# Resistance of Erythrocytes of Hibernating Mammals to Loss of Potassium during Hibernation and during Cold Storage

S. L. KIMZEY and J. S. WILLIS

From the Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801. Dr. Kimzey's present address is the Preventive Medicine Division, National Aeronautics and Space Administration Manned Spacecraft Center, Houston, Texas

ABSTRACT In two species of hibernators, hamsters and ground squirrels, erythrocytes were collected by heart puncture and the K content of the cells of hibernating individuals was compared with that of awake individuals. The K concentration of hamsters did not decline significantly during each bout of hibernation (maximum period of 5 days) but in long-term bouts in ground squirrels (i.e. more than 5 days) the K concentration of cells dropped significantly. When ground squirrels were allowed to rewarm the K content of cells rose toward normal values within a few hours. Erythrocytes of both hamsters and ground squirrels lose K more slowly than those of guinea pigs (nonhibernators) when stored in vitro for up to 10 days at 5°C. In ground squirrels the rate of loss of K during storage is the same as in vivo during hibernation, and stored cells taken from hibernating ground squirrels also lose K at the same rate. The rate of loss of K from guinea pig cells corresponded with that predicted from passive diffusion unopposed by transport. The actual rate of loss of K from ground squirrel cells was slower than such a predicted rate but corresponded with it when glucose was omitted from the storage medium or ouabain was added to it. Despite the slight loss of K that may occur in hibernation, therefore, the cells of hibernators are more cold adapted than those of a nonhibernating mammal, and this adaptation depends in part upon active transport.

It has been known since the 1940s that human erythrocytes, when stored at 5°C, are unable to maintain an ionic steady state and gradually lose K and gain Na (Harris, 1941; Maizels and Patterson, 1940). Hibernators routinely lower their body temperatures to near freezing, sometimes for many days or even weeks at a time, and the question arises as to what happens to the ionic

regulation in the red cells of these animals during this period of natural hypothermia. Curiously, in view of numerous studies of serum electrolyte concentrations in hibernators, there have been no studies of changes in ion composition of red blood cells of mammals hibernating at such profoundly low temperatures. The results on serum electrolytes are not instructive in this regard since the data are quite varied and even contradictory (for review see Fisher and Manery, 1967; Kayser, 1961; Riedesel, 1960). Moreover, changes in plasma levels of these cations would not necessarily reflect only alterations in erythrocyte concentrations, since all tissues could contribute intracellular ions to this compartment.

Active Na-K transport, of course, underlies the regulation of the ionic balance of cells. In kidney cells of hibernators, adaptation of this process to cold has been demonstrated (Willis, 1966; 1968), and the question has been raised as to whether this adaptation involves metabolism or the mechanism of transport itself. Similarily, in red blood cells the primary cause for the loss of steady state with respect to cation concentration has generally been assumed to be a failure of glycolysis resulting in a depletion of energy for the Na-K pump. Evidence for the alternative possibility, the failure of utilization of energy by the pump, has also been presented (Wood and Beutler, 1967).

Erythrocytes are perhaps the most favorable tissue for a detailed analysis of the cold resistance (or sensitivity) of electrolyte metabolism. As a first step, however, it is necessary to ascertain whether erythrocytes of hibernators in fact show a cold adaptation with respect to ion transport. In this study we have undertaken to accomplish this by determining the net change in ion concentrations of red cells in vivo during hibernation and in vitro during cold storage. A preliminary report of these data has been published (Kimzey et al., 1966).

## METHODS

Two species of hibernating mammals, the Syrian hamster and the 13-lined ground squirrel, and one nonhibernator, the guinea pig, were used in this study. The ground squirrels were trapped on the Urbana campus of the University of Illinois. The hamsters were bred in the laboratory and selected for their hibernating ability. All animals were supplied with food and water *ad libitum* and maintained at 22°–25°C on a 14 hr light: 10 hr dark photoperiod, except when the hibernators were placed in the cold room at 4°–7°C in constant darkness.

