Temperature Adaptation of Active Sodium-Potassium Transport and of Passive Permeability in Erythrocytes of Ground Squirrels

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ABSTRACT Unidirectional active and passive fluxes of ^{42}K and ^{24}Na were measured in red blood cells of ground squirrels (hibernators) and guinea pigs (nonhibernators). As temperature is lowered, "active" (ouabain-sensitive) K influx and Na efflux were more greatly diminished in guinea pig cells than in those of ground squirrels. The *fraction* of total K influx which is ouabain sensitive in red blood cells of ground squirrels was virtually constant at all temperatures, whereas it decreased abruptly in guinea pig cells as temperature was lowered. All the passive fluxes (i.e., Na influx, K efflux, and ouabain-insensitive K influx and Na efflux) decreased logarithmically with decrease in temperature in both species, but in ground squirrels the temperature dependence (Q_{10} 2.5–3.0) was greater than in guinea pig (Q_{10} 1.6–1.9). Thus, red blood cells of ground squirrel are able to resist loss of K and gain of Na at low temperature both because of relatively greater Na-K transport (than in cells of nonhibernators) and because of reduced passive leakage of ions.

Unlike human red blood cells, erythrocytes of hibernating species lose K only gradually during exposure to low temperatures either in vitro during cold storage or in vivo during hibernation (Kimzey and Willis, 1971). Observations of net concentration changes in intracellular K during cold exposure, however, do not differentiate the relative effects of low temperature on the active and passive components of ion transport. Thus, the cold resistance of hibernator cells could be due to a maintenance of active transport or the result of a reduced membrane permeability to cations (see Willis, 1966, 1967).

In kidney and muscle, the tissues in which this problem has been studied previously, measurements of unidirectional fluxes of Na and K are complicated by heterogeneity of cell types and complexity of tissue compartmenta-

tion. In red cells, on the other hand, the unidirectional fluxes of Na and K can be accurately measured, and the active and passive components of these fluxes can also be distinguished by inhibiting the active ion movements with the cardiac glycoside, ouabain. Thus, red blood cells may offer the opportunity for a finer degree of definition of the mechanism of cold resistance of ion balance in hibernators than has been possible with the other cell types. Consequently, further insight into the precise cause of failure of ion regulation in human erythrocytes at low temperatures would also be gained.

A preliminary study of the effects of low temperature on ion transport in hibernator erythrocytes has been reported (Kimzey and Willis, 1968).

METHODS

Preparation of Erythrocytes for Incubation

Two species were used in this study: the 13-lined ground squirrel, a hibernator, and the guinea pig, a nonhibernator.

Blood samples were obtained by heart puncture from anesthetized animals and placed in heparinized Pyrex centrifuge tubes chilled in an ice bath. Within 30 min of withdrawal, the samples were centrifuged at 1600 *g* at 2°–3°C for 3 min, and the plasma and buffy coat were removed by aspiration. The red cells were then resuspended in ice-cold incubation medium, and the washing procedure was repeated three times. By taking care to remove the topmost layer of cells each time, the white cells were almost completely eliminated (Glynn, 1956).

Following this initial washing procedure, the cells were normally used immediately for measurement of unidirectional fluxes, but occasionally the cells were stored overnight at 5°C in standard incubation medium.

Composition of Incubation Media

The standard incubation medium contained 154.0 mM NaCl, 5.0 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.0 mM Na_2HPO_4, 0.85 mM NaH_2PO_4 (pH 7.4), and 11.1 mM glucose. Preliminary experiments indicated no significant change in the active component of K influx at either 38° or 20°C in the range of 5–20 mM K concentration. All subsequent experiments were performed with 5 mM K.

INFLUX EXPERIMENTS For influx experiments, the red cell sample was divided into two equal volumes (2–4 ml each) and added to paired incubation vessels (25-ml Erlenmeyer flasks) containing unlabeled standard incubation medium equilibrated to the proper test temperature. One flask of each pair also contained 0.5 mM ouabain. The cell suspension (with hematocrit of 15–20) was then gently shaken in a controlled-temperature water bath for 10–20 min before the radioisotope was added. After the addition of ^{24}Na or ^{42}K, duplicate samples (about 1 ml) of the cell suspension were removed from each flask at timed intervals and placed in Wintrobe tubes cooled in an ice bath. The samples were immediately centrifuged (1600 *g*, 2°–3°C) for 4 min and the radioactive supernatant was removed. The cells were washed three times in an isotonic solution of NaCl, choline chloride, or standard incubation medium to

remove the remaining extracellular radioactivity. The choice of the washing medium was related to whether or not the Na or K content of the cells was to be measured. Following this washing procedure, the activities of the cells relative to the incubation media were determined as described below.

