# Further Studies of Sodium Transport in Feline Red Cells

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ABSTRACT The transport of radioactive sodium in high sodium cat red blood cells has been studied under various experimental conditions. It was found that iodoacetate (IAA) and iodoacetamide (IAM) inhibit Na influx by 50% whereas NaF has no effect. Reversible dyes, such as methylene blue (Mb), also inhibit this influx by 60%. Both IAA and Mb effects show a lag period of about 40 min. Cell starvation abolishes the volume-dependent Na influx which is generally observed in these cells. IAA reduces significantly the volume-dependent Na influx but does not inhibit it completely. 5 mM magnesium chloride produces a twofold increase in Na influx. On the other hand, $MgCl_2$ has no effect on Na transport in human red cells or on potassium or sulfate transport in cat red cells. The effect of $MgCl_2$ is quite rapid and does not interfere with the volume-dependent Na influx. This effect is abolished in starved cells. Reincubation of previously stored cells in buffered solutions containing glucose and $MgCl_2$ causes more than one order of magnitude increase in Na influx. These several observations are discussed in terms of the possibility of a link between Na transport and Na-Mg-activated ATPase.

## INTRODUCTION

Red blood cells of some mammalian species, such as cat and dog, have high intracellular concentrations of sodium and low potassium (1). This is in contrast to the majority of mammalian red cells investigated (1, 2). In addition to this unusual ion distribution, the transport mechanism of these univalent ions shows characteristics which differ significantly from those of human and other mammalian red cells. For example, in these high Na red cells, cations, anions, and nonelectrolytes, movements are very sensitive to changes in cell volume (3–6). This phenomenon is of particular interest since it appears to be unique to the above two species. Recently, however, Pozansky and Solomon have shown that K influx in human red cells is reversibly dependent on cell volume (7). In addition, electrolyte transport mechanisms in these cells exhibit certain peculiarities which are not found in other red cells (3, 6). There

is no measurable ouabain-sensitive component for Na efflux or K influx (8). Membrane fragments do not show any significant Na-K-dependent ATPase (9).

It is postulated (3, 6) that volume changes lead ultimately, either directly or through a change in the concentration of some metabolic intermediate(s), to a conformational change in the red cell membrane. Furthermore, it has been suggested that some metabolic intermediate, probably triose phosphate or an intermediate which depends on triose phosphate for its production, regulates Na and K transport in these cells (6).

The present studies were undertaken to characterize further the Na transport mechanism in these cells and also to try to identify the metabolic intermediate which may be responsible for regulating Na and K movements in these and probably other high Na cells, such as dog.

## MATERIALS AND METHODS

Blood was withdrawn into a heparinized syringe (10,000 U/ml, 4 ml/liter) from anesthetized cats by heart puncture. The blood was centrifuged for 15 min at 1500 *g*, and the plasma and buffy coat were carefully removed. The red cells were then washed three times in 4 vol of incubation medium, which had the following composition in millimoles/liter: NaCl, 150; KCl, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 0.25; $Na_2HPO_4$, 5.0; $NaH_2PO_4$, 10; glucose, 11.1 (pH = 7.4). The washed red cells were resuspended in the incubation medium at 10% hematocrit and were incubated for 15 min in a water-bath shaker at 38°C before tracer was added. After addition of the tracer, the suspension was thoroughly mixed for approximately 60 s and then portions were removed for hematocrit determinations and medium radioactivity analysis. At specified intervals thereafter, portions of the suspension were removed. The erythrocytes were separated from the labeled medium by centrifugation and washed three times in ice-cold isotonic NaSCN. NaSCN was used to prevent the escape of $^{22}Na$ during the washing process (8). The amount of $^{22}Na$ lost during the washing is less than 5%. The packed red cells were then hemolysed with distilled water (1 ml packed cells to 20 ml water). A known volume of this hemolysate was taken for hemoglobin measurement and another for isotope counting. Samples were prepared for counting and for hemoglobin measurement as discussed previously (3). This general procedure was followed unless otherwise specified.

Lactate production was measured in samples of red cell suspension at the start of the experiment and after 4 h of incubation. The lactate content of perchloric acid treated samples were determined enzymatically by conversion of NAD to NADH in the presence of lactic dehydrogenase. Cell water content was measured by drying the cells for 48 h at 60°C. Intermittence measurements were carried out at 24, 40, and 48 h. No change in the value of cell water content was observed after 40 h of drying.

Cellular volume was decreased by adding increasing amounts of sucrose to the isotonic buffer, and increasing cell volume was affected by reducing NaCl concentration. The relative cell volume was calculated from the following relation (10–12):

$$V_c/V_{co} = 1 + W_{\text{eff}}\left(\frac{T_o}{T} - 1\right),$$

in which $V_{co}$ and $V_c$ are the cell volume at osmolality $T_o$ and any test osmolality $T$. $W_{eff}$ is the cellular water which apparently participates in an osmotic phenomenon. The value of $W_{eff}$ was determined by plotting hematocrits vs. $T_o/T - 1$. In five experiments the relative hematocrits vary linearly in the range from 0.8 to 1.2 with $T_o/T - 1$. The slope of this linear relation has a slope of 0.715 ± 0.05. It is interesting to note that if NaCl was used instead of sucrose to decrease cell volume the relation was still linear but the slope was 0.65 ± 0.03. The later value is in good agreement with the value determined previously for these cells by Rich et al. (12). The difference between the two values 0.715 and 0.65 is probably due to the change in chloride ion (13). The possible inherent error in the above equation is well recognized particularly in light of the recent novel explanation proposed by Gary-Bobo and Solomon for the apparent anomalous osmotic behavior of human red cell (13). In spite of this we felt that Eq. 1 is quite adequate for our purpose for two reasons: First, the apparent fraction of osmotically inactive water, if present, in these cells is definitely less than 10% of the total water. This is certainly small in view of the large variations in Na influxes in these cells. Second, the exact quantitative variation of Na influx with cell volume is not of central importance in these studies.