### *Collection of Blood Samples*

Blood samples from normothermic individuals were obtained by heart puncture after the animal had been anesthetized with intraperitoneal injections of pentobarbital sodium (dosage: 28, 70, and 85 mg/kg body weight for guinea pig, hamster, and ground squirrel, respectively). After the animals had reached a state of deep anesthesia, blood was drawn into cooled, heparinized syringes through disposable hypo-

dermic needles (22 gauge, 1 inch). The blood was immediately ejected into heparinized 15-ml Pyrex centrifuge tubes chilled in an ice bath. Blood samples from hibernating ground squirrels and hamsters (body temperature, 5°–7°C) were obtained in the same manner, but without the use of anesthesia. The mechanics of this operation initiated arousal in all cases. The cells were then prepared for cold storage by one of two methods described below.

In view of the known physical effects of anesthetics on cell membranes (for recent example and review see Seeman et al., 1969), the possibility existed that increased leakage might have been induced by anesthetic in erythrocytes of normothermic animals, thus decreasing their capacity for retaining K in comparison with cells of hibernating individuals which had not been anesthetized. This possibility was rendered remote, however, by the subsequent observation that rate of K loss during storage was essentially the same in hibernating and normothermic animals of the same species and that there was no difference in K retention between washed and unwashed cells.

### *Conditions of Cold Storage*

In initial cold storage experiments the red cells were not washed after removal from the animal, but were stored in their plasma diluted with an equal volume of a solution containing 150 mM NaCl, 11 mM glucose, and approximately 500 units/ml sodium-heparin (hereafter referred to as saline-glucose medium). Before storage the cells were separated from the medium by centrifugation and samples were removed for analysis of Na and K. The remaining cells were resuspended in plasma-saline-glucose medium, and the tubes were capped with Parafilm (American Can Co., Green Bay, Wis.) and placed in a water bath in a refrigerator maintained at 4°–6°C. Samples of cells were removed periodically during the storage period for analysis of intracellular cations.

In later series of storage experiments the plasma was removed immediately and replaced by an artificial medium. The composition of this medium was 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 ± 0.1.

The cells were washed three times with the appropriate storage medium at 2°–5°C using centrifugation for 3 min at 1600 *g* to separate the cellular and aqueous phases. The supernatant was then removed by aspiration and the cells were resuspended in the medium. After the initial washing process the cells were not subjected to the stress of centrifugation again. Cellular K was determined by measuring the K concentration of the suspension and of the medium and calculating the difference in these two values.

### *Analyses*

Na and K were measured by emission flame photometry using an internal Li standard, and they are expressed as milliequivalents per liter of cells. Hemoglobin determinations were made by spectrophotometric analysis of cyanomethemoglobin after treatment with Drabkin's solution and were used as an index of cell volume. Concentrations were expressed in terms of original volume.

## RESULTS

### *Loss of K from Erythrocytes during Hibernation*

Initial investigations involved measuring the K content of erythrocytes from hibernating hamsters. Blood was removed by heart puncture from hamsters which had been hibernating continuously for periods of 3–5 days at an ambient temperature of 4°–7°C. The body temperature, measured by placing a thermistor in the animal's cheek pouch, was always within 0.5°-1.0°C of the ambient temperature at the time blood was withdrawn. After the initial sample was removed for K determination, the remaining cells were resuspended in their plasma plus an equal volume of saline-glucose medium and stored at 5°C.

The K content in hamster erythrocytes after 3–5 days of deep hibernation (101 ± 3 meq/liter RBC) was not statistically different ($P > 0.05$) from that of cells of active hamsters (106 ± 3).

The question still remained as to what would happen to intracellular K levels during longer periods of hibernation. The 13-lined ground squirrel hibernates continuously under laboratory conditions for 10–14 days (Fisher, 1964). Blood samples (1–2 ml) were taken from nine individual ground squirrels after various lengths of time in hibernation at an ambient temperature of 4°–7°C. The body temperature of these animals immediately before withdrawal of the blood sample ranged from 5° to 7°C. The K concentration after various periods of in vivo exposure to low temperatures is given in Table I. A decline in intracellular K content was observed as time in hibernation increased.