OUTFLUX EXPERIMENTS For outflux measurements, the washed cells were preincubated at 38°C for about 2 hr in standard incubation medium containing either ^{42}K or ^{24}Na. In later experiments, the cells were not washed before this preincubation, but the plasma was supplemented with radioactive standard incubation medium. This variation had no effect on the measured outflux of either Na or K from the cells. Following the initial incubation, the radioactive supernatant was removed after centrifugation and the cells were washed three times in chilled standard incubation medium. The labeled cells were then added to paired incubation flasks containing standard incubation medium equilibrated to the proper test temperature. As in the influx experiments, one flask contained 0.5 mM ouabain. The vessels were gently shaken in a water bath and duplicate samples were removed as described above, except that the first centrifugation was for 5 min and the supernatant was saved for measurement of its radioactivity. The outflux from the cells was determined from the appearance of radioactive Na or K in the medium.

Preparation of Radioactive Isotopes

The isotopes used, ^{42}K and ^{24}Na, were prepared by neutron bombardment of their respective carbonate salt in the University of Illinois reactor (TRIGA Mark II). The purities of these preparations were checked by measuring the decay constants which confirmed the presence of a single radioactive species in each case.

The cyanomethemoglobin method (Wintrobe, 1961) was used to measure hemoglobin content. Hematocrits were measured by spinning flame-sealed, heparinized capillary tubes containing the cell suspension for 15 min at 6700 *g* (International microcapillary centrifuge, International Equipment Company, Needham Heights, Mass.). From these data, the relationship between hemoglobin content and cell volume was determined and used in calculating the volume of samples obtained for measurement of radioactivity.

Measurement of Radioactivity

DETECTION INSTRUMENTS Two types of instruments were used in this investigation to measure radioactivity. In the initial experiments samples were counted in a well-type solid scintillation counter with an NaI(Tl) crystal. In later experiments, a gas ionization system was used (Beckman Lowbeta II, Beckman Instruments, Inc., Palo Alto, Calif.) with a background level of less than 1 cpm.

PREPARATION OF SAMPLES FOR COUNTING For the well scintillation counting, samples of whole cells were counted after the final washing and their volume (0.1–0.2 ml) was subsequently determined by measuring their hemoglobin content. Planchets were prepared with 1-ml samples of red cell hemolysate for counting in the gas ionization system. Duplicate samples were counted in all cases.

Calculation of K Influx

K influx was calculated from the following equation

$$M_i = \frac{d(S/\bar{P}_o)}{dt}$$

where

M_i = influx (meq K/liter RBC per hr),
S = activity of the cells (cpm/liter RBC),
$\bar{P}_o$ = initial specific activity of the incubation medium (cpm/meq K),
t = time in hours.

M_i then represents the slope of a linear relationship between $(S/\bar{P}_o)$ and time (Fig. 1 illustrates the result of a typical determination).

Other Influx Calculations

In the case of Na, the influx was calculated from the equation:

$$M_i = \frac{m(\mathrm{Na})_o S_\infty}{P_o}$$

where

M_i = steady-state flux (meq Na/liter RBC per hr),
m = slope (hr^{-1}) of the relationship between ln $(1\text{-}S/S_\infty)$ and time (hr),
$(\mathrm{Na})_o$ = sodium concentration of medium (meq/liter),
S_∞ = equilibrium cellular activity of ^{24}Na (cpm/liter RBC),
P_o = initial ^{24}Na activity of medium (cpm/liter incubation medium).

For Na or K outflux the following variation of the above equation was used

$$M_o = \frac{m(C)_{in}[(1 - H)/H]}{(C)_{in}/(C)_o + [(1 - H)/H]}$$

where

M_o = outflux of Na or K (meq/liter RBC per hr),
m = slope (hr^{-1}) of the linear relationship between ln $(1\text{-}P/P_\infty)$ and time,
$(C)_{in}$ = intracellular concentration of Na or K (meq/liter RBC),
$(C)_o$ = extracellular ion concentration,
H = hematocrit.

These equations have been derived previously (Lieb, 1967; Sha'afi, 1965; Sha'afi and Lieb, 1967; Solomon, 1952), and they correct for reverse flux of the radioactive ions. Such a correction is necessary for calculating the rate of movement of an ion from a region of high concentration to one of much lower concentration.

