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FACTORS INFLUENCING Na^+ TRANSPORT IN DOG RED CELLS

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SUMMARY

Na^+ movements in dog red cells have been measured in a study of the relationship between cell volume, Na^+ permeability and glycolysis. When dog red cells are shrunken by 20 % at 38 °C the apparent Na^+ influx increases by a factor of about fifty, and the effect remains when cells are deprived of glucose for 2–2.5 h. Flux returns to normal when the cells are restored to their initial volume. Glycolysis is required for the volume effect and we have studied the effect of glycolytic modifiers such as fluoride, sulfate, bisulfite and pyruvate on these glucose depleted dog red cells. The results indicate that the volume effect is associated with a change in the concentration of 3-phosphoglycerate and may be mediated by phosphoglycerate kinase, the membrane-associated enzyme which forms 3-phosphoglycerate from 1,3-diphosphoglycerate. The state of high Na^+ permeability persists for several hours in the absence of glucose and it appears that shrinking the cells has opened a Na^+-specific channel through which this cation can exchange easily.

INTRODUCTION

The transport of cations in red cells of the dog and cat differs in several respects from that in human red cells. For example, although the ionic composition of the plasma of these species is similar, the red cells of the dog and cat contain low concentrations of K^+ and high concentrations of Na^+, and there is little difference between the intra- and extracellular concentrations of these ions [1]. Hence, the energy required for the active transport of cations is low and consequently the (Na^+–K^+)-dependent ATPase activity [2, 3] is considerably less than in human red cells. Although the activity of many, but not all, of the enzymes involved with glycolysis in dog red cells is less than that in humans [4], the rate of lactate production [5] is within the range of values reported in man (Glader, B. E., private communication) though at the lower end.

Another characteristic of cation transport in dog erythrocytes is that Na^+ and K^+ fluxes are reciprocally dependent on cell volume. This relationship, which was

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first studied by Davson and Reiner [6, 7] (in cat red cells), has been shown by Parker and Hoffman [8, 9] to be influenced by the metabolic state of the cells. More recently Romualdez et al. [5] reported that phloretin, which is known to inhibit membrane ATPase activity [10] and glucose transport [11], almost totally inhibits the increase in Na^+ influx that is normally produced when dog red cells are shrunken in hyperosmolal media. These investigators proposed that the volume effect changed the Na^+–K^+ specificity of a site on the cell surface, and that this process was mediated by a glycolytic intermediate at or below the triose phosphate level. In the present work, several aspects of Na^+ movements in dog red cells have been investigated in an attempt to define more specifically the relationship between Na^+ permeability, cell volume and glycolysis.

METHODS

Materials

Blood was drawn from dogs into syringes wetted with heparin, and within 5 min of collection it was chilled on ice and centrifuged at 4 °C. The plasma and buffy coat were aspirated and the cells were alternately washed and spun down three times at 4 °C in a medium of the following composition (in mM): 142.2 mM NaCl, 5.0 mM KCl, 5.2 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, 1.0 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 5.0 mM glucose (when present). The osmolality of this solution generally agreed with that of dog plasma to within 5 mosmoles/kg water. Hyperosmolal media were made up to contain 100 mM sucrose and all solutions contained 1 % (w/v) bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, Mo. 63178) to act as a carrier for the [^{131}I]albumin (RISA 131 H, Abbott Laboratories, North Chicago, Ill. 60064) which was used to estimate trapped volume in packed cells during the uptake of ^{24}Na. The pH of the bathing medium was reduced from 7.4 to 6.8 by the albumin but the buffering capacity of the red cells at about 12 % hematocrit was sufficient to raise the pH of the cell suspensions to 6.9–7.0.

Substrate depletion

Cells were washed in glucose-free media at 4 °C and resuspended at a hematocrit of approx. 50 % in a stoppered Erlenmeyer flask which was shaken in a water bath at 38 °C. Cells were partially depleted of substrate in one of two ways: (1) incubation for 2 h in the standard glucose-free isosmolal medium with and without fluoride (5 mM) or bisulfite (10 mM) (made up as 5 mM sodium metabisulfite, which in solution gives 10 mM bisulfite); (2) depletion for an initial 2-h period in the standard glucose-free medium without additional anions, after which the cells were divided into two groups, one of which was washed and resuspended in the same medium while the other group was washed and resuspended in an isosmolal medium containing either pyruvate (10 mM), bisulfite (10 mM) or sulfate (5 mM). Both groups of cells were then incubated for a further 30 min at 38 °C after which each group was divided again and centrifuged at 4 °C. 5-ml aliquots of packed cells were washed several times at 4 °C in the appropriate isosmolal or hyperosmolal solution and finally resuspended at a concentration of 5 ml of packed cells (at their isosmolal volume) to 25 ml of bathing medium in a 50-ml Erlenmeyer flask. The appropriate amount of NaCl was omitted from the bathing media containing addi-

tional anions in order to maintain a constant extracellular concentration of Na^+ at approximately the same osmolality.