## RESULTS AND DISCUSSION

### *Effects of Iodoacetate, Iodoacetamide, and Fluoride on $^{22}Na$ Uptake*

In these experiments the cells were incubated for 1 h with the compound under study before $^{22}Na$ was added. Influx was calculated from the amount of $^{22}Na$ present in the cells 2 h after the addition of the isotope. As was observed in previous studies, there was considerable variation among blood samples obtained from different cats. $^{22}Na$ influx varied from 8 meq/liter RBC·h to 17 meq/liter RBC·h. The effects of iodoacetate, 1 mM, and sodium fluoride, 1 mM, on Na influx in cat red cells are shown in Table I. It is quite clear from the table that inhibitors of the enzyme triose phosphate dehy-

TABLE I

EFFECT OF IODOACETAMIDE (IAM), IODOACETATE (IAA), *p*-CHLOROMERCURIPHENYLSULFONATE (PCMBS), AND SODIUM FLUORIDE (NaF) ON $^{22}Na$ UPTAKE IN CAT RED CELLS

| Condition | Concentration | Relative $^{22}Na$ influx* |
|---|---|---|
| | *mM* | |
| Control | | 1.00 (9) |
| IAM | 1 | 0.48±0.06 (9) |
| NaF | 1 | 0.90±0.08 (7) |
| IAA | 1 | 0.40±0.07 (7) |
| PCMBS‡ | 0.1 | 3.00±0.40 (3) |

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

‡ 1 mM of PCMBS causes significant hemolysis in these cells.

drogenase (IAM, IAA) significantly reduce Na influx. On the other hand, the enolase inhibitor, NaF, has very little effect on the transport of Na. Increasing the NaF concentration to 10 mM produces a slight increase in the Na influx. This is probably due to a direct effect of fluoride on the membrane and is independent of intracellular events. This is similar to the effect on passive cation transport found in human red cells (14).

Both IAA and NaF interfere with energy production in mammalian red cells as shown by the marked reduction in cellular ATP (15). However, of these two compounds only IAA produces a significant inhibition of $^{22}Na$ uptake. This suggests that the major component of Na flux in cat red cells is directly dependent on the integrity of some portion of the glycolytic chain.

The time-course of the onset of inhibition of $^{22}Na$ uptake in the presence of IAM or IAA was also studied. In these experiments $^{22}Na$ and IAA were added to the red cells simultaneously. The results of a typical experiment are shown in Fig. 1. Even though inhibition was evident as early as 10 min, the full inhibitory effect of IAA was observed 40 min after its addition. This compound is highly permeable across red cell membrane. Accordingly, this observed delay in the onset of inhibition cannot have been due to the permeability of the cell membrane to IAA. The permeability coefficient of iodoacetamide, IAM, in these cells was measured and found to be very high ($4 \pm 0.5 \times 10^{-5}$ cm/s). In addition, IAM has a very low reflection coefficient, $\sigma = 0.33$ (high permeability), in rabbit gallbladder (16). The permeability coefficient of IAA was not measured in the present studies, but it is well known that 1 mM IAA inhibits lactate production in intact red cells by 80% in less than an hour. It is also well known that this compound induces its effect by inhibiting triose phosphate dehydrogenase which is located within the cell interior. Based on these data, it is quite reasonable to conclude that these two compounds

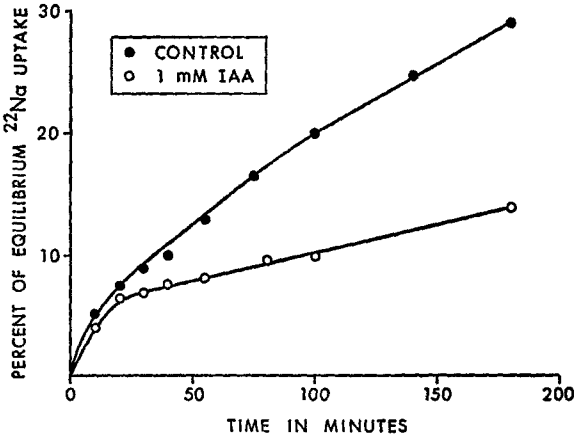


FIGURE 1. Time-course of the effect of IAA on $^{22}Na$ uptake by cat red cells.

readily permeate red cell membrane. The presence of an appreciable time lag in the kinetics of the onset of inhibition strongly suggests that IAA induces its effect by interacting with cell interior, possibly by changing the concentration of active intermediate metabolite(s) important for Na regulation in these cells. However, the possibility that this compound induces its effects by interacting directly with the membrane SH-groups cannot be completely ruled out. The presence of a time delay may be a manifestation of the time required for IAA to interact with some important groups in the cell membrane such as sulfhydryl groups. This is quite unlikely since, as shown in Table I, 0.1 mM of *p*-chloromercuriphenylsulfonate (PCMBS), a compound which interacts primarily with membrane SH-groups (17), significantly increases rather than decreases $^{22}$Na influx in cat red cells. The effect of PCMBS on $^{22}$Na influx in these cells is similar to its effect on Na transport in human red cells (17).

