Sodium Movement in High Sodium Feline Red Cells

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ABSTRACT   The transport of Na in the cat red cells has been studied under various experimental conditions. The unidirectional radioactive Na influx increased with increasing temperature until it reached a maximum value at $37°C \pm 2°C$ and then decreased with a further increase in temperature. Errors stated in this paper represent 1.0 standard errors of the mean. The apparent activation energy was calculated in the region between 25 and $37°C$ and was found to be 4.9 ± 0.5 kcal/mole. Copper at a concentration of 0.04 mM inhibited this influx by 65%. When cells were suspended in isosmotic KCl buffer, cell volume was found to decrease initially with time. This unusual behavior is discussed in terms of Na to K preference of the cell membrane. In cat red cells, Na influx was found to increase about 13-fold when cell volume was decreased from 1.16 normal to 0.87. This effect could not be reproduced when the medium osmolarity was changed only by the addition of urea, a permeating molecule. On the other hand, K influx was found to decrease from 0.24 ± 0.03 mEq/liters RBC, hr at a relative cellular volume equal to 1.0 to 0.11 ± 0.01 mEq/liters RBC, hr at a cell volume of 0.75. Na influx in human red cells did not show any significant dependence on cell volume. The properties of Na movement in the cat red cells are compared to those of human red cells.

INTRODUCTION

The majority of mammalian red cells investigated maintain significant concentration gradients of Na and K across their plasma membranes (1). These gradients are maintained by the "sodium pump" which is driven by metabolic energy, through the hydrolysis of ATP by membrane-associated ATPase. Some animal species, such as the cat and dog have red cells whose cellular Na and K approach that of the plasma; i.e., high Na low K cells (1). In addition, electrolyte transport mechanisms in these cells exhibit certain peculiarities which are not found in other red cells. For example, there is no measurable ouabain-sensitive component for Na efflux or K influx (2). Membrane fragments do not show any significant Na-K–dependent ATPase (3). Moreover,

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Na movement in these cells is very critically dependent on cell volume (4, 5) and on the nature of the monovalent anion present in the bathing medium (2, 6). A less than 10% change in cell volume causes a significant change in Na fluxes. Replacement of Cl ion by any member of the lyotropic series will significantly change both the influx and efflux of Na.

In a previous paper Sha'afi and Lieb (2) reported experiments which dealt with ^{24}Na and ^{42}K fluxes in cat red cells under steady-state conditions. The present paper deals with the transport of Na in these cells under various experimental conditions.

MATERIALS AND METHODS

Blood was withdrawn into a heparinized syringe (10,000 units/ml; 4 ml/liter) from anesthetized cats by heart puncture. It was immediately centrifuged for 15 min at 1500 g and the plasma and buffy coat were discarded. Special care was taken to remove white cells. The red cells were then washed three times in 4 volumes of a saline medium which had the following composition in mM: NaCl, 150; KCl, 5.0; MgCl$_2$, 1.0; CaCl$_2$, 0.25; Na$_2$HPO$_4$, 5.0; NaH$_2$PO$_4$, 1.0; glucose, 11.1 (pH = 7.4). The washed red cells were then resuspended in the incubation medium. After 15 min of equilibration radio-active tracer was added. After thorough mixing for approximately 1 min, aliquots were removed for hematocrit determination and radioactivity analysis. The suspension was then incubated in a shaker in a water bath at the desired temperature. The temperature was controlled to within 1°C. At specified intervals thereafter, samples were removed for various analyses. The details of flux calculations, trapped space measurements, counting of radioactivity, and sample handling were thoroughly discussed in a previous paper (2). This general procedure was followed unless otherwise specified.

Cell volume was altered either by the reduction of NaCl in the medium to make it hypotonic or by the addition of sucrose to make it hypertonic. Relative cell volume was calculated from the resulting final osmolarity of the medium. The osmolarity was measured by means of a Fiske Osmometer (Osmometer Model G-62, Fiske Associate Inc., Bethel, Conn.). Hemoglobin was determined from the optical density at 540 mμ after conversion of hemoglobin to cyanmethemoglobin.