It should be emphasized that equations used to calculate these ion fluxes are for equilibrium conditions. The red cells examined were not always in a strict steady state with respect to Na-K exchange during the incubation period. But, with the

exception of Na outflux in ground squirrel cells at 38°C, any deviation from equilibrium was not significant and could not under any circumstances have caused more than a 3% error in the calculated flux. In all cases actually measured, net Na and K changes in either the media or the cells during the incubation period were not detectable by flame photometry. This apparent lack of net change of ion concentration

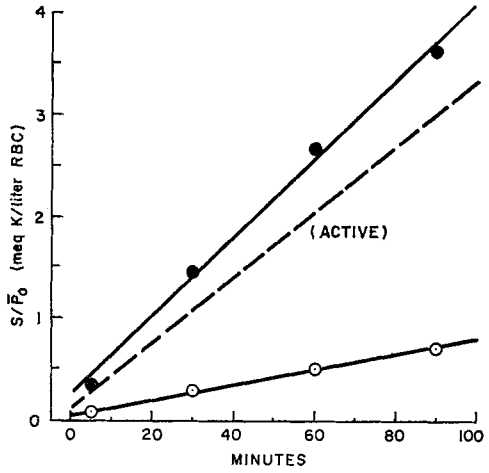

FIGURE 1

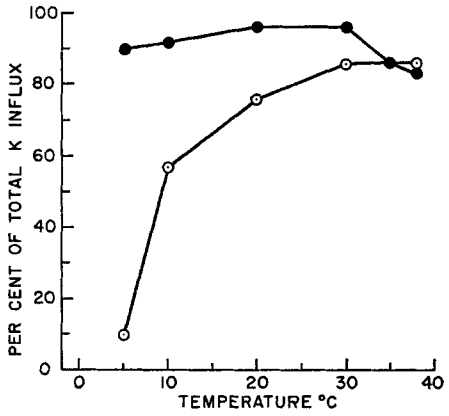

FIGURE 2

FIGURE 1. Typical experiment for measuring K influx into erythrocytes. The activity of the cells (S, cpm/liter RBC) at specific times was divided by the specific activity of the medium at time zero ($\bar{P}_o$, cpm/meq K), and this value ($S/\bar{P}_o$) was plotted against time. The slope of the linear regression line through these points represents the influx of K (meq/liter RBC per hr). At least three, and usually four, determinations were made in each experiment. The regression lines were fitted by the method of least squares, but in most cases, the increase in K content over a given time interval was virtually constant. The closed circles represent the influx of K into cells incubated in a complete medium, and the open circles represent that portion of K influx remaining in the presence of 0.5 mM ouabain. The difference between the two regression lines (broken line) is the ouabain-sensitive K influx and is considered to be attributable to active transport (Post et al., 1967).

FIGURE 2. The influence of temperature on the active K influx relative to the total K influx. The values in this figure were calculated from the data in Table I. The ouabain-sensitive influx was divided by the total influx at each temperature. The solid circles represent relative values of K influx in ground squirrel erythrocytes, and the open circles are data from guinea pig cells.

may be attributed to the brief periods of incubation (60 min at temperatures above 20°C, 90 min at 20°C, and 120 min at temperatures below 20°C). Changes in relative cellular volume, hematocrit, and the appearance of hemoglobin in the incubation medium were also not evident during the time of incubation. Calculation of slopes was always based upon duplicate values at each of three or more time periods and deviation from linearity was not observed. Assumption of steady-state conditions for the calculation of fluxes therefore seemed justified.

RESULTS

Effect of Temperature on Ouabain-Sensitive Fluxes

K INFLUX The K influx into erythrocytes of the ground squirrel and of the guinea pig were measured at temperatures between 5° and 38°C. The cells were washed in standard incubation medium and incubated in paired flasks containing ^{42}K standard incubation medium. The media in the flasks were

TABLE I
POTASSIUM INFLUX

Species	Temp	N	K influx		
			Total	Ouabain	Active
	°C		*meq/liter RBC per hr*		
Ground squirrel	38	3	2.25±0.10	0.39±0.10	1.86±0.20
	35	2	2.50±0.14	0.35±0.08	2.15±0.22
	30	3	3.34±0.27	0.14±0.04	3.20±0.31
	20	3	1.14±0.13	0.05±0.01	1.09±0.14
	10	3	0.39±0.06	0.03±0.01	0.36±0.07
	5	4	0.14±0.01	0.01±0.01	0.13±0.02
Guinea pig	38	5	3.32±0.29	0.48±0.19	2.84±0.48
	30	4	3.36±0.23	0.45±0.14	2.91±0.37
	20	4	0.85±0.09	0.20±0.10	0.65±0.19
	10	2	0.14±0.01	0.07±0.01	0.07±0.02
	5	4	0.09±0.01	0.09±0.01	0.01±0.01

Washed erythrocytes were incubated in standard incubation medium containing ^{42}K for 1–2 hr at each test temperature. Samples were removed periodically, washed to remove the extracellular radioactivity, and the activity of the cells was measured. K influx was calculated from the increase in activity of the cells (cpm/liter RBC) relative to the specific activity of the incubation medium (cpm/meq K) during a period of 1 hr.