*Effect of Iodoacetamide on the Volume-Dependent Sodium Influx*

It is well known that Na transport in cat red cells is very sensitive to changes in cellular volume. Na influx is highest when the cells are shrunken and lowest when they are swollen. We, therefore, examined the relation between the cell volume effect and the IAM effect. The cellular volume was decreased by adding increasing amounts of sucrose to the isotonic buffer, and increasing cell volume was affected by reducing NaCl concentration. The relative cell volume was determined as discussed in the Method section. The results of a representative experiment are shown in Fig. 2. It is quite clear that 1 mM IAM does not abolish the volume-dependent $^{22}$Na influx. On the other hand, the degree of interdependence is dramatically reduced. Such a behavior will

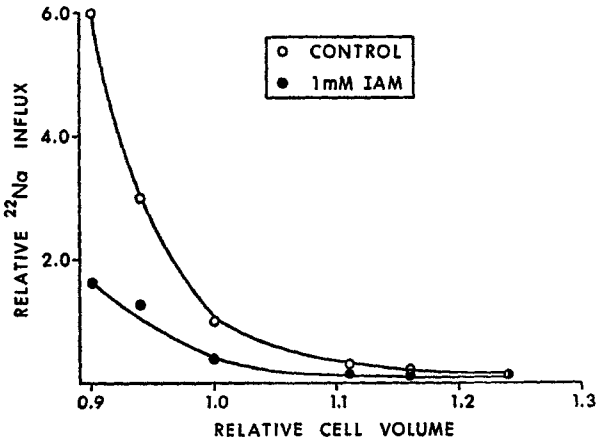


FIGURE 2. Effect of 1 mM IAM on $^{22}$Na influx in cat red cells as a function of cell volume.

be expected in the case where IAM reduces significantly but does not abolish the concentration of some important metabolic intermediate. The absolute Na uptake at a given volume will be reduced; however, changing cell volume will still produce changes in the concentration of the metabolic intermediate, and this will in turn affect Na transport systems. These results are consistent with the view that the primary event in the volume-dependent Na movement is a change in the concentration of some intracellular metabolite(s) which in turn produce conformational change in the cell membrane. This may be contrasted with the other view which considers the primary event to be a conformational change arising as a result of deformation of the cell membrane on shrinking (6).

*Effect of Reversible Dyes on $^{22}Na$ Uptake*

It has been long known that certain reversible dyes, such as methylene blue, cause a shift in the course of carbohydrate metabolism away from the anaerobic formation of lactic acid and toward the oxidative path (18). For example, 0.005% methylene blue when added to mammalian red cells alters the carbohydrate metabolism in such a way that the velocity of sugar transformation is increased, while at the same time less lactic acid is formed (18). Coincidental with this shift in the path of the metabolism is a marked increase in oxygen consumption and carbon dioxide production. Such a shift may alter the concentration of some intermediate metabolite(s) which are important for the regulation of Na and K in cat red cells. Accordingly, we studied the effect of seven dyes on $^{22}Na$ uptake in these cells.

In these experiments red cells were incubated with the dye in a temperature-controlled bath and shaken for 2 h before the isotope was added. The concentration of each dye was 1 mM. This time and concentration were sufficient to cause a significant change in the metabolic state of various red cells (19). The results of these experiments are shown in Table II. It is evident from the table that all of these dyes inhibit $^{22}Na$ transport, with methylene blue being the strongest inhibitor. It appears that there is no obvious correlation between the reduction potential of the dye and its inhibitory action. This is in contrast to the case in human red cells where methylene blue has no effect on passive cation transport (14). Since the characteristics of cation transport system in dog red cells resemble very much those found in cat red cells, we investigated the effect of this dye on $^{22}Na$ uptake in the former cells. In six different experiments Na influx in dog red cells was inhibited by 50 $\pm$ 10% in the presence of 1 mM methylene blue. The inhibitory action of methylene blue and the other reversible dyes was initially thought to be due to a change in pH since this compound, a known electron acceptor, is capable of stimulating the oxidation of glucose to carbon dioxide which does not

TABLE II
EFFECT OF REVERSIBLE DYES ON $^{22}Na$ UPTAKE IN CAT RED CELLS

| Name of dye | Relative uptaek | Inhibition |
|---|---|---|
| | | % |
| Control | 1.00 | — |
| Methylene blue | 0.40 | 60 |
| Phenol indophenol | 0.60 | 40 |
| Cresyl violet | 0.50 | 50 |
| Neural red | 0.55 | 45 |
| Janus green | 0.50 | 50 |
| Safranine | 0.84 | 16 |
| Brilliant crystal blue | 0.50 | 50 |

normally occur in mammalian red cells. This is, however, not the case because the maximum observed change in pH of the suspension was less than 0.2 U. $^{22}Na$ uptake in cat red cells is not sensitive to pH in the range of 7.1–7.6 (20).