RESULTS

Dependence of Na Influx on Temperature

Radioactive Na influx in cat red cells has been measured over the temperature range of from 25° to 45°C. The flux increases with increasing temperature until it reaches a maximum value in the neighborhood of 37°C ± 2°C, and then decreases as the temperature is increased further. The results are shown in Fig. 1. Each point is the average of at least three experiments. Note that the flux passes through a maximum, a behavior which is characteristic of enzymes. In the rising phase of the curve, from 25° to 37°C, the logarithm of the flux varies linearly with the reciprocal of the absolute temperature. The apparent activation energy calculated from this linear region is 4.9 ±

0.5 kcal/mole. This value is three to four times less than the corresponding value for human red cells (7, 8).

Effect of Copper on ²⁴Na Uptake

In these experiments, packed red cells were added to buffered solution containing 0.04 mM of cupric chloride. The suspension was shaken for about
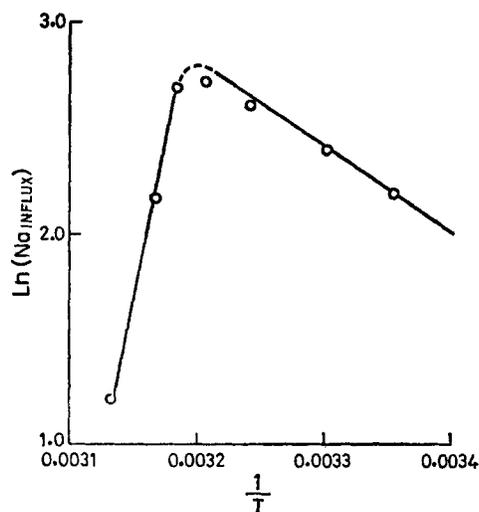

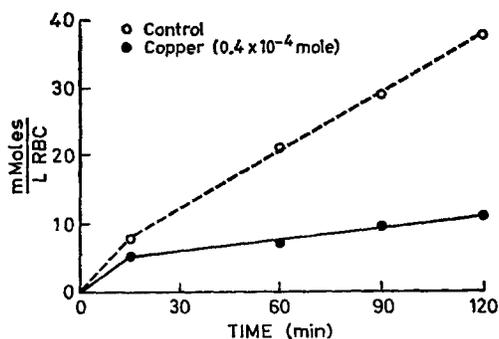FIGURE 1. Temperature dependence of Na influx in cat red cells.

FIGURE 2. Effect of copper on Na influx.

a minute and then radioactive Na was added. This concentration was chosen because it produced maximal inhibition of Na influx without producing any cell hemolysis. This concentration was found to inhibit the uptake of ²⁴Na in these cells by 65% (four experiments). The results of a typical experiment are shown in Fig. 2.

Dependence of Na Influx on Cellular Volume

Na influx in the cat red cell is critically dependent on cellular volume. The flux increases with decreasing volume and decreases with increasing volume. The interdependence between Na fluxes and cellular volume was studied. Hypertonic solutions were obtained by adding different amounts of sucrose to the incubation medium. Cat red cells have been shown to be impermeable to
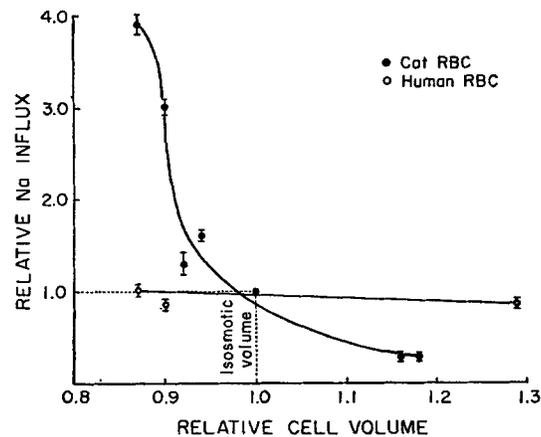

FIGURE 3. Dependence of Na influx on cellular volume.