$^{24}Na^+$ uptake

^{131}I-labeled albumin (RISA 131 H, Abbott Laboratories, Chicago, Ill. 60064) was passed through an anion exchange resin AG1-X8 (Biorad Laboratories, Richmond, Calif.) and made up to an activity of 0.2 mCi/ml in isosmolal saline. 0.5-ml volumes of this solution were added to each suspension of red cells which were then shaken for about 15 min at 38 °C. 0.2 ml of a $^{24}Na^+$ solution (1m Ci/ml, Cambridge Nuclear Corp., Billerica, Mass. 01821) was then added at 10-s intervals to each flask in turn. The flasks were then vigorously agitated for a few minutes. 4-ml samples of the red cell suspensions were taken from each flask at appropriate times and pipetted into centrifuge tubes standing in a tray of ice water. The samples were centrifuged at 2500 rev./min in a cold room for 5–10 min, after which samples of the supernatants were removed and saved. The remaining supernatant was aspirated and 100-μl volumes of the packed cells were sampled with a gas tight Hamilton syringe with a Chaney adaptor (Hamilton Co., Reno, Nev. 89502). The packed cells were pipetted into 2 ml of water, dispensed from a Cornwall pipettor (Beckton, Dickinson, Corp., Rutherford, N. J. 07070), in a small test tube (10 mm $\times$ 75 mm). The Hamilton syringe was washed several times with a detergent solution followed by water between each pipetting and the same syringe was used throughout each experiment.

The uptake of $^{24}Na^+$ was usually measured in duplicate at 30-min intervals for about 3 h at 38 °C. At the end of this period, the radioactive supernatants were diluted 25:1 and 200-μl aliquots of the diluted samples were added to 2 ml of water in small test tubes as with the packed cells. The activities of $^{24}Na^+$ and ^{131}I in the lysates of packed cells, diluted samples of supernatant and cell suspensions were measured in a two-channel γ-counter (System 4222, Nuclear-Chicago Corp., Des Plaines, Ill. 60016).

Subsequently, the hemoglobin concentration in each lysate was estimated from the absorbance at 540 nm after conversion to cyanmethemoglobin; Na^+ and K^+ concentrations were measured on a flame photometer (Model 143, Instrumentation Laboratory, Watertown, Mass. 02172).

All Hamilton syringes and Cornwall automatic pipettes were calibrated by weighing samples of water issued by the devices. Simple calculations then produced: (1) the time-dependence of the uptake of $^{24}Na^+$ into red cells in terms of cpm of $^{24}Na^+$/unit volume of cells, (2) a simultaneous record of the relative volume of the cells as estimated from the concentration of hemoglobin/unit cell volume and (3) actual intracellular concentrations of Na^+ and K^+ from which net fluxes could be estimated.

RESULTS AND DISCUSSION

Apparent $^{24}Na^+$ fluxes in fresh cells

Fig. 1 shows the effect of increasing the osmolality of the medium on the time course of the uptake of $^{24}Na^+$ in cells at 38 °C. As Lange et al. [12] have shown, three compartments are required to describe Na^+ flux in dog red cells under isosmolar conditions. However, as Fig. 1 shows, the uptake of $^{24}Na^+$ by cells of normal volume