The values under the "Total" and "Ouabain" columns represent the experimentally measured influxes in paired experiments. The former represents the influx in a complete incubation medium while the latter is the flux observed in the presence of 0.5 mM ouabain. The "Active" column is computed from the difference in the "Total" and "Ouabain" data.

The variation represents the standard error of the mean.

identical except that 0.5 mM ouabain was present in one of each pair. The data from these experiments are given in Table I. The active influx was computed by taking the difference of the two experimentally measured quantities—total influx and the influx remaining in the presence of ouabain. (This definition of "active flux" as that flux inhibited by ouabain does of course rest upon two assumptions: namely, that the only measurable effect of ouabain was inhibition of the active component of K influx [or Na efflux], and that the concentrations used were totally inhibitory. While the first assumption is based upon a large body of precedent the possibility always does exist in any

previously unexplored system that ouabain, especially at such a high concentration, might also exert an effect either to increase or to decrease passive leakage of ions. With respect to the second assumption, no dose-response relationship was determined in the course of this study, but subsequent observations have shown that 20 μM ouabain causes 75% inhibition and that 100 μM is maximally inhibitory in erythrocytes of guinea pigs. Sensitivity to ouabain of other ground squirrel tissues is similar to that of guinea pig and the essentially complete inhibition of K influx at 5°C noted below indicates that this was no less so for the erythrocytes.)

As the incubation temperature decreased, the active component of K influx in guinea pig erythrocytes was unaffected from 38° down to 30°C. Below 30°C, there was a rapid decline in the active influx until at 5°C there was no significant ouabain-sensitive K influx detectable under these experimental conditions (Table I).

In ground squirrel cells, the response of the active K influx to a change in temperature was quite different from those in guinea pig cells. In the temperature range of 38°–30°C, there was a substantial increase in the active component. Below 30°C, the ouabain-sensitive influx declined as a function of the incubation temperature, but was significantly greater than the active influx in guinea pig red cells at all temperatures tested (Table I). At 5°C, there was still a small ouabain-sensitive K influx significantly greater than zero ($P < 0.01$).

In Fig. 2, the difference between the two species is summarized by comparing the relative effect of temperature on the active component with respect to the total K influx. In guinea pig red cells, the proportion of the total K influx sensitive to ouabain decreased markedly with temperature, so that at 5°C it was less than 10% of the total K influx. In ground squirrel erythrocytes on the other hand, the relative amount of K influx due to active transport remained essentially constant or even increased slightly as the temperature was reduced.

NA OUTFLUX To estimate the effect of temperature on the active extrusion of Na, cells were first loaded with radioactive Na by preincubation in ^{24}Na-standard incubation medium at 38°C for 1–2 hr and the outflux was determined over the next 1–2 hr period at the appropriate test temperature (Table II). As with K influx, the active component of Na outflux in guinea pig cells was more sensitive to a lowering of the temperature than in ground squirrel. At 5°C, only about 1% of the ouabain-sensitive outflux present at 38°C remained in the guinea pig red cells. In this nonhibernating species, the reduction in the active component at low temperatures was disproportionately greater than the decrease in total outflux, while in ground squirrel cells, the active outflux of Na relative to total influx increased slightly at lower temperatures (Table II). Thus, the energy-dependent Na outflux in guinea

pig erythrocytes was more sensitive to decrease of temperature than in ground squirrel erythrocytes.

Effect of Temperature on Passive Fluxes

K OUTFLUX To characterize the effects of temperature on the outward movement of K, cells were preincubated for 1–2 hr at 35°–38°C in ^{42}K-standard incubation medium. After three washes in unlabeled incubation medium to remove the radioactivity from the extracellular compartment, the cells were incubated in standard incubation medium for 1–2 hr at different test temperatures ranging from 38° to 5°C. The outflux of K was determined

TABLE II

EFFECT OF TEMPERATURE ON Na OUTFLUX IN ERYTHROCYTES

Species	Temp	N	Na Outflux			Active/total
			Total	Ouabain	Active	
	°C		*meq/liter RBC per hr*			
Ground squirrel	38	3	3.21±0.13	0.98±0.02	2.32±0.15	0.72
	30	2	3.66±0.29	0.71±0.10	2.95±0.39	0.80
	20	4	1.39±0.15	0.14±0.03	1.25±0.18	0.90
	5	2	0.20±0.03	0.03±0.01	0.17±0.04	0.85
Guinea pig	38	3	4.29±0.35	0.82±0.10	3.47±0.45	0.81
	20	3	1.40±0.27	0.52±0.15	0.88±0.37	0.63
	5	2	0.18±0.01	0.12±0.02	0.06±0.03	0.33