TABLE I

DEPENDENCE OF K INFLUX ON CELL VOLUME*

Relative cell volume	⁴²K influx
	mEq/liters RBC, hr
1.0	0.24 ± 0.03 (3)
0.95	0.17 ± 0.03 (4)
0.86	0.16 ± 0.02 (4)
0.82	0.14 ± 0.05 (4)
0.75	0.11 ± 0.01 (3)

* The numbers in parentheses refer to the number of determinations. Errors are standard errors of the mean.

sucrose (4). In the present experiments, cells were incubated in these solutions for 25 min before radioactive Na was added. This time was chosen since no significant changes in suspension hematocrits were observed after 25 min of incubation. The results of these experiments are shown in Fig. 3. Each point represents the average of at least four determinations. The relative cell volume was calculated from the following relation (9):

$$\frac{V}{V_o} = 1 + 0.72 \ (T_o/T - 1)$$

in which V_o and V are the cell volumes at 290 milliosmols and any test osmolality, respectively, and T_o and T are the osmolalities of isosmotic solution (290 milliosmols) and the test solution. It is clear from the graph that the behavior of Na influxes with respect to changes in cell volume for human and cat red cells is quite different. Lieb has shown that Na efflux in cat red cells is also dependent on cell volume (4).

The variation of ^{42}K influx with cellular volume is quite the reverse. Decreasing cell volume causes a decrease in ^{42}K influx. The results are shown in Table I.
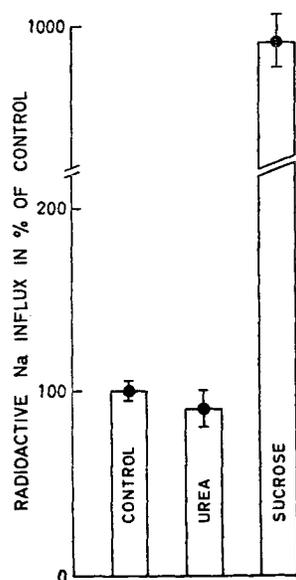

FIGURE 4.   Effect of changing medium tonicity by permeating molecule on Na influx.

One other facet of the cell-shrinking effect was investigated. Parker and Hoffman (5) have shown that the volume effect on Na and K movements in dog red cells depended only on cell water content. These experiments suggested that osmolarity itself does not influence the volume effect. We have confirmed this idea in cat red cells by showing that an increase in osmolarity, through the use of urea, a molecule to which the cell is very permeable, does not produce an increase in Na flux. In these experiments, two buffered solutions of identical osmolarity were prepared by the addition of (a) 60 mM urea and (b) 60 mM sucrose to the usual isotonic buffer. Samples of cells were suspended in each of these solutions for 25 min before the addition of radioactive Na. By using urea it is possible to change medium tonicity without changing cell volume. The results of three experiments are shown in Fig. 4.

It is clear that increasing only the tonicity is not sufficient to produce an increase in Na influx.

Effect on Cell Volume of Replacement of the Outside Na by K

A great deal can be learned about the nature of Na movement in these cells by studying the variation of radioactive Na influx with medium Na concentration. Unfortunately, replacement of Na by either Li or K causes significant changes in cell volume. This in turn complicates the interpretation of the results, since changes in cell volume will influence Na fluxes in these cells.

The time course of cell volume change, when cells were placed in isosmotic buffered saline in which NaCl was replaced by KCl, was measured using the hematocrit method. Hematocrit was determined by spinning a small sample of the red cell suspension at 13,000 g for 5 min using a capillary centrifuge (model MB). This combination of speed and time was sufficient to ensure
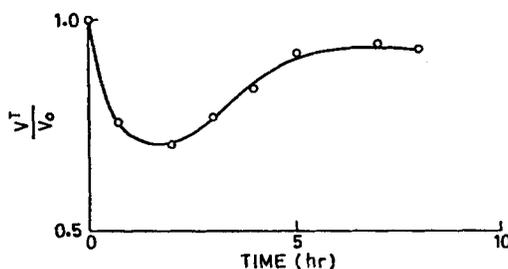

FIGURE 5. Time course of cell volume. The cells were suspended in isotonic medium in which NaCl was replaced by KCl.

maximum packing. The length of the packed column relative to that of the whole column was measured with a capillary tube reader (model CR). When the cat red cells were placed in an isosmotic medium in which the NaCl was replaced by KCl it was found that the cell volume initially decreased to about 70% of its initial value and then increased. Fig. 5 shows the results of three experiments. The initial decrease in cell volume suggests that the net outward movement of Na is greater than the simultaneous inward movement of K.