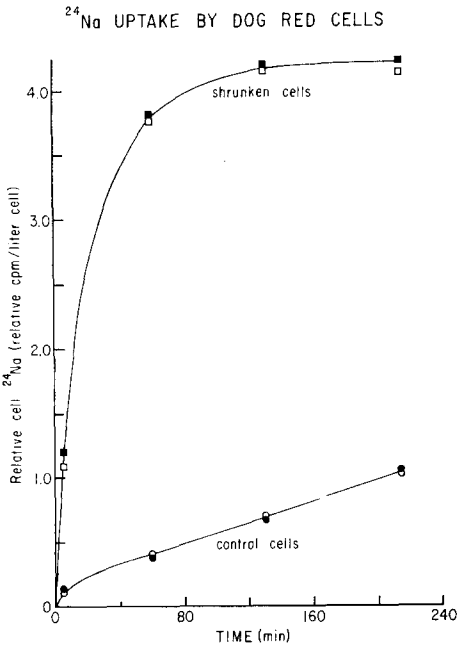

Fig. 1. Uptake of $^{24}Na^+$ by dog red cells incubated with 5 mM glucose. The ordinate is the intracellular radioactivity of $^{24}Na^+$ in cpm/l cell relative to an arbitrary unit of cpm. The cells in the upper curve were shrunken in 100 mM sucrose. Points shown are for a typical experiment with duplicate suspensions of red cells. In this and subsequent figures the specific activities of $^{24}Na^+$ in the different supernatants were the same within a few %. Results are expressed in terms of measured cell volume for both control and shrunken cells.

was linear from about 30–200 min. We have therefore computed the apparent Na^+ flux (mmoles per l cell/h) in the control cells from the least squares regression line fitted to the slope of the linear portion of the lower curve divided by the specific activity of Na^+ in the medium. As a result of the high Na^+ content of these cells and the relatively low flux in cells of normal volume, less than 20% of the cell Na^+ was labelled

TABLE I

APPARENT Na^+ FLUXES IN DOG RED CELLS

Errors are S.E. and the number of experiments is given in parenthesis.

Conditions	Apparent Na^+ influx (mmoles per l cell/h)		Volume change
	Normal cells	Shrunken cells	(%)
38 °C (+ glucose)	5.4±0.1 (6)	273±23	20±1
38 °C (substrate depleted for 2 h)	3.9±0.05 (6)	142±25	21±1
38 °C (substrate depleted for 2 h + extra 30 min)	4.0±0.3 (4)	133±21	24±1

at the end of 4 h so that we have neglected back diffusion of the isotope. Table I shows that the apparent influx of Na^+ in fresh cells in isosmolar media containing glucose was 5.4 mmoles per l cell per h at 38 °C.

Fresh cells shrank by 20–25 % when incubated in media containing 100 mM sucrose in addition to the usual constituents. As the top curve in Fig. 1 shows, $^{24}Na^+$ appeared to equilibrate exponentially in these shrunken cells. However, the asymptotic value of $^{24}Na^+$ activity in the shrunken cells was always less than that expected on the basis of equal specific activity inside and outside the cells at true equilibrium; in fact, $^{24}Na^+$ did not exchange with 17.7 ± 1.2 ($n = 19$) mmoles Na^+/l cell out of a total Na^+ content of 102.7 ± 2.1 mmoles/l cell at their final volume (errors are S.E.). The apparent flux in shrunken cells has been computed from the half-time of the exponential curve on the basis of a two-compartment model [13].

A complication arises from the continuing net loss of about 7 mmoles per l cell/h of the Na^+ in the shrunken cells which, as Fig. 2 shows, is accompanied by a further reduction in cell volume. The apparent flux computation from the exchange of Na^+ has been made on the basis of the average cell Na^+ content and volume during the experimental period, and is expressed in terms of the original volume of the cells in isosmolal medium. Fig. 1 shows that the difference in $^{24}Na^+$ uptake under the