Washed erythrocytes preloaded with ^{24}Na were incubated in standard incubation medium, and Na outflux was determined from the appearance of radioactivity in the medium. The "Total" and "Ouabain" columns represent the outflux of Na in the absence and presence of 0.5 mM ouabain, respectively. The "Active" column was calculated from the difference in these two measurements and represents the ouabain-sensitive outflux. The ratio, "Active/total," represents the fraction of the total outflux due to active processes at each temperature and is comparable to the data for K influx presented in Fig. 2.

from the appearance of ^{42}K in the medium. The presence of ouabain in the incubation vessel had no effect on the outflux of K from the red cells. The results of these experiments are shown in Table III and Fig. 3. The K outflux decreased exponentially with a lowering of the incubation temperature. The Q_{10} for the decrease between 38° and 5°C was greater in the ground squirrel (2.56) than in the guinea pig (1.56) (Table IV).

SODIUM INFLUX For determination of Na influx, washed erythrocytes of the guinea pig and ground squirrel were incubated at 38°, 20°, or 5°C in flasks containing ^{24}Na-standard incubation medium. The data were corrected for reverse movements of the radioisotope as described in the Methods section. Na influx into red cells of either of the species was not altered by the presence of 0.5 mM ouabain in the incubation medium. Na influx into guinea

pig erythrocytes decreased with a Q_{10} of 1.62 when the temperature was lowered from 38° to 5°C (Fig. 4). In ground squirrel red cells, the experimental value for Na influx at 38°C was quite large (7.09 ± 0.26 meq/liter RBC per hr) compared with that of guinea pig (2.84 ± 0.29; Table III) and human cells (about 2; Glynn, 1956; Solomon, 1952). Even so, at the lowest temperature tested (5°C), the passive entry of Na into ground squirrel cells was substantially lower than in guinea pig. Consequently, the Q_{10} for the decline in Na influx in ground squirrel cells over the temperature range of 38°–5°C was 2.81 (Fig. 4), substantially greater than that for guinea pig cells (1.62; Table IV).

TABLE III

POTASSIUM OUTFLUX AND SODIUM INFLUX IN ERYTHROCYTES

Species	Temp	N	Flux	
			K outflux	Na influx
	°C		*meq/liter RBC per hr*	
Ground squirrel	38	3, 5	6.20±0.37	7.09±0.26
	35	5	4.67±0.07	
	30	4	3.24±0.20	
	20	3, 4	1.16±0.08	1.36±0.08
	7	3, 3	0.35±0.03	0.23±0.04 (5°C)
Guinea pig	38	4, 3	3.43±0.07	2.84±0.29
	20	4, 4	1.14±0.20	1.68±0.18
	7	4, 3	0.92±0.07	0.60±0.11 (5°C)

Cells preloaded with ^{42}K were incubated in standard incubation medium, and the increase in radioactivity of the medium measured as an index of K outflux. Na influx was measured as described for K influx (Table I). In all experiments, the presence of 0.5 mM ouabain had no detectable effect on the outflux of K from the cells during the short incubation period, nor on the influx of Na.

OUABAIN-INSENSITIVE K INFLUX AND NA OUTFLUX As described earlier, the proportion of K influx and Na outflux due to active processes remains relatively consistent in ground squirrel cells, but virtually disappears in guinea pig when the temperature is reduced to 5°C. An obvious corollary of this observation is the decline in the proportion of passive cation flux in ground squirrel cells and the increase in guinea pig cells. The effect of temperature on the absolute values of these passive ion movements is illustrated graphically in Figs. 5 and 6. As was the case with the other passive movements, the decline in K influx and Na outflux in ground squirrel cells was greater than in guinea pig erythrocytes. The temperature coefficients for these rates between 38° and 5°C were comparable to those observed for K outflux and Na influx in each species (Table IV).

Effect of Temperature on K Balance

It seemed, therefore, that the passive permeability of the red cell membrane to K flux in either direction was more influenced by temperature in ground squirrel than in guinea pig. At 5°C, the outflux of K from ground squirrel

