

Cation Transport in Dog Red Cells

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ABSTRACT Studies have been made on the cation transport system of the dog red cell, a system of particular interest because it has been shown that there is a marked dependence of cation fluxes on the cell volume. We have found that a 10% decrease in cell volume causes a large increase in 1 hr uptake of ^{24}Na as well as a considerable inhibition of ^{42}K uptake. This effect cannot be produced by a difference in medium osmolality but rather requires the cell volume to change. Dog red cell uptake of ^{24}Na is not inhibited by iodoacetate. Phloretin inhibits ^{24}Na uptake and lactate production, and virtually abolishes the volume effect on Na uptake. These several observations may be accounted for in terms of a working hypothesis which presupposes a cation carrier complex which pumps K into and Na out of cells of normal volume. When the cells are shrunk the carrier specificity shifts to an external Na-specific mode and there is a large increase in ^{24}Na uptake, driven by the inwardly directed Na electrochemical potential gradient.

Some animal species, such as the cat and the dog, have red blood cells the electrolyte composition of which approaches that of the plasma (1), in contrast to human and most other mammalian red cells. Other characteristics of the electrolyte transport mechanism in dog and cat red cells also differ significantly from human and other mammalian cells. For example, there appears to be no measurable ouabain-sensitive component to the Na or K fluxes in these cells (2, 3) and membrane fragments do not show any significant ouabain-inhibitable Na-K-dependent adenosine triphosphatase (ATPase) (4).

A phenomenon of particular interest in this system is the volume dependence of the Na and K fluxes initially reported by Davson (5) and subsequently studied by Parker and Hoffman (6). These investigators and others (7) demonstrated that osmotic shrinking of the dog red cell is accompanied by a dramatic increase in Na flux and a significant decrease in the flux of K. Conversely, when the cells are swollen, Na flux falls while K flux rises. This phenomenon does not occur when the cells are starved to the point at which

lactate production is almost absent (6), which suggests that the volume dependence of the cation transporting mechanism is related to the metabolic state of the dog red blood cell. We have investigated the effect of phloretin on the volume dependence of cation transport since phloretin has been shown to inhibit glucose transport (8) in human erythrocytes and also to interfere with metabolic enzymes in other tissues (9). We have also studied lactate production and the effect of iodoacetate on Na flux. The results of these experiments have led to a working hypothesis in which the volume effect on cation fluxes is related to conformational changes on an acceptor located at the cell surface.

MATERIALS AND METHODS

Blood was obtained by venipuncture from adult dogs anesthetized with Na Nembutal (30 mg/kg body weight). Heparin (10,000 units/ml, 4 ml/liter) was used to prevent clotting. Freshly drawn blood was used for all experiments. The red blood cells were suspended at pH 7.4 in a buffered solution of the following composition (in millimoles per liter): NaCl, 150; KCl, 5.0; Na₂HPO₄, 5.2; NaH₂PO₄, 0.8; CaCl₂, 1.0; MgCl₂, 0.25; glucose, 5.0. The resulting suspension was placed in a vessel designed to give efficient mixing (10), and shaken in a water bath at 38° ± 0.5°C. After 30 min of equilibration, ²⁴Na or ⁴²K (Cambridge Nuclear Corp., Princeton, N. J.) was added and samples were drawn at time intervals appropriate to the various determinations.

Cell volume was altered by changing the osmolality of the buffer. Hypoosmolar solutions were prepared by removal of NaCl alone and hyperosmolar ones by the addition of sucrose, all other solutes remaining constant. Relative cell volume was calculated from the final measured osmolarity of the medium using the data of Rich et al. (11), which showed the apparent "osmotic water" content of the dog red cell to be 0.57 ± 0.029 of total cell volume. The osmolarity was measured by means of a Fiske osmometer (Osmometer Model G-62, Fiske Associates Inc., Uxbridge, Mass.). Hematocrits were determined in duplicate by centrifuging at 10,000 *g* for 50 min in a constant bore hematocrit tube. The length of the packed column relative to that of the whole column was measured by an optical device mechanically coupled to a machinist's dial gauge (12). Hemoglobin was determined from the optical density at 540 μ after conversion of hemoglobin to cyanmethemoglobin. The same method of determination was used to measure hemolysis. Na and K were measured by a flame photometer (Model 143, Instrumentation Laboratory Inc., Lexington, Mass.).