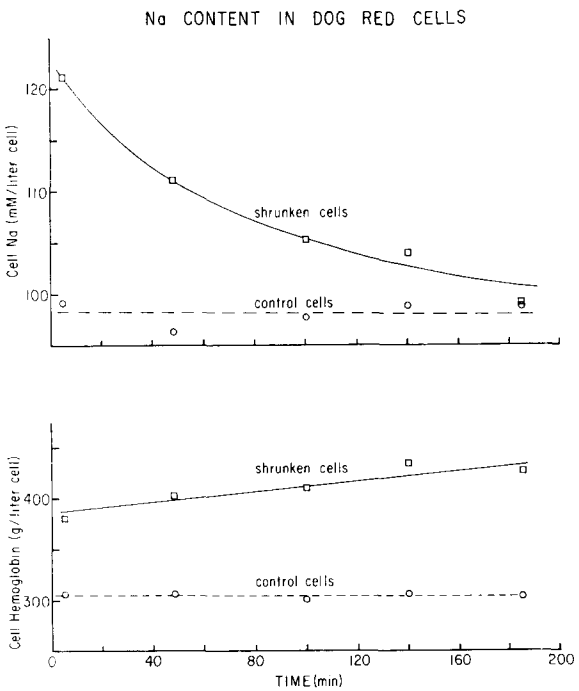

Fig. 2. Na^+ content in dog red cells after 2 h substrate depletion. Inthe control cells the mean Na^+ is 98.2 ± 0.5 mmoles/l cell and the mean hemoglobin concentration is 304 ± 1 g/l cell. The cells were shrunken in 100 mM sucrose and the average change in cell volume in this experiment was 25.8 %; the slope of the hemoglobin concentration line is 0.24 ± 0.14 g/l cell per min. The cells in this figure had been depleted of substrate for 2 h, the treatment used for the experiments with the anion inhibitors.

two conditions is very great; hence, the approximations involved in the apparent flux calculations are unimportant compared to the size of the effect.

In order to establish the reversibility of the effects of the volume change on Na^+ flux, cells were exposed for 30 min to a hyperosmolal solution at 38 °C and resuspended in an isosmolal medium. Table I shows that the flux of Na^+ in shrunken cells rose sharply to an average of 171 mmoles per l cell/h at 38 °C. After the cells were washed and resuspended in an isosmolal medium the Na^+ flux returned to 7.4 ± 1.2 and 8.3 ± 0.8 mmoles per l cell/h compared with control values of 5.3 ± 0.3 and 4.95 ± 0.45 mmoles per l cell/h in duplicate suspensions of cells maintained in isosmolal conditions. This shows that the change in the structure of the cell membrane which leads to the increase in Na^+ permeability is a reversible one, thereby confirming the observations of Romualdez et al. [5] who showed that a transient change in dog red cell volume, produced by incubating cells in media containing permeant non-electrolytes, had no lasting effect on the permeability characteristics of the cell. A similar observation was made by Sha'afi and Hajjar [14] on cat red cells.

Relationship with glycolysis

A link between the volume effect and the metabolic state of the cell was established by Hoffman [9], who found that the response to a given change in volume of cells depleted of substrate for 12 h was partly restored, together with lactate production, when the cells were incubated in media containing adenosine. Subsequently Romualdez et al. [5] showed that the volume effect in depleted cells could be restored by glucose, but not by 2-*O*-methylglucose.

A duplicated experiment in the present study indicated that when dog red cells were washed free of glucose there was little effect either on the rate of $^{24}Na^+$ uptake under isosmolal conditions or on the response of Na^+ to the change in cell volume. This implies that the first step in the glycolytic sequence involving hexokinase is not implicated in the response of Na^+ influx to a change in cell volume and that Na^+ and glucose transport are not tightly coupled in the dog red cell as they are in other systems discussed by Schultz and Curran [15]. This in turn means that phloretin, which almost totally inhibits the volume effect [5], must be exerting its influence on a site other than the first step in glucose transport, and may be interfering with the cation transport process itself.

Experiments were carried out on substrate depleted cells in an attempt to localize the part of the glycolytic cycle responsible for the volume effect. Feig et al. [16] have shown that, after 1 h without glucose, human red cells are depleted of all intermediates up to and including the triose phosphates. When dog red cells were depleted of glucose for 2 h prior to studies of the volume effect, there were no significant changes in Na^+ and K^+ content or the volume of the cells as measured by hemoglobin content. As Table I shows, this procedure reduced the Na^+ influx in normal cells by about 28 % and in shrunken ones by about 50 %. The table also shows that substrate depletion for a further 30 min had no added effect.

We had hoped that the triose phosphates in dog red cells would have disappeared completely in 2 h. However, a few estimations in Dr Nathan's laboratory on dog red cells indicated that consumption of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate was only about 80–100 % complete after 2 h and so there was some residual glyceraldehyde-3-phosphate dehydrogenase activity in the 2-h