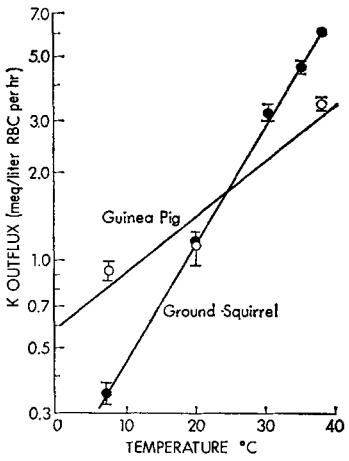

FIGURE 3

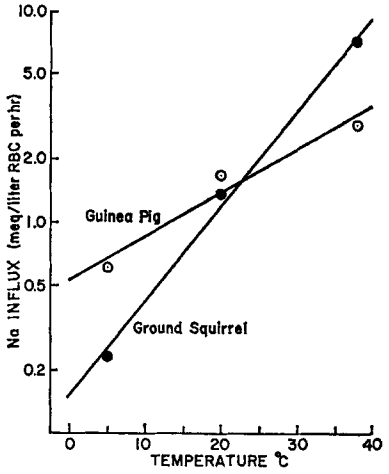

FIGURE 4

FIGURE 3. The effect of temperature on K outflux from erythrocytes of the ground squirrel and the guinea pig. Washed red cells, preloaded with ^{42}K, were incubated in unlabeled standard incubation medium for 1–2 hr at different test temperatures. At timed intervals, samples of the suspension medium were removed and their activity was determined. The solid circles are data from ground squirrel cells and the open circles from guinea pig, with each point being the mean of three to five experiments. The vertical bars represent the standard error of the mean. The regression line was calculated by the method of least squares. The temperature coefficients for K outflux were calculated to be 2.56 for ground squirrel and 1.56 for guinea pig.

FIGURE 4. The effect of temperature on Na influx in erythrocytes of the ground squirrel and the guinea pig. Washed red cells were incubated in ^{24}Na-standard incubation medium for 1–2 hr at various test temperatures. Although 0.5 mM ouabain was present in half of the incubation flasks, in none of the experiments at any temperature was there a significant effect of this glycoside on Na influx. Each point (closed circles represent ground squirrel and open circles represent guinea pig) represents the mean of two to four determinations. Variation in the data is presented in Table III. The regression lines were drawn by the method of least squares.

erythrocytes was only 0.29 meq/liter RBC per hr compared to 0.74 for guinea pig cells. This greater reduction in ground squirrel red cell permeability is unquestionably an important factor in determining the rate of net K loss from these cells during long-term cold exposure.

In a steady state, the K influx of red cells equals the K outflux. It was of interest to see whether cells would have been in a steady state at different

TABLE IV

TEMPERATURE COEFFICIENTS FOR PASSIVE CATION MOVEMENTS IN ERYTHROCYTES

Species	K influx	K outflux	Na influx	Na outflux
Ground squirrel	2.95	2.56	2.81	2.91
Guinea pig	1.85	1.56	1.62	1.71

The temperature coefficients were calculated from the slope of the regression lines through experimental points. The regression lines were drawn by the method of least squares.

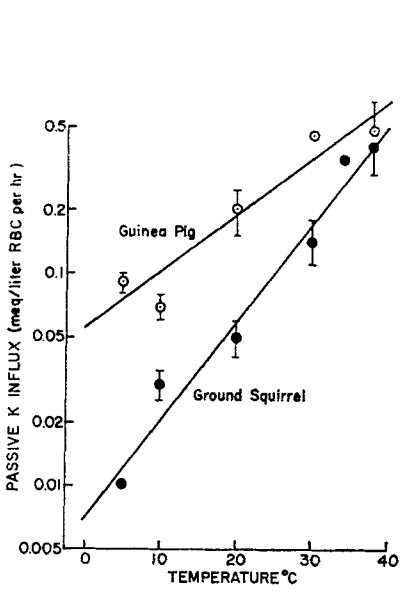

FIGURE 5

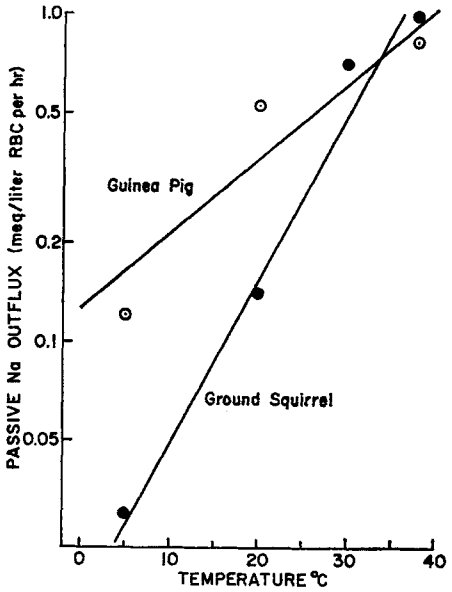

FIGURE 6

FIGURE 5. The effect of temperature on the ouabain-insensitive K influx into erythrocytes. The data in this figure represent K influx into red cells of ground squirrels (solid circles) and guinea pigs (open circles) incubated in standard incubation medium containing ^{42}K and 0.5 mM ouabain. The influx remaining in the presence of the cardiac glycoside is considered to be governed by passive diffusion. Each point represents the mean of two to five experiments, and the vertical bars represent the standard error of the mean. The regression lines were calculated by the method of least squares. The calculated Q_{10}'s for the change in rates with temperature were 2.95 for ground squirrel and 1.85 for guinea pig.