In order to characterize further the action of methylene blue, the kinetics of inhibition of $^{22}Na$ inward movement by 0.8 mM methylene blue were also studied. In these experiments packed red cells were added to buffered solution containing Mb and $^{22}Na$. Portions were taken every 10 min for the first 60 min and at longer intervals thereafter. The results of a representative experiment are shown in Fig. 3. The kinetics of $^{22}Na$ inhibition by Mb resembles very much that of IAA effect. This gives further support to the idea that Mb induces its effect by interacting with some intracellular component.

*Effect of Cell Starvation on Volume-Dependent $^{22}Na$ Uptake*

In order to characterize the interdependence of Na transport and metabolism in these cells, we have studied the effect of iodoacetate and glucose on the volume-dependent Na transport in starved cells. In these experiments, cells were centrifuged and the plasma was replaced by equal volumes of isotonic buffered solution (identical to the incubation medium minus glucose). This suspension was incubated for 24 h at 25°C. Later the cells were centrifuged free of the supernatant and were equally divided into four groups: group A contains no glucose; B contains 10 mM glucose; C, 1 mM IAA; and D, 1 mM IAA + 10 mM glucose. Each group was divided into three flasks and each flask had incubation medium of different osmolarity. Hypertonic solution was made by adding sucrose to the incubating medium. The suspensions were then incubated at 38°C in a temperature-controlled water-bath shaker for 30 min before isotope was added. The results are summarized in Table III, and they show that:

(*a*) Preincubation of cells in the absence of glucose reduces Na uptake

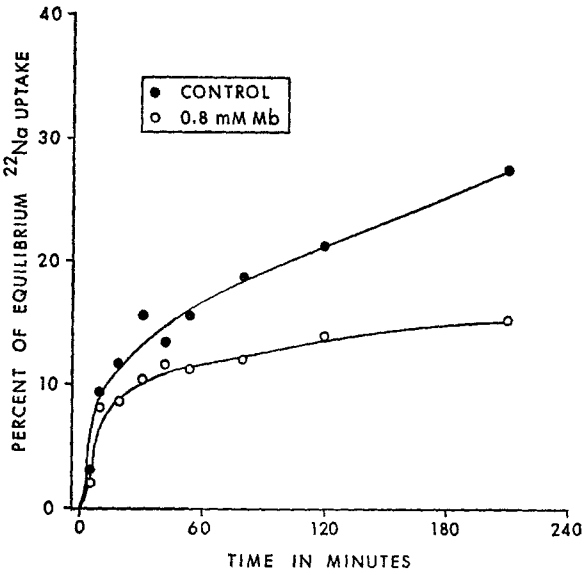


FIGURE 3. Time-course of the effect of Mb on $^{22}$Na uptake in cat red cells.

TABLE III

EFFECT OF GLUCOSE AND IODOACETATE ON THE VOLUME-DEPENDENT SODIUM UPTAKE IN STARVED CAT RED CELLS

| Experimental conditions | Relative cell volume | Relative uptake* | |
|---|---|---|---|
| Fresh cells | 1.22 | 0.25 | 0.88 |
| | 1.00 | 1.00 | 3.50 |
| | 0.90 | 3.00 | 10.50 |
| Group A (no glucose) | 1.22 | 1.15 | 0.86 |
| | 1.00 | 1.00 | 0.70 |
| | 0.90 | 1.12 | 0.77 |
| Group B (10 mM glucose) | 1.22 | 0.91 | 0.91 |
| | 1.00 | 1.00 | 1.00 |
| | 0.90 | 1.53 | 1.53 |
| Group C (1 mM IAA) | 1.22 | 1.05 | 0.82 |
| | 1.00 | 1.00 | 1.00 |
| | 0.90 | 0.94 | 0.73 |
| Group D (1 mM IAA + 10 mM glucose) | 1.22 | 1.10 | 0.93 |
| | 1.00 | 1.00 | 0.84 |
| | 0.90 | 1.07 | 0.91 |

* In the first column the results are expressed relative to the uptake at iso-osmotic condition, whereas in the second column they are expressed relative to iso-osmotic condition in the presence of glucose.

considerably (fresh cells and group A, B, C, D; column 3), and also abolishes the volume-dependent Na influx (fresh cells and group A; column 2). (*b*) Addition of 10 mM glucose to previously starved cells restores significantly the volume-dependent Na influx (group B; column 2).

(*c*) IAA inhibitory action on Na influx is practically abolished in starved cells (groups A and C; column 3).

(*d*) Addition of IAA and glucose simultaneously to previously starved cells does not restore the volume-dependent Na influx (group D; column 2).

(*e*) Addition of 10 mM glucose to starved cells containing 1 mM IAA increases slightly Na influx (groups C and D; column 3).

In the case of the dog red cells which are also high sodium cells, volume-dependent cation flux is also inhibited in starved cells and can be restored upon incubation with adenosine or glucose (6, 21).