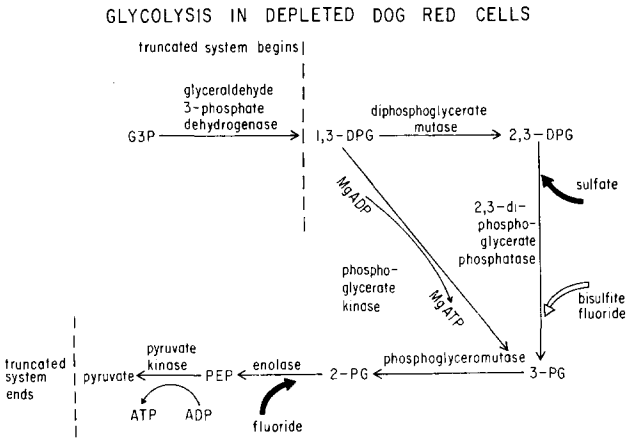

Fig. 3. Schematic showing truncated glycolytic sequence with major components remaining in dog red cells after 2-h substrate depletion. Symbols: G3P, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 2,3-DPG, 2,3-diphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate.

depleted cells used in this study. If triose phosphate disappearance were complete, the glycolytic cycle could be considered to be partially truncated as shown in Fig. 3 since glyceraldehyde-3-phosphate dehydrogenase regulates the supply of NADH in the cell which in turn controls the final step (pyruvate–lactate) in glycolysis. The main reactions remaining after 2 h depletion start with 1,3-diphosphoglycerate, which is present at a concentration of approx. 1 μM under normal conditions [8, 17] and end with pyruvate, which is present at concentrations of the order of 50 μM in the steady state. The primary substrate is 2,3-diphosphoglycerate which, as Table II shows, is present at a concentration of 5.3 mmoles/l cell in 2-h depleted cells. Since the triose phosphates in depleted dog red cells are reduced to 20 % or less of their normal concentration, we will neglect the influence of the glycolytic reactions outside of the truncated system.

Hoffman [9] showed that, for a given change in cell volume, Na^+ influx increased by approximately the same factor in non-depleted, and repleted dog red cells; though the increase remained after 12 h depletion, its absolute magnitude was de-

TABLE II

2,3-DIPHOSPHOGLYCERATE CONCENTRATION IN DOG RED CELLS

Fresh cells were washed in glucose-free media at 4 °C and immediately extracted in $HClO_4$. Estimations were made on red cells from one animal using the method of Keitt [18] and the errors are the range of values from duplicate suspensions.

	2,3-Diphosphoglycerate (mmoles/l cell)
Fresh cells	5.45 ± 0.05
Control, 2 h depleted	5.30 ± 0.12
2 h depleted + 10 mM bisulfite	3.31 ± 0.04

TABLE III

EFFECT OF ANIONS ON APPARENT Na^+ INFLUX IN SUBSTRATE DEPLETED DOG RED CELLS AT 38 °C

The experiments with fluoride and 4 of the experiments with bisulfite were carried out with anion present during the 2-h substrate depletion. The remainder of the experiments, including 2 with bisulfite, were carried out in the absence of anions during the 2-h substrate depletion; the anions were added in a subsequent 30-min incubation period, as described under Methods. Since there was no significant difference in the bisulfite experiments, the controls have been averaged and the results of all the anion experiments have been combined.

	Number of experiments	Apparent Na^+ influx (mmoles per l cell/h)	
		Normal cells	Shrunken cells
Control	10	3.9±0.4	139±16
+ 5 mM fluoride	2	6.1±0.3	16.9±0.5
+ 5 mM sulfate	2	3.84±0.03	176± 5
+ 10 mM bisulfite	6	13.5±0.9	11.3±0.9
+ 10 mM pyruvate	4	4.5±0.2	154±8

creased. Similarly, in the present study the ratio of Na^+ fluxes (hyperosmolar : isosmolar) was 50±4 ($n = 6$) in fresh cells compared with 36±4 ($n = 10$) in cells depleted of substrate for 2 h. Since the difference between these values is barely significant ($0.02 < P < 0.05$) the volume effect is not altered drastically by this partial depletion of substrate.

Table III shows the effect of various anions on apparent Na^+ flux in 2-h depleted cells. Addition of these anions had no effect on cell shrinking as determined by measurements of hemoglobin concentration. The effects of fluoride were studied initially because it is known to inhibit enolase activity [19]; however, like other inhibitors, fluoride is not specific, and Rose [17] has shown that in the human red cell it also activates 2,3-diphosphoglycerate phosphatase. We suggest that the overall effect in the dog red cell is probably an accumulation of 3- and 2-phosphoglycerate produced from hydrolysis of the large pool of 2,3-diphosphoglycerate (Table II). Fluoride (5 mM) caused a marked reduction in the increase in apparent Na^+ flux when substrate depleted cells were shrunken as shown in Line 2 of Table III whereas the flux under isosmolar conditions increased significantly.