Samples for measurement of radioactivity in the red blood cells were washed three times in 5 vol of cold 150 mM NaSCN. NaSCN was used to prevent the escape of cell cation during the washing procedures since it has been shown to inhibit Na movement in cat red cells (3). The error in red cell radioactivity due to trapped plasma was less than 1%. The packed cells were then placed in a tube containing 2 ml of distilled water. ²⁴Na and ⁴²K were measured in an automatic gamma counter (System 4222, Nuclear-Chicago Corp., Des Plaines, Ill.), and subsequently the amount of hemoglobin in each tube was determined so that specific activity could be computed as the ratio of the observed counts per minute to the amount of hemoglobin per milli-

equivalent of cell Na or K. The equilibrium specific activity was calculated in duplicate from the relationship of the ^{24}Na or ^{42}K concentration of a fraction of the whole suspension to the total Na or K concentration in the fraction.

Lactate production was measured in duplicate samples of the suspension at the start of the experiment and after 2 hr of incubation. The lactate content of a perchloric acid-treated sample was determined enzymatically by conversion of nicotinamide adenine dinucleotide (NAD) to reduced NAD (NADH) in the presence of lactic dehydrogenase (13). The mean recovery of added lactate was better than 90% and duplicate samples were within 5% of one another. The lactate contents of the samples were related to the hemoglobin concentration of the suspension and subsequently expressed as millimoles of lactate per liter of original cell volume.

Phloretin was made by the hydrolysis of phlorizin (Aldrich Chemical Co., Inc., Milwaukee, Wis.), in the presence of H_2SO_4 according to the method of Bach (14). After the final crystallization, a 2 M solution of phloretin in ethanol was prepared. In all phloretin experiments, both control and test suspensions contained the same amount of ethanol, which was 0.5×10^{-3} M for most experiments.

RESULTS

Preliminary experiments with both phlorizin and phloretin showed that the latter was more effective in reducing net Na efflux from dog erythrocytes suspended in a high K buffer. These results are in conformity with the discrimination of human red cells between phloretin and phlorizin as reported by LeFevre and Marshall (15). In subsequent measurements of ^{24}Na uptake, it was found that, although 1 mM phlorizin had no effect, 1 mM phloretin reduced the 1 hr uptake of normal cells by 40%.

Fig. 1 shows a dose-response curve obtained in three experiments on the effect of phloretin on the ^{24}Na uptake by dog red blood cells in 1 hr. The degree of inhibition increased as the concentration of phloretin was increased from 10^{-5} to 10^{-3} M. The highest concentration provides 10^7 molecules of phloretin/red cell which inhibits ^{24}Na uptake by about 40%. Measurements at higher concentrations could not be made since hemolysis was greater than 10% at a concentration of 2 mM phloretin. All subsequent experiments were carried out at a concentration of 1 mM phloretin.

Phloretin is a relatively specific inhibitor of sugar transport in the red cell (15) at concentrations in the range 10^{-4} – 10^{-8} M. However, at higher concentrations, in the range 10^{-3} – 10^{-4} M, phlorizin has been shown by Britten and Blank (16) to inhibit Na-K-activated ATPase from rabbit kidney. As already pointed out there is no significant Na-K ouabain-sensitive ATPase activity in dog red cells so that this particular additional mechanism of phloretin action does not affect ion transport in our system. There may possibly be other, as yet unknown, actions of phloretin on dog red cells, although the smooth shape of the dose-response curve from 10^{-5} to 10^{-3} M

phloretin shown in Fig. 1 does not suggest any new modes of action in the 10^{-4} – 10^{-3} M range.

Figs. 2 and 3 show time-courses, typical of three experiments with each isotope, for the uptake of ^{24}Na and ^{42}K by dog red cells in the presence and in the absence of 1 mM phloretin. Phloretin accelerates the uptake of ^{42}K

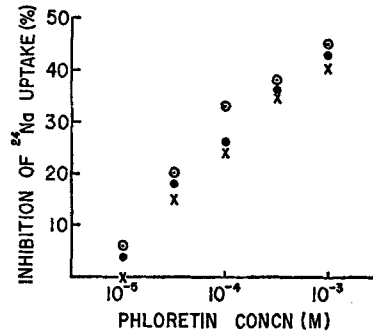


FIGURE 1. Effect of phloretin on the amount of ^{24}Na taken up by dog red cells in 1 hr at 38°C . Each symbol refers to a separate experiment.