When we know the inside and outside concentrations of Na and K and when we assume that the membrane potential is given by the chloride ratio, we can verify this assumption by direct measurement of the membrane potential in human red cells (10), and can calculate the difference in electrochemical potential, $\Delta\mu$, for both Na and K using the following relation:

$$\Delta\mu_j = RT \ln(C_j)^o/(C_j)^i + ZF\Delta\psi$$

in which $\Delta\mu_j$ is the electrochemical potential difference for the jth ion, $(C_j)^i$ and $(C_j)^o$ are the concentrations of the ion inside and outside the cell, respectively, R and T have their usual meanings, Z is the valence, F is the Faraday constant, and ψ is the electrical potential difference across the membrane. With the use of the above equation the absolute value for the electrochemical potential difference for K ion, $|\Delta\mu_K|$, under the present experimental condition is 3.3 RT driving the K ion into the cell and the corresponding value for Na, $|\Delta\mu_{Na}|$, is 2.3 RT driving Na outward. Since $|\Delta\mu_K|$ is greater than $|\Delta\mu_{Na}|$ and the cell still shrinks initially, then the rate of outward movement of Na must be greater than the inward movement of K. These observations are consistent with the earlier findings using [24]Na and [42]K (2). This behavior is not found in most other mammalian red cells. Tosteson showed that both types of sheep red cells (HK, LK) would swell under similar experimental conditions (11).

The data in Fig. 5 can be used to calculate a rough estimate of the ratio of Na permeability, P_{Na}, to K permeability, P_K, in these cells. However, a better and more useful approach will be to calculate the ratio, $P_{Na}:P_K$, in these cells from unidirectional flux measurements reported in reference 2. Because of minimal differences in experimental conditions the $P_{Na}:P_K$ ratio thus calculated can be directly compared to the corresponding ratios obtained in the red cells of other species. The $P_{Na}:P_K$ in cat red cells is 3 whereas the corresponding values for human, high potassium sheep, and low potassium sheep red cells are: 0.06, 0.10, 0.36, respectively. These last three values were calculated from the values of fluxes reported by Solomon (8) and Tosteson and Hoffman (12).

Dependence of the Rate of Na Escape from the Cell on Inside Na Concentration

Since the concentration of Na cannot be varied either at the inside or the outside without a significant change in cell volume, the tracer method is not very helpful. Therefore, another method must be used to investigate the dependence of fluxes on Na concentration. The alternative approach employed was based on measuring the time course of cell Na concentration when these cells were placed in isosmotic buffer in which NaCl was replaced by KCl. Na concentration was measured by an internal standard type flame photometer. Trapped fluid was measured as discussed in a previous paper (2). Since the cells were placed in high K medium the contribution of trapped fluid to the measurement of Na was negligible (less than 1%). The suspension hematocrit was made very low (5%) in order to minimize changes in Na and K concentrations of the suspending fluid during the course of the experiment. At the end of the experiment, Na concentration of the bathing medium increased from 5 to 11 mM. Cell Na concentration was measured every 20 min starting from the time when the cell was placed in KCl-buffered solution.

Initially, two ways were used to calculate the rate of net Na exit. The first method used was to draw a curve by eye through the experimental points and then to calculate the tangent of the curve at each desired internal Na concentration. Even though this method was quite easy it was not very satisfactory. The second method was to fit empirically the time course of cell Na by an equation of the following form (13):

$$C_1 \ln[(Na)_i^o/(Na)_i^t] + C_2[(Na)_i^o - (Na)_i^t] = t$$

in which $(Na)_i^o$ and $(Na)_i^t$ are the cell Na concentrations initially and at any given time respectively, t is time, and C_1, C_2 are two adjustable constants.
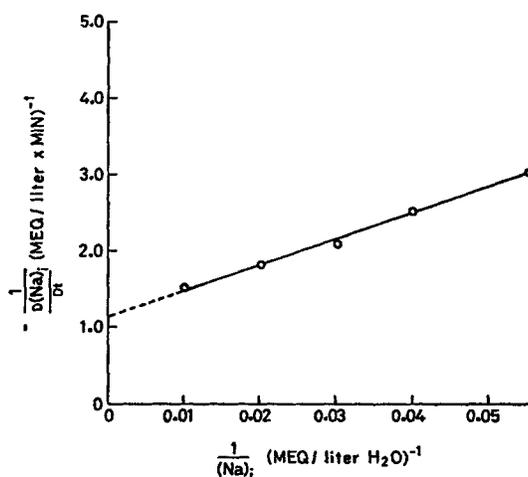

FIGURE 6.   Dependence of cellular Na concentration on the rate of Na exit in cat red cells.