The mean K concentration of erythrocytes from three ground squirrels after 3–5 days of hibernation (83 meq/liter RBC) was only slightly lower than that of cells from active animals (89 ± 3). In ground squirrels after longer periods of hibernation (9–13 days) the intracellular concentration of K (74 ± 2) was significantly lower than in active animals ($P < 0.01$).

### *Reaccumulation of K After Arousal from Hibernation*

In three of the ground squirrels from which blood was taken during hibernation, a second sample was obtained 2–10 hr after the animal had rewarmed to a body temperature of 36°C. Because of the stress of the initial heart puncture the ground squirrels were removed from the cold room and allowed to rewarm under a heat lamp. This accelerated the arousal process, and the body temperature of the animals tested reached 36°C within 0.5–1.0 hr after being removed from the cold. From the data (Table I) it can be seen that 6 hr at a body temperature of 36°C was sufficient time for the major portion of K lost during long-term hibernation to be replaced. The greatest total variation

observed in K measurements among multiple samples from the same heart puncture was less than 2%, and, therefore, the smallest increase observed due to rewarming (10%) was greater than the expected experimental error.

*Loss of K from Erythrocytes during Cold Storage*

Although hibernator erythrocytes did lose K during hibernation the rate of loss was substantially less than that reported for human cells during cold storage (Ponder, 1949; Prankerd, 1961). It is not possible to subject erythro-

TABLE I

CHANGES IN K CONTENT OF GROUND SQUIRREL ERYTHROCYTES DURING HIBERNATION AND REACCUMULATION UPON AROUSAL

| Animal No. | Days in hibernation | K content | Time at 36°C | K content |
|---|---|---|---|---|
| | | *meq/liter RBC* | *hr* | *meq/liter RBC* |
| | 0* | 89 ± 3 | | |
| GS-H9 | 3 | 90 | | |
| GS-8 | 4 | 82 | | |
| GS-H10 | 5 | 77 | 10 | 85 |
| GS-H1 | 9 | 78 | | |
| GS-H5 | 10 | 78 | | |
| GS-H3 | 10 | 75 | | |
| GS-H2 | 12 | 76 | 6 | 85 |
| GS-H4 | 12 | 70 | | |
| GS-H6 | 13 | 67 | 2 | 74 |

* Average (± SE) of samples from nine animals.

Blood samples were obtained by heart puncture on unanesthetized ground squirrels which had been hibernating at an ambient temperature of 4°–7°C for 3–13 days. The body temperature of the squirrels immediately before withdrawing the blood ranged from 5° to 7°C. In three animals (GS-H10, 2, 6) a second sample was taken after the animal had rewarmed (in 0.5–1 hr) to a body temperature of 36°C for 2, 6, or 10 hr.

cytes of normothermic mammals to low temperatures in vivo for any sustained period of time since body temperatures much below 20°C are fatal for most mammals. An alternative approach was to compare the effects of temperature on ionic balance in erythrocytes of hibernators and nonhibernators under in vitro conditions. The principal criterion for cold resistance in these studies was chosen to be the retention of intracellular K during cold storage at 5°C.

GUINEA PIG Erythrocytes of the guinea pig were suspended in their own plasma plus an equal volume of a saline-glucose medium and stored at 5°C for 10 days. Samples were removed periodically for determination of cellular K concentrations by flame photometry.

The guinea pig cells exhibited a rapid loss of K during the 10 day storage period at an average rate of 4.7 meq/liter RBC per day. The relationship between K content and day of storage was not linear but could be approximated by an exponential expression, as would be expected from the fact that the concentration gradient of K was decreasing. A complimentary increase in intracellular Na accompanied the net loss of K during cold storage in these cells (Fig. 1).