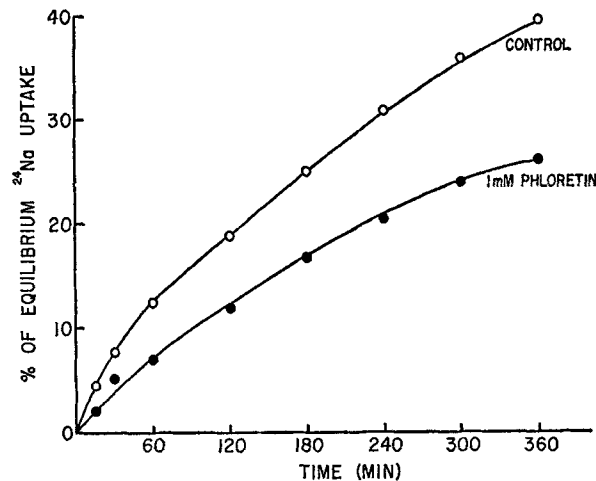


FIGURE 2. Time-course of the effect of phloretin on ^{24}Na uptake by dog red cells.

while it inhibits that of ^{24}Na , exactly opposite to the effect of cell shrinking on cation movement. We, therefore, examined the relation between the cell volume effect and the phloretin effect in the two experiments, the results of which are shown in Fig. 4. Although inhibition of 1 hr ^{24}Na uptake occurs at all cell volumes, the degree of inhibition by 1 mM phloretin increases with decreasing cell volume. This is clearly demonstrated in Fig. 5, in which the volume-dependent uptake is shown relative to normal cell volume.

In view of inhibitory effect of phloretin on the volume-dependent Na flux we studied the effect of cell shrinking on glycolysis by dog red cells and obtained the results given in Table I. Although there appears to be a consistent increase in lactate production as the cells shrink, the differences

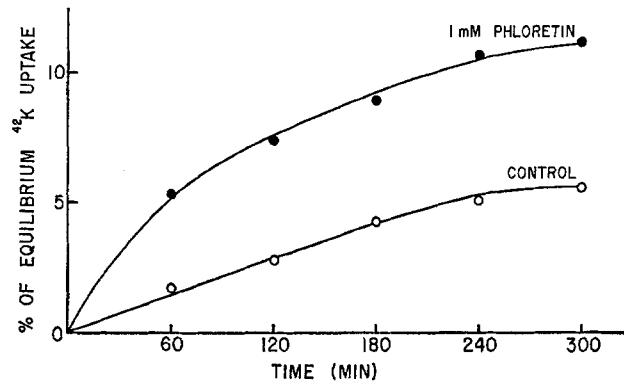


FIGURE 3. Time-course of the effect of phloretin on ⁴²K uptake by dog red cells.

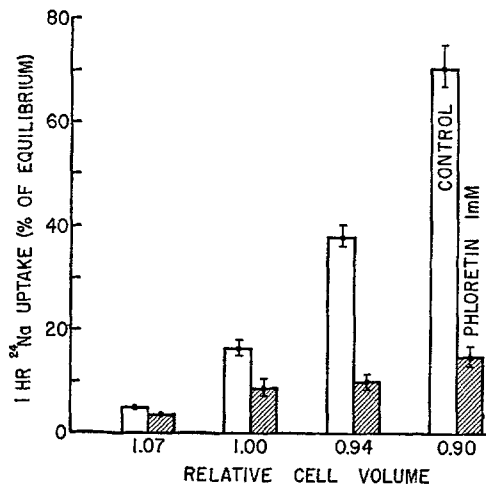


FIGURE 4. Effect of 1 mM phloretin (shaded bars) on 1 hr ²⁴Na uptake as a function of cell volume.

are too small to be significant, particularly when compared to the more than threefold increase in ²⁴Na uptake which occurs when cell volume is decreased by 10%, as shown in Fig. 4. The absence of any significant difference in lactate production as a result of cell shrinking is consistent with the idea that the observed volume effect on Na transport is not the result of a general inhibition of glycolysis. Furthermore, the large quantitative difference between the effect of cell shrinking on lactate production and Na uptake shows that the two processes are very loosely coupled, if at all.