It is known that the transport of Na in these cells is extremely sensitive to changes in cell volume (3). Accordingly, it is possible that the observed effects in Na influx by these various treatments are caused indirectly by causing volume changes. In order to guard against this possibility we have routinely measured the hematocrit of each suspension at the start, the middle, and the end of each experiment. The hematocrit value of each suspension was equal to the value of the control suspension and remained constant with time. In order to check this further, cell water content was measured after the cells had been incubated for 3 h in the presence of 1 mM IAA, 1 mM NaF, 1 mM Mb, and 0.1 mM PCMBS. The water content of the cells which had been starved for 24 h was also measured. The results which are summarized in Table IV clearly indicate that no significant change in cell water content was observed. Based on these two criteria we concluded that the observed changes in $^{22}Na$ influx were not due to changes in cell volume.

Romualdez et al. (6) has proposed a model for Na transport in dog red cells in which Na ions are combined with glucose or adenosine to form a complex, and this complex is transported across the cell membrane. It is evident from these studies that addition of glucose does not restore the volume effect in the presence of IAA. This strongly suggests that it is not the transport of glucose, as is the case in Romualdez's model, but the integrity of some metabolic intermediate(s) which is important for Na transport in these cells. The same mechanism probably holds true for dog red cells.

TABLE IV

CELL WATER CONTENT UNDER VARIOUS EXPERIMENTAL CONDITIONS

| Experimental condition | Water content in % by weight* |
|---|---|
| Control cells | 63.1±0.2 (9) |
| IAA (1 mM) | 63.2±0.3 (6) |
| NaF (1 mM) | 63.2±0.3 (3) |
| Mb (1 mM) | 63.0±0.2 (3) |
| PCMBS (0.1 mM) | 64.0±0.5 (3) |
| Cell starved for 24 h | 62.5±0.2 (6) |

* The number in parentheses refers to the number of determinations. Errors are standard errors of the means.

*Effect of Magnesium Chloride on Sodium Influx*

Recently Lee et al. (22) reported that incubation of dog red cells in isotonic (0.12 M) $MgCl_2$ for about 2 h causes a significant amount of hemolysis. They further have shown that this hemolysis is not a colloid osmotic lysis. During the course of our investigation of the transport of Na and K in cat red cells, we have noticed similar results. Therefore, we have decided to investigate the effect of $MgCl_2$ on Na influx in cat red cells. In these experiments the cells were washed three times in 4 vol of the incubation medium which contains no $MgCl_2$. The cells were divided into two groups. A known amount of $MgCl_2$ was added to one group to give the desired concentration, and an osmotically equivalent amount of NaCl was added to the second group. This was necessary in order to avoid any difference in the osmolarity of the incubation medium between the control set and the experimental set. A change in osmolarity will produce a change in cell volume which in turn affects Na flux. The suspensions of the two groups were incubated at 38°C in a temperature-controlled water-bath shaker for 30 min before the tracer was added. The results of a representative experiment are shown in Fig. 4. It is quite clear that Na influx increases rapidly with increasing concentration of $MgCl_2$ in the incubation medium and then reaches a plateau at 10 mM $MgCl_2$. Once again cell water content was measured after the cells had been incubated for 3 h in the presence of 5 mM $MgCl_2$ and found to be not significantly different from control value

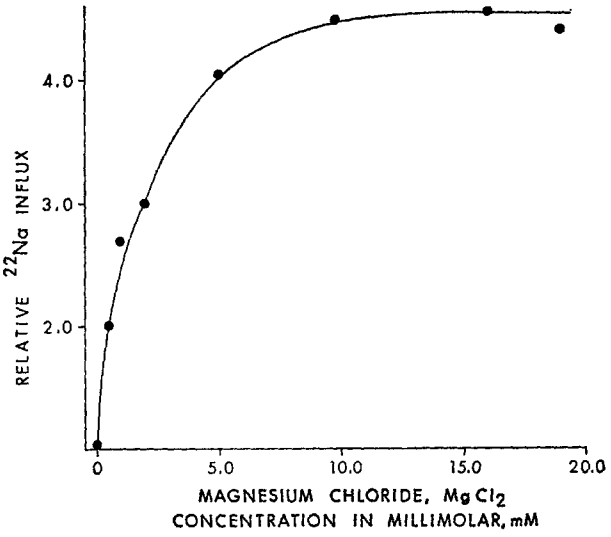


FIGURE 4. $^{22}$Na influx in cat red cells as a function of magnesium chloride concentration in the bathing medium.

(the values of six determinations were: control, 61.5 ± 0.5% and experimental, 62 ± 0.7% by weight).

Initially it was thought that this magnesium effect was due to a difference in surface charge density across the two sides of the cell membrane arising from unequal concentration of $MgCl_2$ between the two sides. A difference in Mg ions concentration across the membrane will generate an electric field within the membrane because of the symmetry of the two surface potentials (Zeta potential). This difference in surface energy may produce an instability in the membrane which may cause an increase in Na influx and lead ultimately to cell hemolysis. Such an effect has been demonstrated in artificial membranes made out of phosphatidyl serine, but not in those made out of neutral phosphatidyl choline (23).