FIGURE 6. The effect of temperature on the ouabain-insensitive Na outflux in erythrocytes of the ground squirrel and the guinea pig. Na outflux from preloaded erythrocytes was measured in the presence of 0.5 mM ouabain as previously described. The variation in these values is given in Table III. The equation of the regression line was calculated by the method of least squares. The calculated temperature coefficients are 2.91 for ground squirrel and 1.71 for guinea pig.

temperatures as revealed by the measurement of unidirectional fluxes of K. To do this, the ratio of total K influx to K outflux was computed as a function of temperature (Fig. 7). In guinea pig cells, K influx declined more rapidly than did the outflux with decreasing temperature, a situation which would have resulted in a net loss of K at temperatures below 30°C. At 5°C, outflux of K was 10 times faster than the total influx of this ion. In ground squirrel red blood cells, the total K influx was equal to K outflux between 30° and 20°C; at 5°C, the influx was still 70% of the outflux. At temperatures above 30°C, however, ground squirrel cells were not in balance with respect

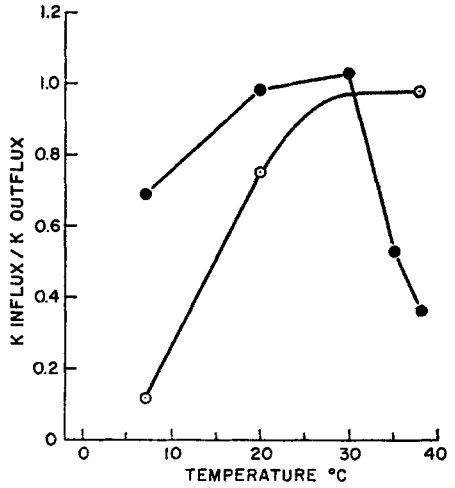

FIGURE 7. The effect of temperature on the K balance in erythrocytes of the ground squirrel and guinea pig. The values for the points in this graph were calculated by dividing the total K influx observed at each test temperature by the corresponding K outflux. When the ratio (K influx/K outflux) is equal to one, there is no net movement of K into or out of the cells. The closed circles represent ground squirrel cells and the open circles represent guinea pig cells.

to K (Fig. 7), and from 30° to 38°C, the rate of K outflux increased almost twofold (Table III), while the total K influx decreased (Table I). These results, based on unidirectional flux measurements, indicated that ground squirrel cells would exhibit a net loss of K at temperatures above 30°C.

DISCUSSION

When red blood cells of humans, guinea pigs, or other nonhibernating mammals are placed at low temperatures and, consequently, lose K and gain Na, the loss of electrolyte steady state is obviously due to an imbalance between active pumping and passive leakage. A priori, therefore, adaptation to cold in hibernators could be accomplished either by a lower passive permeability at low temperature or by a relatively greater rate of active transport at low

temperatures. The results of this study clearly show that ground squirrel erythrocytes employ both of these alternatives. A similar conclusion has already been drawn with respect to the cold adaptation of kidney cells (Willis, 1966) based on observations of changes in net uptake and loss of K.

The present results represent an advance over that earlier study in several quantitative respects. In kidney cells, adaptation of active transport was judged by measuring initial rate of net uptake of K into leached kidney cells. The possibility existed (although it was somewhat unlikely) that the faster uptake observed in hibernator kidney at low temperature was only due to reduced permeability, and that appreciable uptake did not occur in guinea pig kidney cells, not because of failure of transport, but because of excessive leakiness. Determination of unidirectional fluxes in red cells does not suffer from that ambiguity.

Again, passive leakage in kidney cells was judged by loss of K at low temperature from unleached slices in the presence of metabolic inhibitors. The procedure did not allow a quantitative measure of permeability and did not permit a conclusion about the identity of the species of ions to which the cells were less permeable. The present results show that in red blood cells permeability to both K and Na is more steeply dependent upon temperature in ground squirrels than in guinea pigs and that passive permeability in both directions changes by corresponding amounts for each ion.

Finally, the data on kidney cells was, of necessity, obtained on slices which had been subjected to the trauma of leaching with inevitable alterations in cellular structure and function creating the possibility of erroneous interpretation. Red blood cells, by contrast, are not greatly damaged by removal from the body and survive well during incubation. One can be more confident, therefore, that both the qualitative and quantitative alterations with respect to temperature are an accurate reflection of processes occuring in vivo.