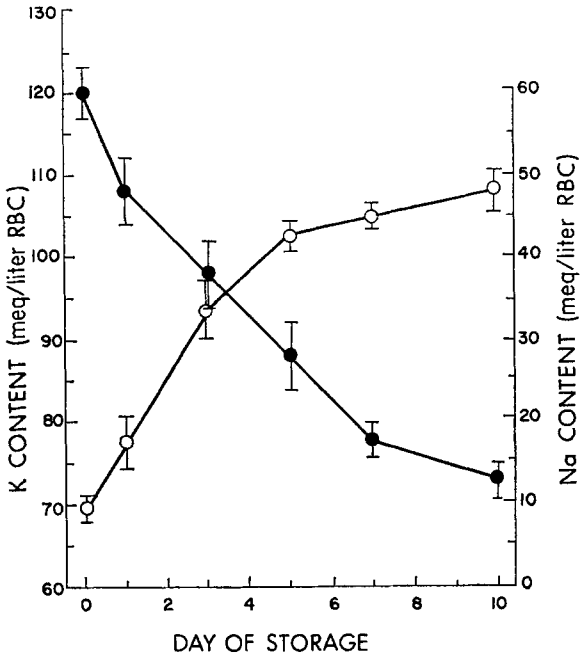


FIGURE 1. Loss of K and accumulation of Na during cold storage in erythrocytes of guinea pigs. Red cells were stored at 5°C in their own plasma supplemented with an equal volume of saline-glucose medium (154 mM NaCl, 11 mM glucose, 500 units/ml heparin). Closed circles, K; open circles, Na. Each point represents the mean of duplicate determinations from three to nine individual animals. The vertical bars are the standard error of the mean.

ACTIVE GROUND SQUIRRELS AND HAMSTERS Erythrocytes from active ground squirrels also lost K when stored at 5°C (Fig. 2), but at a substantially slower rate than did cells of the normothermic guinea pig. The average K loss over 10 days of storage was only 1.3 meq/liter RBC per day compared to 4.7 for guinea pig cells. Loss of K during the initial 24 hr period when the concentration gradient was the largest and hence the most unfavorable, was only 3.0 meq/liter RBC compared to 12.0 for guinea pig. It should be noted, however, that initial K concentration in cells of the ground squirrel was lower than that in the guinea pig. It was also significant that K loss from

ground squirrel erythrocytes during cold storage was the same as that observed after exposure of the cells to low body temperature in vivo for comparable periods of time during hibernation (Fig. 3).

Erythrocytes from active hamsters lost K during cold storage at about the same rate as ground squirrel cells (Fig. 4), with the average loss over the 10 day period being 2.0 meq/liter RBC per day.

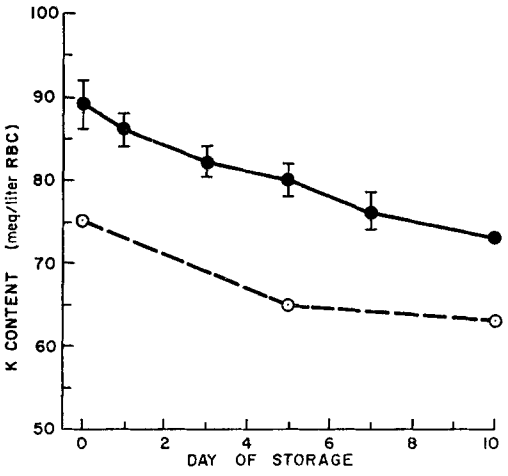


FIGURE 2

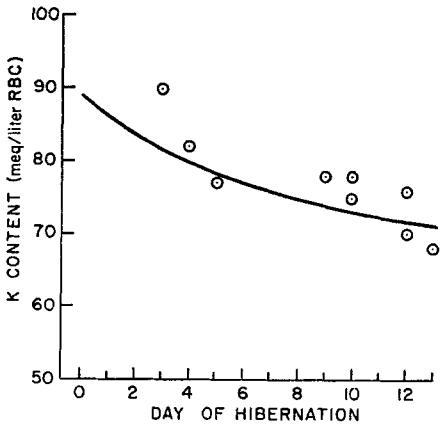


FIGURE 3

FIGURE 2. Loss of K during cold storage by erythrocytes of active and hibernating ground squirrels. The storage conditions for this experiment are the same as those described for Fig. 1. The closed circles represent the K content of cells taken from nine active ground squirrels, i.e., squirrels with a normal body temperature. The open circles connected by the broken line are the means of data from animals (two to five individuals) which had been hibernating for 10–12 days at 4°–7°C. The vertical bars represent the standard error of the mean.