There is no theoretical basis for this equation. The rate of Na exit, $d(Na)_i^t/dt$, can be easily calculated from this equation. The fit was good to within 10% in the region after $t = 45$ min. This is the region which was of interest to us since the cell volume was not changing rapidly (see Fig. 5). Fig. 6 shows a double reciprocal plot between $d(Na)_i^t/dt$ and $(Na)_i^t$. In this case, $C_1 = 30$ min and $C_2 = 1.25$ (mM/liter H_2O, min)$^{-1}$. The rate of Na escape is probably more dependent on the inside Na concentration than appears in the graph because as $(Na)_i^t$ decreases, so does the cell volume. This decrease in cell volume will cause an increase in the rate of Na movement. In other words, the rate calculated in Fig. 6 is the result of two opposite effects, the change in $(Na)_i^t$ and the change in cell volume. This linear variation suggests that the Na transport system in these cells possesses a finite number of receptor sites for Na. The effect of temperature on the relationship observed in Fig. 6 was

also studied. The results are shown in Fig. 7. Each line represents the average of three experiments. These results are quite puzzling since it appears from the lines shown in the figure that the temperature dependence of net Na escape is also concentration dependent. No adequate explanation can be given
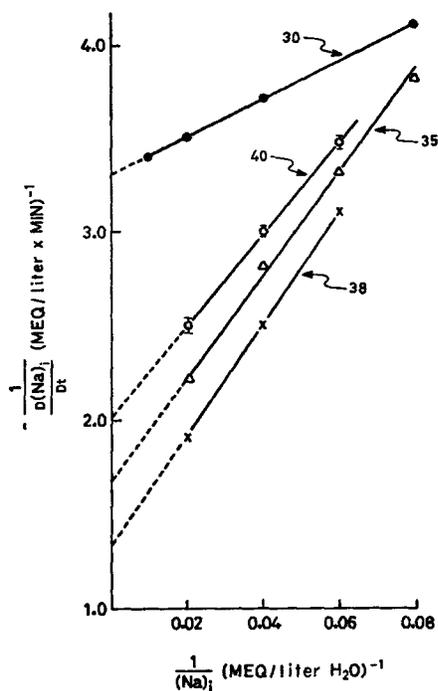

FIGURE 7.   Effect of temperature on the relationship between cellular Na concentration and Na exit.
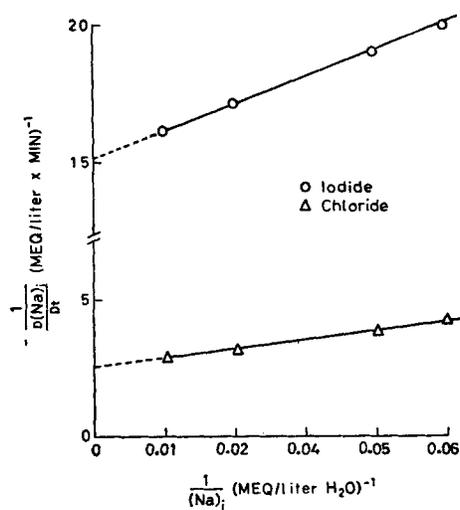

FGIURE 8.   Effect of iodide on the relationship in Fig. 6.

at the present time. One possible explanation which is currently being investigated is that the interdependence between Na flux and temperature is also volume dependent and that what we are seeing in Fig. 7 is the result of this dependence.

In an earlier paper, Sha'afi and Lieb showed that Na influx in cat red cells is very much dependent on the nature of the monovalent anion present in the incubation medium (2). Replacement of Cl^- ion totally or partially by any member of the lyotropic series (I^-, Br^-, CNS^-) inhibited dramatically (>50%) Na influx. Furthermore, these authors showed that this inhibition of the Na transport system was a feature peculiar to the cat and dog red cells. In order to investigate this observation further, the effect of iodide on the dependence of the rate of sodium escape on cellular Na was studied. The result of a typical experiment is shown in Fig. 8. Note that in the presence of iodide, both the slope and the intercept of the line were changed.