Phloretin was also found to reduce glycolysis in dog red cells suspended in isosmolal buffer. In two experiments, lactate production fell from a control value of 1.68 ± 0.03 mmoles/liter cell per hr to 1.09 ± 0.08 mmoles/liter cell per hr in the presence of 1 mM phloretin. This 35% decrease in lactate production corresponds to the 40% decrease in ^{24}Na uptake at the same

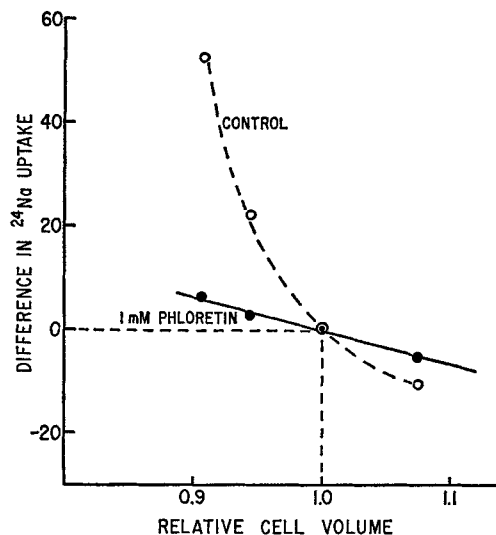


FIGURE 5. Difference in 1 hr ^{24}Na uptake as related to cell volume. The difference is obtained by subtracting the 1 hr ^{24}Na uptake at the control volume from that at the altered volume. ^{24}Na uptakes are expressed as per cent of equilibrium uptake.

TABLE I
EFFECT OF DOG RED CELL VOLUME ON LACTATE
PRODUCTION*

Relative cell volume	Lactate production
	<i>mmoles/liter original cell volume per hr</i>
1.0	1.74 ± 0.17 (5)
0.90	1.95 ± 0.10 (5)
0.80	2.00 ± 0.08 (3)

* The numbers in parenthesis refer to number of experiments. Errors are standard errors of the mean.

phloretin concentration. However, these observations do not necessarily indicate that the phloretin effect on ^{24}Na uptake is mediated through the ordinary route of intermediary metabolism. Three experiments were carried out in which the metabolic inhibitor, iodoacetate, was added to the buffer at a concentration of 0.5 mM, which is sufficient to reduce lactate production in control dog red cells by 80%. The effect on ^{24}Na uptake is small, as shown

in Fig. 6. Although the inhibition at the beginning of the experiment is slight, the difference at 1 hr is clearly observable.

One other facet of the cell shrinking effect was investigated. Hoffman (17) had studied the volume effect by placing dog red cells of different initial volumes in the same medium; he found the volume effect to depend on cell water content and to be independent of cell Na and K contents over quite broad ranges. These experiments show that the volume effect is essentially independent of intracellular Na and K concentration. They also indicate that medium osmolality does not itself influence the volume effect. We confirmed the latter result by showing, through the use of propionamide, a molecule to which the cell is very permeable, that an increase in osmolality could not produce the volume effect. Two buffers of identical osmolality

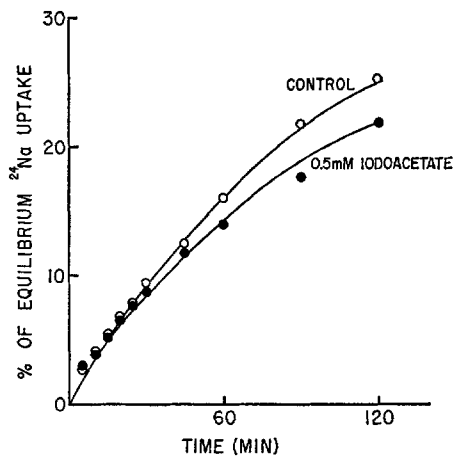


FIGURE 6. Time-course of the effect of 0.5 mM iodoacetate on ^{24}Na uptake by dog red cells.

were prepared by the addition of (a) 100 mM propionamide and (b) 100 mM sucrose to the usual phosphate buffer. Samples of cells were suspended in each of these buffers and after a 15 min preincubation period ^{24}Na was added. The cell uptake at 1 hr was measured in three experiments. The uptakes are compared with the uptake by cells in isosmolar phosphate buffer in Table II, which clearly shows that the effect is to be attributed to changes in cell volume rather than to changes in osmolality.