FIGURE 3. K loss from ground squirrel erythrocytes during cold storage compared with loss in vivo during hibernation. The solid line is based on K loss during cold storage at 5°C (data in Fig. 2). The open circles are measurements of the K content of erythrocytes from nine individual ground squirrels which had been hibernating for 3–13 days at 4°–7°C (Table I).

For comparative purposes the data from these cold storage experiments were normalized by expressing the K content at each day of storage as per cent of the initial intracellular concentration (Fig. 5). The difference in the rate of K loss from cells of the hibernators and the nonhibernating guinea pig is immediately obvious and the difference in relative K content statistically significant ($P < 0.05$ at day 3). After 10 days of cold exposure the K concentration in guinea pig erythrocytes was reduced by 40%, while cells of the ground squirrel and hamster still had a K level that was 82% of the initial concentra-

tion. The rates of loss of K in the red cells of the two hibernating species were almost identical.

HIBERNATING GROUND SQUIRRELS AND HAMSTERS Erythrocytes from hamsters which had been hibernating for 3–5 days were stored under the same

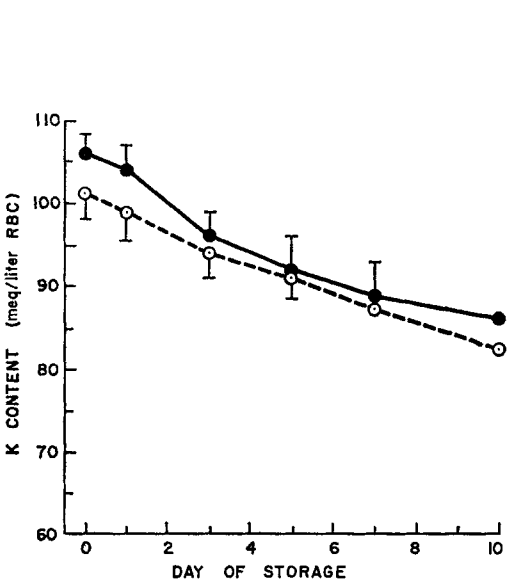


FIGURE 4

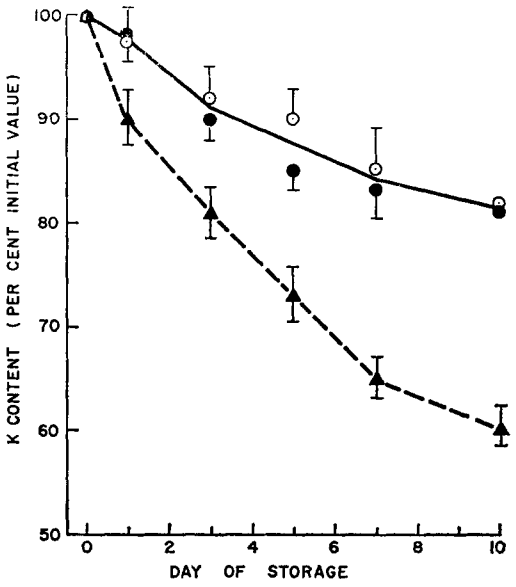


FIGURE 5

FIGURE 4. Loss of K during cold storage from erythrocytes of active and hibernating hamsters. Cells were stored under the same condition described for Fig. 1. The closed circles represent the K content of cells from seven active hamsters. The open circles connected by the broken line represent the K loss in erythrocytes from 10 hamsters which had been hibernating for 3–5 days at an ambient temperature of 4°–7°C. The vertical bars represent the standard error of the mean.

FIGURE 5. Comparison of K loss during cold storage in erythrocytes of the guinea pig, ground squirrel, and hamster. For comparative purposes, the K content for each day of storage at 5°C is expressed as per cent of the initial K concentration in erythrocytes of that species. Data in this illustration were calculated from values obtained in experiments described for Figs. 1–3. The vertical bars represent the standard error of the mean value. The symbols identify each species as follows: open circles, ground squirrel; closed circles, hamster; triangles, guinea pig.

conditions as cells from active animals (Fig. 4). Values for K concentration were slightly lower at each day of storage, but the difference was not significant. More important was the observation that the rate of K loss was identical in cells from hibernating and from awake hamsters.