DISCUSSION

The observed inhibition of Na influx in the presence of 0.04 mM copper can be due to a conformational change in the cell membrane caused either by the direct effect of copper or by a glycolytic intermediate whose concentration has changed due to the presence of copper. Another equally possible explanation is that the presence of copper lowers the level of cell ATP which may decrease Na transport. At first instance, the second hypothesis seems more feasible since copper is known to interfere with cell metabolism. The results in Fig. 2 show clearly that the action of copper is apparent as early as 15 min. During this short time interval the cell would not have exhausted a major portion of its energy supply. Therefore these findings do not support the latter explanation.

It is instructive to compare the characteristics of cat red cell transport with those of the human red cell in order to gain insight into the difference on the molecular level between the two mechanisms. In man the ratios of concentration in cell water to that in plasma water are: Na = 0.12, K = 27.4, and Cl = 0.69 = $(1.45)^{-1}$. The corresponding values for the cat are: Na = 0.9, K = 1.7, and Cl = 0.75 = $(1.33)^{-1}$. The chloride ratio in both the human and the cat fits with the expected passive Cl distribution computed from the observed potential difference of −8 mv (10). In human red cells, the difference of more than an order of magnitude between the Cl ratio and those of Na and K indicates that an active transport mechanism is operating in these cells. This is consistent with the sensitivity of the transport system to ouabain and the presence of Na-K–sensitive ATPase in the membrane (3). The situation in the cat red cell is apparently different since the Na ratio is lower by a factor of 1.5 and the K ratio is higher by a factor of 1.2 than the Cl ratio. These ratios are very much closer to the Cl ratio than is the case in human red cells. This implies that a large component of cation transport in these cells is

passive insofar as an energy requirement to drive the system is concerned. However, there still must be a regulated component of some magnitude to account for the difference from the Cl ratio.

The large $P_{Na}:P_K$ ratio in cat red cells is the result of a high Na flux and an absence of an ouabain-sensitive component for K influx (2). Even though the total value of K influx (see Table I and reference 2) in cat red cells is only about 15% of the total value of K influx in human red cells, the ouabain-insensitive component in the former cells is considerably larger than the corresponding value in the latter cells. It is generally accepted that the selectivity of mammalian red cell membrane to cations and anions is due to the presence of "pores" with positive fixed charges (14). If the diameter of these postulated pores is larger in cat red cell membrane than in human red cell membrane, then one could predict high values of ouabain-insensitive Na and K fluxes since the positive charges would be less effective in hindering cations because the charge density would be smaller. Furthermore, if we assume that Na-K selectivity of these pores depends on how well the ion fits the pore (15), then we would expect the cat red cell membrane to be more selective for Na. There are two lines of evidence which may be taken to support the idea of larger pore size. First, osmotic water permeability in cat red cells is considerably higher than in human red cells (16). Second, sulfate permeability appears to be considerably lower in cat red cells.[1] The low sulfate permeability is taken as a reflection of a decrease in charge density as a result of larger pore size.

A possible working hypothesis is to postulate that Na and K in cat red cells are regulated by means of a finite number of specialized "sites" or pores located in the cell membrane. These postulated pores are quite similar to those controlling ouabain-insensitive components of Na and K fluxes in human red cells. They differ only in that they are adapted more to Na than to K because of their sizes. The degree of selectivity between these two cations is under metabolic control. Furthermore, any perturbation which may affect membrane configuration can produce a shift in Na-K selectivity. According to this hypothesis the volume effect is the result of a change in the concentration of some metabolic intermediate(s) which may cause a generalized conformational changes in the membrane. The effects of the anions of the lyotropic series on the influence of both Na and K (2) are consistent with this hypothesis. More so since the order of effectiveness of these anions appears to be related to their relative adsorbabilities to the cell membrane. The greater the degree of adhesion the greater is the effect on Na and K movements. This adhesion might well cause a weakening of certain electrostatic interactions important for the stability of the membrane. This may change the shapes, sizes, and charge characteristics of these specialized pores. All the other findings which are reported here and in the previous paper (2) can be ex-

[1] Sha'afi, R. I. Data to be published.

plained by this hypothesis. It is well-recognized that this hypothesis is quite general and can be adapted to accommodate a large spectrum of results. Nevertheless, it is fundamentally different from the mechanism proposed by Romualdez et al.[2] These authors postulate a direct coupling between cation transport and glucose metabolism, other than through energy requirement, but by a glucose carrier which transports Na into the cell along with glucose or some of its products similar to the coupling between glucose and Na in the distal rabbit ileum (17). One possible experiment to distinguish between these two mechanisms and to further elucidate the role of changing cellular volume in Na and K transport is to study Na and K movements in reversible ghosts. This experiment has met with failure due to the difficulties in preparing reversible ghosts for cat red cells.