Hoffman (17) also showed that the volume response was inhibited in starved red cells but was restored upon incubation with adenosine. We have shown that the volume effect may also be restored by incubation with glucose. In one such experiment red cells were starved at 37°C for 20 hr and were then incubated with glucose at concentrations of from 0 to 10 mM. The control cells had a ^{24}Na influx of 11 mmoles Na/liter cells per hr in the absence of

glucose and this figure was unchanged when the cells were shrunken by 75 mM sucrose. The addition of glucose led to a consistent increase in ^{24}Na influx in the shrunken cells (with 0.2 mM sucrose the influx was 16 mmoles Na/liter cells per hr, with 4 mM sucrose 20, and with 10 mM sucrose 23 mmoles Na/liter cells per hr). In conjunction with these experiments we also determined that the volume response could not be elicited by 3-O-methyl glucose and confirmed Hoffman's adenosine result.

DISCUSSION

The characteristics of dog red cell cation transport may be compared with those of the human in order to examine the role of metabolism in maintaining the normal cation distribution. 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, 18). The inverse of the Cl ratio is 1.45, which fits with the expected passive Cl

TABLE II
EFFECT OF OSMOLALITY ON VOLUME EFFECT IN DOG
RED CELL

Exp.	Normal buffer (310 mosmols/liter)	Buffer + 100 mM sucrose (410 mosmols/liter)	Buffer + 100 mM propionamide (410 mosmols/liter)
<i>fraction of equilibrium ^{24}Na uptake in 1 hr</i>			
1	0.12	0.59	0.09
2	0.10	0.53	0.09
3	0.08	0.41	0.07

distribution computed from the observed potential difference of -8 mv, given by Lassen and Sten-Knudson (19) and Jay and Burton (20). The difference of more than an order of magnitude between the Cl distribution ratio and those for Na and K indicates that both Na and K are actively transported in human red cells, which is concordant with the sensitivity of the human transport system to ouabain (21) and the presence of an Na-K ouabain-sensitive ATPase in the membrane (18). Glucose metabolism is required in the human red cells to support the active cation transport and to maintain the normal cation distribution ratios.

In the dog, however, the pattern is entirely different since the concentration ratios are Na, 0.88; K, 2.1; and Cl, $0.69 = 1.45^{-1}$ (1, 18). The Na ratio is lower by a factor of 1.65 and the K ratio is higher by a factor of 1.45 than the inverse Cl ratio. These cation ratios are close to one another and almost an order of magnitude closer to the Cl ratio than is the case in human red cells. This significant species difference implies that much of the cation transport in dog red cells is passive. Nonetheless, there still must be a regulated

component to keep the electrochemical potential of cell Na lower and cell K higher than expected according to the inverse Cl ratio. These relatively small electrochemical potential gradients may be presumed to be maintained by an Na-K exchange pump which requires energy to pump Na out and K in, against their respective electrochemical potential gradients.

The most striking observation in the present study is the effect of phloretin in inhibiting Na uptake while accelerating K uptake. The fact that the effects on these two cations are complementary is consistent with a link between them, particularly because the volume effect itself increases Na influx while decreasing K influx. A linkage of this sort would provide general support for the view that the gradients in the normal cell are produced by an Na-K exchange pump.

Our working hypothesis assumes that when the cell shrinks this pump changes its specificity on the outer face from a primarily K-specific mode towards an Na-specific one. This shift causes the observed decrease in K influx and the accompanying increase in Na influx. In the normal cell, the outwardly directed K electrochemical potential gradient is equivalent to a K concentration ratio of 1.45 which has to be surmounted by the inward K flux. When the cation receptor changes its specificity upon shrinking, the uphill K gradient is replaced by a downhill Na ratio of 1.44, assuming no change in electrical potential difference with shrinking. Thus the pump, now carrying Na downhill into the cell, has to do significantly less work than when it pumps K up an electrochemical potential gradient. It is this reversal of driving force, equivalent to a 16 mv change, which accounts for the dramatic increase in Na flux which accompanies a very small change in cell volume.

Metabolic Aspects of Working Hypothesis

Glucose is involved in the volume effect, as indicated by several lines of experiment. Addition of 1 mM phloretin dramatically reduces the volume effect, as shown in Fig. 4, presumably by interference with glucose transport; in the presence of phloretin, inhibition of lactate production parallels inhibition of Na uptake. Parker and Hoffman (6) have shown that the volume effect can no longer be induced in starved dog red cells in which glucose is no longer present. We have found that glucose will restore the volume response, whereas 3-O-methyl glucose, a nonmetabolizable analogue, will not. All of these experiments indicate that the volume effect is dependent upon either the integrity of some part of the metabolic chain or the presence of an intermediate located somewhere along the chain.