Similar experiments were done with red cells from hibernating ground squirrels (Fig. 2). In this case, however, the animals had been torpid for 10–

12 days, and thus the initial value ("day 0") was significantly lower than that of cells from awake animals. However, as was the case with the hamster, the rate of K loss was similar in both sets of cells.

*Ion Transport at Low Temperatures*

Although these experiments did suggest an adaptation to low temperatures in erythrocytes of hibernators, they did not provide any information relating to the metabolic processes involved in adaptation. The following series of experiments were designed to elucidate the relative roles of active cation transport, glycolysis, and membrane permeability in the resistance to low temperatures observed in these cells.

The red cells of the guinea pig and the ground squirrel were washed three times at low temperature and stored in artificial medium with combinations of glucose (11 mM) and ouabain (0.5 mM) added. The rates of K loss in cells of both species stored in this artificial medium with glucose were essentially the same as those measured in earlier experiments in which plasma was the principal constituent of the storage medium (Fig. 6 for guinea pig and Fig. 7 for ground squirrel).

In guinea pig erythrocytes K loss in the absence of glucose was significantly greater than when the substrate was present (Fig. 6), the loss after 10 days being 82 meq/liter RBC compared to 55 in control cells. Ouabain, however, was without effect on the rate of K loss, either in the presence or in the absence of glucose. Thus, metabolism did appear to play a role in regulating K loss at low temperatures, but at some site other than the Na-K pump. In the ground squirrel either the presence of ouabain or the absence of glucose caused more than a doubling of the rate of loss of K from the cells (Fig. 7). The increased rate of K loss, comparable to that observed in the absence of metabolic energy, suggests that continued active accumulation of K in erythrocytes of ground squirrels is significant at low temperature.

An alternative approach for testing this interpretation would be to compare the observed rate of K loss in cold-stored cells with that predicted by a simple two-compartment system lacking active transport. In such a system, if the passive permeability did not change with time and the rate constants relating the rate of K influx and outflux to concentrations are assumed to be equal, the intracellular K loss is predicted by the equation (see Kimzey, 1969 for derivation)

$$[K]_{in} = \left([K]_o - \frac{B}{A}\right) e^{-At} + \left(\frac{B}{A}\right) \quad (1)$$

where $[K]_o$ represents the initial K concentration at time ($t$) zero, $B$ and $A$

represent constants derived from the rate constants, hematocrit, and suspension K concentration, and $B/A$ is the equilibrium K concentration.

The loss of K in guinea pig red cells stored with glucose corresponded closely with that predicted by the above equation (Fig. 8), both with and without ouabain. In contrast the loss of K from ground squirrel erythrocytes under