If this hypothesis is proven to the correct, it would raise interesting questions about the physiological significance of such a transport system. Also why do only the red cells of cats and dogs of all the mammalian red cells investigated, have such a transport mechanism? Lieb in his Ph.D. thesis noted that of the beaver, pig, man, kangaroo, rat, jerboa, *Psammomys* (a desert rodent), cat, and dog only the latter three animals have 100% long-looped nephrons (4). It is well-known that these loops of Henle set up a very hypertonic solution in the medullae of the kidneys of these animals (18). It is also known that these loops penetrate much deeper into the medullae than do short loops (18). If it turns out that the red cells of the *Psammomys* have a transport system similar to that found in the red cells of the dog and cat, one would be tempted to postulate a connection between these two observations.

BIBLIOGRAPHY

1. Bernstein, R. E. 1954. Potassium and sodium balance in mammalian red cells. *Science* (*Washington*). 120:459.
2. Sha'afi, R. I., and W. R. Lieb. 1967. Cation movements in the high sodium erythrocyte of the cat. *J. Gen. Physiol.* 50:1751.
3. Chan, P. C., V. Calabrese, and L. S. Theil. 1964. Species differences in the effect of sodium and potassium ions on the ATPase of erythrocyte membranes. *Biochim. Biophys. Acta.* 79:424.
4. Lieb, W. R. 1967. Interactions of Sodium Transport and Cellular Volume in the Cat Erythrocyte. Ph.D. Thesis. University of Illinois, Urbana.
5. Parker, J. C., and J. F. Hoffman. 1965. Interdependence of cation permeability, cell volume, and metabolism in dog red cells. *Fed. Proc.* 24:589.
6. Davson, H. 1940. The influence of the lyotropic series of anions on cation permeability. *Biochem. J.* 34:917.
7. Clarkson, E. M., and M. Maizels. 1955. Sodium transfer in human and chicken erythrocytes. *J. Physiol.* (*London*). 129:476.
8. Solomon, A. K. 1952. The permeability of the human erythrocyte to sodium and potassium. *J. Gen. Physiol.* 36:57.

[2] Romualdez, A., R. I. Sha'afi, and A. K. Solomon. Data to be published.

9. PONDER, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton Inc., New York. 84.

10. LAY, A. W. L., and A. C. BURTON. 1969. Direct measurement of potential difference across the human red blood cell membrane. *Biophys. J.* **9**:115.

11. TOSTESON, D. C. 1964. Regulation of cell volume by sodium and potassium transport. *In* The Cellular Functions of the Membrane Transport. J. F. Hoffman, editor. Prentice-Hall Inc., Englewood Cliffs, N.J. 3.

12. TOSTESON, D. C., and J. F. HOFFMAN. 1961. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol.* **44**:169.

13. SHA'AFI, R. I. 1965. Kinetics of Ion Movements across the Cell Membrane of Cat Erythrocytes and the Phenomenon of Active Transport. Ph.D. Dissertation. University of Illinois, Urbana.

14. PASSOW, H. 1969. Passive ion permeability of the erythrocyte membrane. *Progr. Biophys. Mol. Biol.* **19**:425.

15. MULLINS, L. J. 1961. The macromolecular properties of excitable membrane. *Ann. N.Y. Acad. Sci.* **94**:390.

16. RICH, G. T., R. I. SHA'AFI, T. C. BARTON, and A. K. SOLOMON. 1967. Permeability studies on red cell membranes of dog, cat, and beef. *J. Gen. Physiol.* **50**:2391.

17. SCHULTZ, S. G., and R. ZALUSKY. 1964. Ion transport in isolated rabbit ileum. II. The interaction between active sodium and active sugar transport. *J. Gen. Physiol.* **47**:1043.

18. PROSSER, C. L., and F. A. BROWN, JR. 1962. Comparative Animal Physiology. W. B. Saunders Company, Philadelphia. 2nd edition. 44.