The adequacy of the present results in describing the adaptation of the cells is summarized by the K balance predicted by the ratio of K influx to K outflux. A slightly disturbing feature of the results shown in Fig. 7 is that the ground squirrel red blood cells did not appear to be in a steady state above 30°C. Since K outflux is more difficult to determine than influx, involving as it does a long preincubation, it might be supposed that the absence of steady state at high temperatures is a result of some deterioration of the cells in the medium. That such is not the case and that the observation is, in fact, valid is suggested by several additional observations:

(*a*) It is actually the K influx and, in particular, the ouabain-sensitive component of the K influx which exhibits a maximum at 30°C (Table I). K outflux exhibits a monotonic logarithmic change with temperature (Fig. 3).

(*b*) The ouabain-sensitive component of Na outflux also shows a maximum

at 30°C (Table II) and Na influx varies with temperature in a manner similar to K outflux (Fig. 4).

(*c*) In a series of long-term incubations (5 hr) at 38°C, erythrocytes of ground squirrels did lose about 15 meq K/liter cells.

It would seem, therefore, that the lower temperature optimum for ground squirrel erythrocytes is probably real and may be a reflection of the greater lability of body temperature of this species (30°–39°C) characteristic of some hibernators (Williams and Heath, 1970).

The main point of interest, however, is that a steady state of ion balance for ground squirrels is predicted between 30° and 20°C and that at 5°C they would be far closer to a balanced state than erythrocytes of guinea pigs.

A subsidiary point of interest is that, although adaptations of both active transport and permeability exist, they do not allow for "perfect" adaptation. This conclusion was already apparent from observations of net changes in K content of stored cells (Kimzey and Willis, 1971) and it is satisfying that analysis of unidirectional fluxes should confirm it. Indeed, the rate of net loss could probably be predicted fairly accurately from the flux results. Thus, the difference between outflux and influx shown in Tables I and III would lead to a loss of 5 meq/liter cells per day in ground squirrel cells while the observed initial rate was 3 (Kimzey and Willis, 1971). In guinea pig, the corresponding values are 19 and 12, respectively. The lower values actually observed in stored cells could be attributed to a declining rate of loss due to decreasing gradients.

Further analysis of the cold adaptation of hibernators' erythrocytes must now focus on either the membrane mechanisms themselves or upon the glycolytic function of the cell. Both in the field of erythrocyte storage at low temperature and in the field of hibernator cold resistance, much attention has been directed to the sensitivity or insensitivity of metabolic, energy-releasing processes. It has been suggested, however, that the cold sensitivity of non-hibernator kidney cells with respect to ion transport is a result of the direct effect of cold on the mechanism inself and not on metabolism (Willis, 1968). In accordance with this notion, the Na,K–adenosine triphosphatase (ATPase) of kidney of hamsters (a hibernating species) has been shown to be less sensitive to temperature than that of rats (Willis and Li, 1969; Fang and Willis, 1970). The Na,K–ATPase of brain of hedgehogs and hamsters exhibits a similar adaptation but, curiously, only when the animal is actually hibernating or about to hibernate (Bowler and Duncan, 1969; Goldman and Willis, 1970). Wood and Beutler (1967) have shown that the Na,K–ATPase of human red blood cells is reduced 1000-fold at 4°C from its activity at 37°C and that loss of K in stored cells is independent of the ATP content of the cells. Determination of the effect of temperature on Na,K–ATPase of hibernator erythrocytes remains to be done, but would be a fruitful undertaking

since, in these cells, the enzyme activity may also be measured *in situ* in the membrane.

Control of passive permeability is even less well understood than active transport. With respect to cold adaptation a principal question must be whether the greater temperature sensitivity in hibernators depends upon metabolism, in other words, whether the difference between hibernators and nonhibernators is merely structural, resulting from, perhaps, different lipid or protein composition, or whether it is actively maintained. One result from the preceding study of stored cells suggests the former interpretation. Stored erythrocytes of ground squirrels lost K at the same rate in cells deprived of glucose as in cells with ouabain, whereas, in guinea pig cells, in which there is no evidence for active transport at 5°C (Table I), glucose deprivation caused an increased loss of K during storage (Kimzey and Willis, 1971)

On the other hand, it is becoming clear that low K permeability depends upon the maintenance of low cellular ionic Ca content which, in turn, requires a metabolically supported Ca pump (Whittam, 1968; Lew, 1970). Hence, part of the permeability adaptation of ground squirrel erythrocytes might consist of the continued function of that pump at low temperature. Thus, while the more detailed nature of cold resistance in hibernators and sensitivity in nonhibernators is still conjectural, the probability is good that the questions can be solved experimentally in erythrocytes. Since erythrocytes possess both aspects of cold resistance exhibited by other hibernator cells, they constitute an excellent model for further study.

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