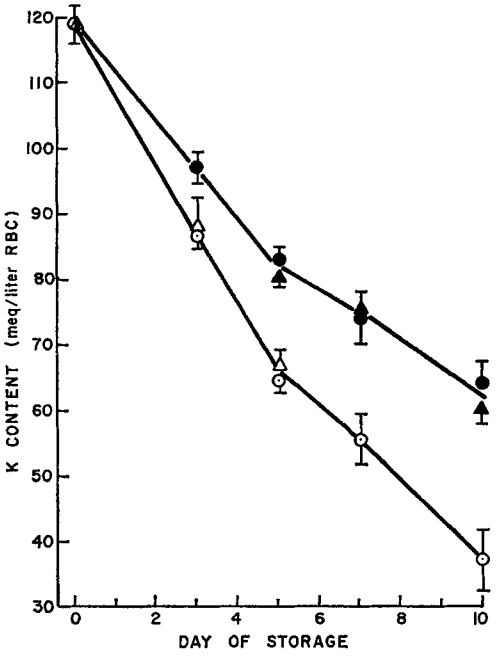


FIGURE 6

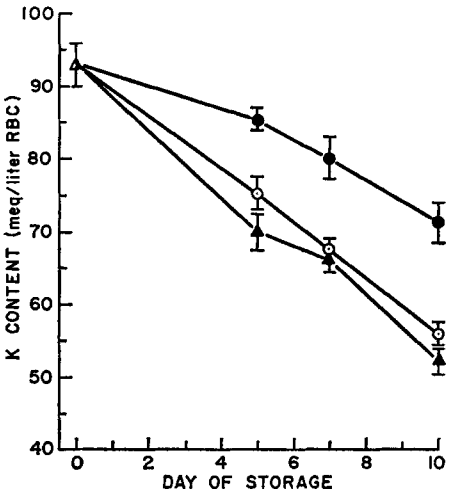


FIGURE 7

FIGURE 6. Effect of glycolysis and ouabain on K loss in guinea pig erythrocytes during cold storage. Immediately before storage, the erythrocytes were washed three times in approximately 10 vol of the appropriate medium chilled to 2°–4°C. The basic medium used (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 $NaH_2PO_4$, and 11.1 mM glucose. The storage temperature was 5°C as in previous experiments. The composition of this medium was altered only by the omission of glucose or by the addition of 0.5 mM ouabain. K loss from cells in the various storage media are represented by the following symbols: closed circles, standard incubation medium; open circles, glucose omitted; closed triangles, standard incubation medium containing 0.5 mM ouabain; open triangles, 0.5 mM ouabain present in medium with no glucose. Vertical bars represent the standard error of the mean of five determinations.

FIGURE 7. Effect of glycolysis and ouabain on K loss in ground squirrel erythrocytes during cold storage. The conditions of this experiment were identical to those described for Fig. 6. K loss from cells in the various storage media is presented by the following symbols: closed circles, standard incubation medium; open circles, standard incubation medium without glucose; closed triangles, standard incubation medium containing 0.5 mM ouabain. The vertical bars represent the standard error of the mean of five determinations.

conditions favorable for transport (i.e. glucose present, no ouabain) was much slower than that predicted by the equation (Fig. 9). In ground squirrels the loss of K fell close to the computed line only when cells were stored without glucose or with ouabain.

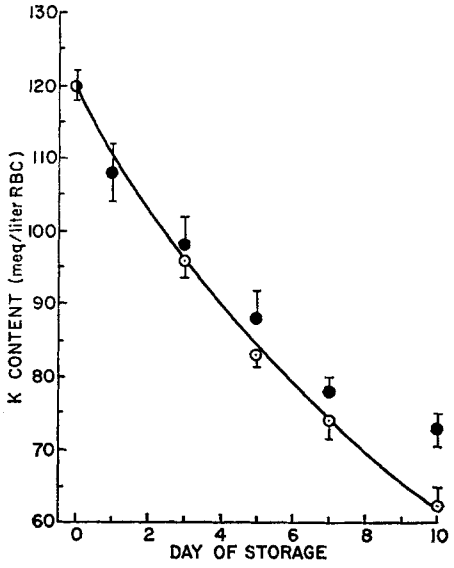


FIGURE 8

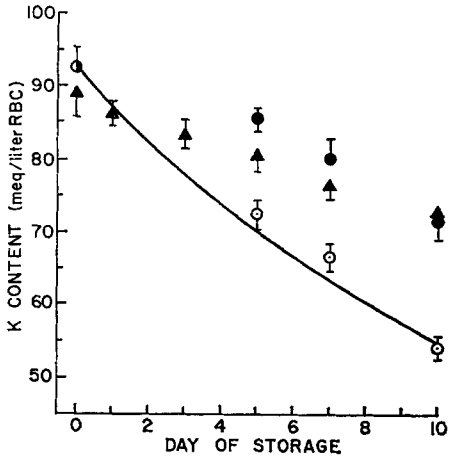


FIGURE 9

FIGURE 8. Comparison of the observed K loss from guinea pig erythrocytes during cold storage with that predicted from a model system. The solid line represents the loss of K predicted from a two-compartment model (for details see text of Results and Kimzey, 1969). Data from cold storage of guinea pig erythrocytes in plasma (closed circles) and standard incubation medium (open circles) are the same as those in Figs. 1 and 6, respectively.