In order to check this possibility, the effect of calcium and manganese ions on Na influx in cat red cells was also investigated. In these experiments the phosphate ions in the incubation medium were removed and pH was adjusted to 7.4 by Tris buffer. This was necessary in order to avoid any precipitation of calcium phosphate. Replacement of phosphate by Tris buffer had no effect on Na influx. The results of these studies are summarized in Table V. It is quite evident from the table that only Mg ion produces any significant acceleration of Na influx. Furthermore, the effect of $MgCl_2$ on the transport of $K^+$ and $SO_4^=$ in these cells were also investigated. It has been previously reported that the transport of both $K^+$ and $SO_4^=$ are sensitive to changes in cell volume (3, 4). The results which are given in Table VI, clearly show that only Na influx is significantly changed by $MgCl_2$. In addition, we found that 10 mM $MgCl_2$ has no significant effect on Na transport in human red cells. Although these results do not support the view that $MgCl_2$ produces its effect by generating an asymmetry potential across, they do not rule it out completely. It is possible that the instability produced by the difference in the surface energy of the two surfaces is not a generalized alteration but may be specific conformational change.

TABLE V

EFFECT OF CALCIUM CHLORIDE ($CaCl_2$), MAGNESIUM CHLORIDE ($MgCl_2$), AND MANGANESE CHLORIDE ($MnCl_2$) ON SODIUM INFLUX

| Compound | Relative $^{22}Na$ influx* |
|---|---|
| Control | 1.00+0.10 (8) |
| $CaCl_2$ (5 mM) | 0.96±0.08 (4) |
| $MnCl_2$ (5 mM) | 0.95±0.09 (3) |
| $MgCl_2$ (5 mM) | 2.00±0.15 (8) |

* The numbers in parentheses refer to number of experiment. Errors are standard errors of the means.

It is possible that Na movement in cat red cells is energized through the breakdown of ATP by membrane-associated ATPase which is activated by Mg ions. It is known that ATPase activity in cat red cell membranes in the absence of Na and K ions, but in the presence of Mg ions, is almost twice as high as in human red cell membranes (9). In order to test this possibility, the effect of $MgCl_2$ on Na influx was studied in the presence of IAA and NaF. Samples of cells were suspended in the presence of either 1 mM IAA or 1 mM NaF for 1 h. The results are summarized in Table VII. Both IAA and NaF interfere with energy production in mammalian red cells which is evident by

TABLE VI

EFFECT OF 5 mM $MgCl_2$ ON $^{42}K$, $^{35}SO_4$ INFLUX AND $^{22}Na$ INFLUX

| Condition | $^{42}K$ influx | $^{35}SO_4$ influx | $^{22}Na$ influx |
|---|---|---|---|
| Control | 1.00±0.05 (2)* | 1.00±0.10 (3) | 1.00±0.08 (3) |
| 5 mM $MgCl_2$ | 0.94±0.05 (2) | 1.10±0.10 (3) | 1.94±0.10 (3) |

* The numbers in parentheses refer to number of experiment. Errors are standard errors of the means.

TABLE VII

EFFECT OF IODOACETATE (IAA) AND SODIUM FLUORIDE (NaF) ON $MgCl_2$-INDUCED $^{22}Na$ INFLUX

| Condition | Relative influx* |
|---|---|
| Control (no $MgCl_2$) | 1.00±0.10 (4) |
| $MgCl_2$ (5 mM) | 2.10±0.10 (4) |
| IAA + $MgCl_2$ (1 mM IAA, 5 mM $MgCl_2$) | 0.40±0.05 (4) |
| NaF + $MgCl_2$ (1 mM NaF, 5 mM $MgCl_2$) | 1.90±0.10 (3) |

* The numbers in parentheses refer to number of experiment. Errors are standard errors of the means.

the marked reduction in cellular ATP (15). However, of these two energy inhibitors only IAA abolishes Mg effect on Na transport.

The kinetics of acceleration of $^{22}Na$ inward movement by magnesium were also studied. In these experiments packed red cells were added to medium containing 5 mM magnesium and $^{22}Na$ maintained at 38°C. Samples were taken every 10 min for the first 40 min and at longer intervals thereafter. The results of a typical experiment are shown in Fig. 5. It is clear from the graph that acceleration was evident as early as 10 min. Even though $^{28}Mg$ inward movement is one order of magnitude higher in cat red cells than in human, the rate constant of magnesium transfer is still small and measured in terms of hours. In other words very little magnesium had entered the cell during the 10 min period (24). Accordingly, this lack of any delay suggests that magnesium induces its effect by interacting directly with some compo-

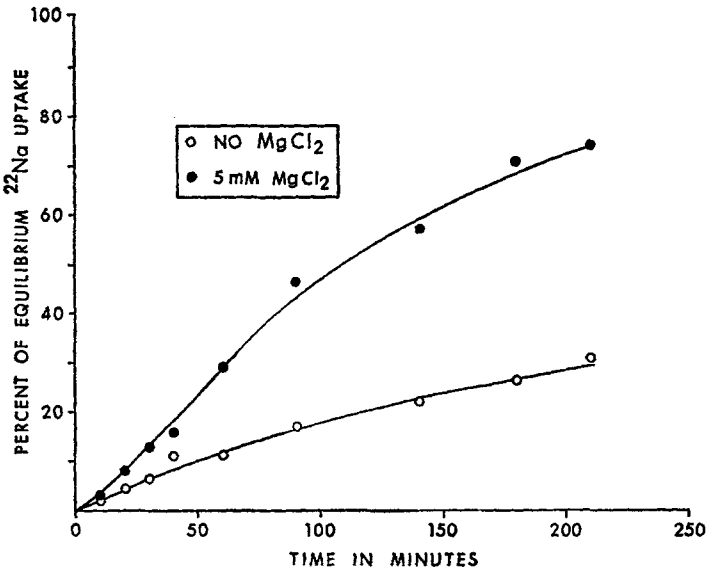


FIGURE 5. Time-course of the effect of $MgCl_2$ on $^{22}Na$ influx in cat red cells.

nents of the cell membrane, possibly Mg-activated ATPase, and not by altering the concentration of some intermediate metabolite(s).

