggest that their Na pumps simply slow down without favoring any specific confirmation. However, these conclusions are somewhat surprising in the light of work on partial reactions of Na-K-ATPase preparations which suggests that cooling may stabilize an $E_2(E_2[K])$ form of the enzyme (Barnett and Palazzotto, 1974; Swann and Albers, 1979; 1981).

Because the affinities for the two ions increase *symmetrically* in both the cold-sensitive and cold-resistant cells, we must entertain the possibility that a similar stabilization of E_1 occurs with cooling. Other evidence from differential temperature effects could also be interpreted in terms of temperature affecting E_1-E_2 conversion in either direction such that any partial reaction involving *either* E_1 or E_2 without interconversion is less sensitive to cold than the overall ATPase reaction (Kimelberg, 1975; Gruener and Avidor, 1966; Blostein, 1970; Neufeld and Levy, 1970). Further resolution of this issue will require determination of the effect of temperature on the so-called "partial fluxes" (ouabain sensitive Na:Na exchange, Na:nothing, and K:K exchange).

The ouabain binding results, as discussed, also argue against a differential pile up of either E_2 or E_1 . It is possible, however, that our prime criterion of linearity between binding and inhibition could have been misleadingly reassuring due to our sole reliance upon K influx as the measure of pump activity. Some K influx could in fact have been occurring at low temperature through an increased proportion of ouabain-sensitive K:K exchange. Determination of ouabain-sensitive K efflux would test this possibility.

The present results thus do not rule out the possibility that partial fluxes are reduced less at low temperatures in guinea pig red cells than the overall pump rate. They do, however, suggest that if such a change does occur, it is a symmetrical one, probably involving a maintained balance between K:K and Na:Na exchange with little change in the E_2/E_1 ratio.

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