FIGURE 9. Comparison of the observed K loss from ground squirrel erythrocytes during cold storage with that predicted from a model system. The solid line represents the loss of K predicted from a two-compartment model (for details see text of Results and Kimzey, 1969). Actual storage data is represented by the following symbols: closed triangles, cells stored in plasma medium (same data as Fig. 2); closed circles, cells stored in standard incubation; open circles, cells stored in standard incubation medium without glucose or in presence of 0.5 mM ouabain (see Fig. 7).

## DISCUSSION

The relatively straightforward conclusions that can be drawn from the results of this study are that:

(*a*) during cold storage erythrocytes of the hibernating species, hamsters and ground squirrels, lose K at a rate substantially slower than that of cells of the guinea pig, a nonhibernating species;

(*b*) in hibernation, erythrocytes of ground squirrels and hamsters lose K

very slowly and at a rate corresponding to that in the cells stored in vitro at a comparable temperature;

(*c*) in ground squirrels K which is lost during hibernation is quickly reaccumulated by the red cells upon arousal of the animal to a normothermic state.

Thus, red blood cells of hamsters and ground squirrels resemble cardiac and diaphragm muscle and kidney cortex cells of those species in retaining K during hibernation and exhibiting a cold adaptation of the transport mechanism (Willis, 1967). The only other previous investigation of the Na-K balance in red blood cells involving a hibernator was done with the pocket mouse, *Perognathus fallax* (Andrus et al., 1965), and indicated a significant loss of K and accumulation of Na during torpor. Those findings are not strictly comparable to the results of this study, however, because hibernation in that species is only for short periods of time and involves relatively moderate drops in the body temperature (to 17°–20°C).

Sustained ion transport is essential for the regulation of red cell volume and therefore for the prevention of osmotic hemolysis (Tosteson, 1964). In addition, it is important for the continued function of excitable membranes that increases in extracellular K resulting from loss of that ion from red blood cells as well as other cells be minimized or prevented. It is interesting that, in spite of the adaptation, there is nevertheless a slow leak of K from hibernator red cells. If this pattern is characteristic of other cells within the body, then the hibernator may in fact face the problem of an elevated serum K after several days in hibernation. It is conceivable, therefore, that alteration in K balance could be both the trigger for arousal and a factor whose correction necessitates periodic arousal (Willis, 1970).

The finding that the loss of K from ground squirrel and hamster erythrocytes during cold storage either in plasma or artificial medium was essentially identical to the loss normally occurring during natural hibernation at a comparable temperature (Fig. 5) is significant for two reasons. In the first place it shows that conditions of storage, aside from the low temperature, were not especially disruptive. Hence, this finding validates the use of in vitro storage as a means of comparing the effects of cold on erythrocytes of hibernators with red cells of guinea pig and human. Secondly, the correspondence of the K loss in plasma medium and in totally artificial medium indicates that the cold adaptation is intrinsic to the cells and does not depend on the continued presence of plasma-borne factors.

The inhibitory effect of ouabain on K loss during cold storage in ground squirrel red cells but not in those of the guinea pig suggests that active transport of K persists at low temperatures in the hibernator cells, but probably not in the guinea pig. This conclusion is strengthened by the close correspondence to the values predicted by a two-compartment model of the ob-

served K changes in guinea pig erythrocytes with or without ouabain and in ground squirrel cells with ouabain.

Despite the conclusion that energy-dependent cation transport in guinea pig cells is inactive at low temperatures, it is apparent that glucose does play a role in determining the rate of K loss in these cells. It seems reasonable that this result could be explained by metabolism having a role in maintaining low K permeability of the red cell membrane even at low temperature. This result is in accord with several recent investigations emphasizing a regulative role for glycolysis in the passive permeability of red cell membranes (Hoffman, 1962; Passow, 1963, 1964; Whittam, 1968). The finding that in ground squirrel erythrocytes glucose deprivation increases the rate of K loss only to the level of that caused by ouabain inhibition raises the possibility that different factors may be involved in regulating the passive permeability of red cells of this species.

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