*Effect of Magnesium on the Volume-Dependent Sodium Influx*

It is well known that Na transport in cat red cells is very sensitive to changes in cellular volume. Na influx is highest when the cells are shrunken and lowest when they are swollen. We, therefore, examined the relation between the cell volume effect and the Mg effect. The cellular volume was decreased by adding increasing amounts of sucrose to the isotonic buffer, and increasing cell volume was effected by reducing NaCl concentration. The relative cell volume was determined as discussed in the Method section. The results of a representative experiment are shown in Fig. 6. The degree of acceleration of Na influx by 5 mM magnesium chloride does not change with variation in cell volume. This is clearly illustrated in Fig. 7. In this figure we have normalized each set of points (control points and 5 mM magnesium chloride points) to their corresponding iso-osmotic condition. It is clear that all the points fall on the same curve. This indicates that Mg ion merely changes the base line and does not interfere with the nature of the volume-dependent $^{22}Na$ movement. This indicates that the two effects are not antagonistic to each other.

*Effect of Mg Ion on $^{22}Na$ Influx in Starved Cells*

In order to characterize further the role of magnesium ions in the regulation of sodium transport in these cells, we have studied the effect of magnesium

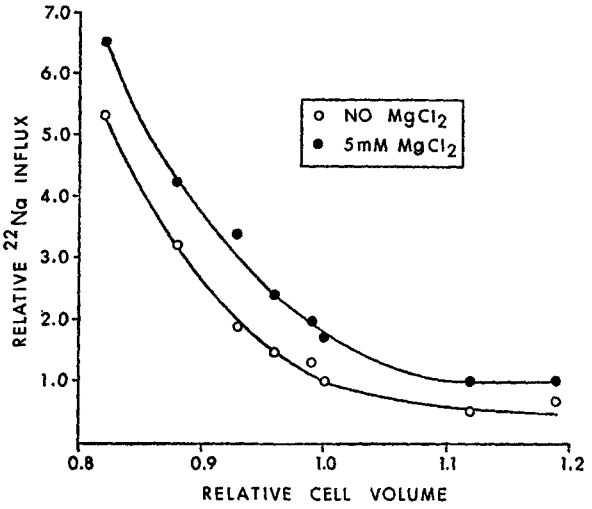


FIGURE 6. Effect of 5 mM $MgCl_2$ on $^{22}Na$ influx as a function of cell volume.

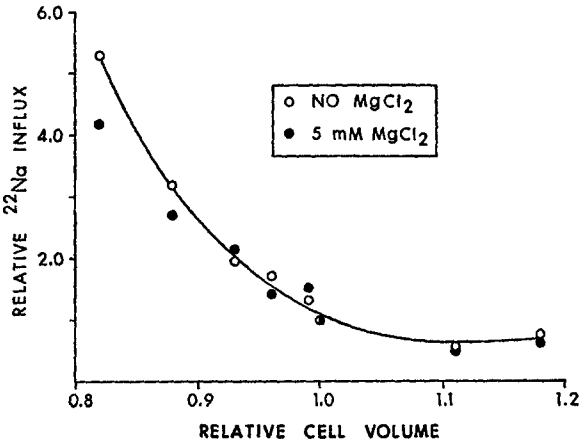


FIGURE 7. Effect of 5 mM $MgCl_2$ on $^{22}Na$ influx as a function of cell volume. In this figure we have taken the results from Fig. 6 and transported the curve passing through the solid circles onto the curve passing through the open circles. This can be done by dividing the flux value of each relative cell volume of the experimental set (solid circles) by the flux value at relative cell volume equal to unity.

and glucose on $^{22}Na$ influx in starved cells. In these experiments, cells were centrifuged and the plasma was replaced by an equal volume of isotonic buffered solution (identical to the incubation medium minus glucose and magnesium). This suspension was incubated for 24 h at 25°C. Later on the cells were centrifuged free of the supernatant and were equally divided into four groups: group A contained no glucose or magnesium; B contained 10 mM $MgCl_2$, C contained 10 mM glucose, and group D contained 10 mM glucose and 10 mM $MgCl_2$. An osmotically equivalent amount of NaCl was added to

groups A, B, and C to maintain the constancy of medium osmolarity. This is necessary in order to eliminate differences in cell volume which otherwise will arise from differences in the incubation medium osmolarity as the result of adding glucose and $MgCl_2$. The suspensions were then incubated at 38°C in a temperature-controlled water-bath shaker for 3 h before isotope was added. As discussed previously, hematocrits were always determined in order to check for any variation in cell volumes. In all these studies the results of the experiment were discarded if the hematocrit of the experimental suspension was either different from control or varied with time. The results are summarized in Table VIII. It is evident from the table that 10 mM $MgCl_2$ does not induce any significant increase in $^{22}Na$ influx when glucose is deleted. Furthermore, 10 mM glucose without magnesium ions has only a small effect on Na transport. On the other hand, when both $MgCl_2$ and glucose are added together there is more than a 10-fold increase in $^{22}Na$ influx. The stimulation