Parker and Hoffman (6) have also shown that the volume effect in starved red cells is restored by adenosine, which also restores the production of lactate. In view of the ability of adenosine to substitute for glucose, an observation which we have confirmed, the portion of the glycolytic chain required for

the volume effect must be located below the intersection of the glucose path and the adenosine path which Whittam (18) places at the triose phosphate level.

This leads to the implication in the Na transport system of an intermediary in the glycolytic cycle, presumably at the triose phosphate level, or lower. The next problem is to account for the action of iodoacetate which, as Fig. 6 shows, has little or no effect on ^{24}Na uptake while strongly inhibiting lactate production. Although the experiments were carried out on normal cells and not on shrunken ones, the results suggest that ^{24}Na uptake is not dependent on the activity of all the elements in the metabolic chain, but only on that part of it that lies above the level of iodoacetate inhibition. Iodoacetate, according to Whittam (18), inhibits triose phosphate dehydrogenase which is just below the triose phosphate level. This evidence leads to a tentative identification of the metabolic effector with triose phosphate or an intermediate which depends upon triose phosphate for its production.

We next need to consider the observation that the volume effect exercises little or no influence on lactate production as shown in Table I. This raises a question as to how the metabolic effector works. The experiments on the starved cells indicate that the volume effect depends upon the presence of a substance which is destroyed when the cells are starved. Once sufficient glucose or adenosine is provided to bring the concentration of the metabolic effector back to its steady-state value, the volume effect is restored. There is no obligatory requirement for shrunken cells to increase their lactate production and no significant increase is found.

The basic features of the working hypothesis are shown schematically in Fig. 7. We have used "Na complex" to denote the receptor site on the outer cell surface which changes its selectivity from K to Na when the cell shrinks. The volume effect, as previously discussed, also depends upon a metabolic effector formed at or below the glycolytic level at which the glucose and adenosine paths converge. This feature has been shown in the schematic by the intersection of these dotted paths at "Na complex." The site of iodoacetate action is shown on the dotted line which represents further steps along the glycolytic path. The details of the molecular mechanism involved in the formation of "Na complex" are unknown, as is the mechanism of Na passage across the membrane, and we have indicated this latter system simply by an arrow.

In a recent study of the volume effect in cat red cells, Sha'afi and Hajjar (22) have proposed a generalized hypothesis, according to which the selectivity between Na and K is under metabolic control, and have attributed the volume effect to "the result of a change in the concentration of some metabolic intermediate(s) which may cause a generalized conformational change in the membrane." This may be contrasted with the present view which

considers the primary event to be a conformational change arising as a result of deformation of the cell membrane on shrinking. Conformational changes have also been suggested by others as, for example, by Whittam and Ager (23), who proposed a cation-induced conformational change in the fit of ATP to an ATPase involved in cation transport. Since the differences between the properties of Na and K are relatively small, small changes in cell area and shape could easily effect important specificity differences, possibly by an allosteric mechanism. In the case of lipid-soluble ion-binding macrocyclic molecules such as the nactins and the enniatins, ion binding depends on the existence of an optimal ring size for complexing. Shemyakin et al. (24) have stressed the exceptional sensitivity of these complexing reactions to the conformational characteristics of the molecule.

The most interesting feature of the volume effect in dog red cells is the amplification by which a small change in cell volume is responsible for a very large change in Na uptake. The present hypothesis ascribes the observed

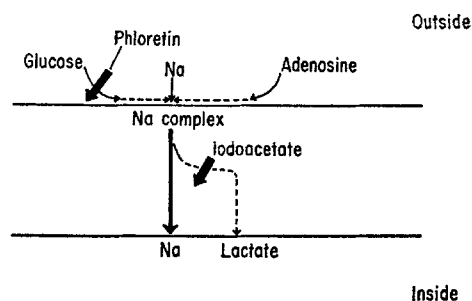


FIGURE 7. Working hypothesis for cation transport in dog red cells.

behavior to the new driving force which comes into play when the carrier complex becomes Na specific so that the inwardly directed Na electrochemical potential gradient can be used immediately to transport Na into the cell. This provides the physical-chemical force necessary to drive the system.

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