TABLE VIII

EFFECT OF GLUCOSE AND MAGNESIUM CHLORIDE ON SODIUM INFLUX IN CAT RED CELLS

| Experimental condition | Relative influx* |
|---|---|
| Group A (no glucose and no $MgCl_2$) | 1.00±0.06 (5) |
| Group B (10 mM $MgCl_2$) | 0.99±0.05 (4) |
| Group C (10 mM glucose) | 1.36±0.15 (5) |
| Group D (10 mM glucose + 10 mM $MgCl_2$) | 12.40±1.50 (5) |

* The numbers in parentheses refer to the number of experiment. Errors are standard errors of the means.

of $^{22}Na$ influx by Mg in starved cells is significantly higher than that seen in fresh cells. One possible explanation for this difference is that the intracellular Mg ion concentration is different in the two conditions. Cells which are incubated for 24 h in magnesium-free solution will definitely lose more magnesium than those which are washed three times in 4 vol of the incubation medium which contains no $MgCl_2$. These results confirm the view arrived at earlier that it is not the transport of glucose, as it is the case in Romualdez's model, but the integrity of the metabolic chain which is important for Na transport in these cells.

When previously starved cells (no glucose and no $MgCl_2$) were reincubated in a solution containing 10 mM glucose, the effect of 10 mM $MgCl_2$ on Na influx was not immediate as in the case of fresh cells. There was a delay of 1 h before significant acceleration of Na influx by $MgCl_2$ was observed. This indicates that time is needed to build up the concentration of some intracellular component which is important for Na transport.

There are a minimum of three possible ways by which magnesium chloride can produce acceleration in Na influx in cat red cells: (*a*) Addition of $MgCl_2$ to the bathing medium can create a difference in surface energy across cell

membrane which in turn produces an instability in the cell membrane. This instability will lead to a nonselective increase in transport across the membrane. There is overwhelming evidence which argues against this possibility. (*b*) Increase in $MgCl_2$ concentration will ultimately lead to an elevation of the intracellular concentration of this ion. This may accelerate the rate of glycolysis, thus possibly increasing the concentration of some intermediate(s) which is important for Na transport. (*c*) It is possible that Na movement in cat red cells is energized through the breakdown of ATP by membrane-associated Na and Mg-dependent ATPase. Although the results which are presented so far strongly support either of the last two possibilities, they do not distinguish between them.

In order to differentiate between these two possibilities we have investigated the effect of glucose and magnesium on lactate production in a previously starved cell. In these experiments cells were centrifuged and the plasma was replaced by an equal volume of isotonic buffered solution (identical to the incubation medium minus glucose and magnesium). The suspension was incubated for 24 h at 25°C. Later the cells were centrifuged free of the supernatant and were equally divided into three groups: group A contained no glucose or magnesium; B contained 10 mM glucose; and C contained 10 mM glucose and 10 mM $MgCl_2$. An osmotically equivalent amount of NaCl was added to groups A and B to maintain the constancy of medium osmolarity. Lactate production was measured at the start of the experiment and after 4 h of incubation. The results of these studies are shown in Table IX. It is quite clear from the table that the glycolytic chain is still intact as evident from lactate production in both groups B and C. However, Na influx is accelerated only in group C. This suggests that Na transport in these cells is very much dependent on both the integrity of the metabolic chain and magnesium. One possible explanation for this behavior is that Na transport in cat red cells is energized through the breakdown of ATP by membrane-associated Na and Mg-dependent ATPase. This postulate can be extended to include not only the transport of Na in high Na red cells but also the "passive" transport of Na and K in high K cells. The exact nature of this possible coupling cannot be deduced from these studies. However, one possibility is that "contractile" proteins may be involved in the regulation of "passive" transport of Na and K in mammalian red cells. There is some evidence to indicate that there are actin- and myosin-like proteins in human red cell membrane (25). There is one piece of evidence which does not seem to fit with this general idea. As shown in Table IX, there is a tremendous increase in Na influx without a concomitant increase in lactate production. On the surface this appears to contradict the hypothesis that Na transport in cat red cells is energized through the breakdown of ATP by membrane-associated Na and Mg-dependent ATPase. It would certainly have been nice if there were an increase

TABLE IX

EFFECT OF GLUCOSE AND MAGNESIUM CHLORIDE ON LACTATE PRODUCTION IN STARVED CAT RED CELLS

| Experimental condition | Lactate production* | Relative Na influx* |
|---|---|---|
| | *mmol/liter RBC·h* | |
| Group A (no glucose and no $MgCl_2$ | 0 | 1.00±0.06 (5) |
| Group B (10 mM glucose) | 1.40±0.30 (4) | 1.36±0.15 (5) |
| Group C (10 mM glucose and 10 mM $MgCl_2$) | 1.30±0.30 (4) | 12.40±1.50 (5) |

* The number in parentheses refers to the number of experiment. Errors are standard errors of the means.

in lactate production. But, first, lactate production does not truly measure ATP hydrolysis; second, ATP may be produced at a faster rate than it is hydrolysed. Accordingly, the lack of any significant increase in lactate production should not be surprising.

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