In order to study the enzymes involved in the formation of 3-phosphoglycerate, we investigated the effects of sulfate and bisulfite. These anions not only stimulate glycolysis in fresh human red cells but also, as Gerlach et al. [20] have shown, interfere in at least two reactions in the glycolytic sequence. Sulfate activates phosphofructokinase but inhibits 2,3-diphosphoglycerate phosphatase. The result in intact human red cells according to Gerlach et al. [20] is a dramatic increase in the levels of fructose 1,6-diphosphate and the triose phosphates but virtually no change in the content of 3-phosphoglycerate. In the 2-h depleted dog red cell we can assume that the increase in fructose 1,6-diphosphate probably does not take place because there is little, if any, substrate for phosphofructokinase to react with. However, the sulfate inhibition of 2,3-diphosphoglycerate phosphatase probably plays a significant role in the truncated glycolytic system since the concentration of its substrate, 2,3-diphosphoglycerate, remains at a high level in depleted cells (Table II). Nonetheless Table III (Line 3)

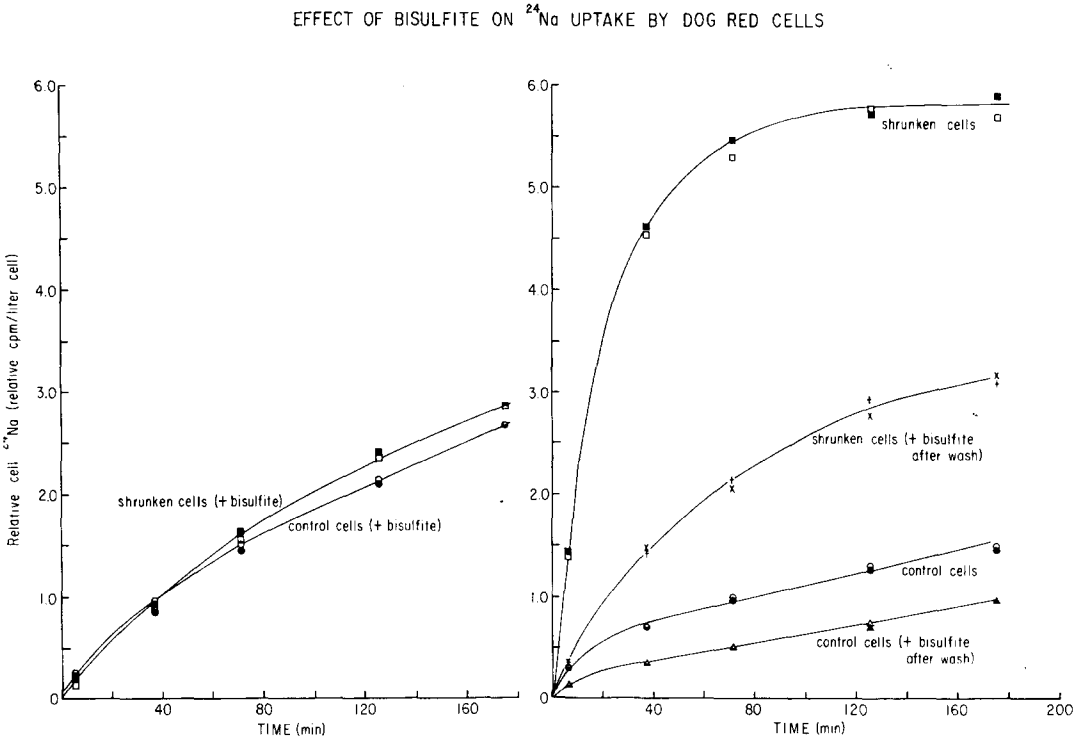

Fig. 4. Left hand: effect of 10 mM bisulfite on $^{24}Na^+$ uptake in normal dog red cells and cells shrunken in 100 mM sucrose. Right hand: partial recovery of the volume response after washing the cells in bisulfite-free media. All these cells were first substrate depleted in isosmolar conditions for 2 h in glucose-free media containing 10 mM bisulfite.

shows that sulfate has little effect on apparent Na^+ fluxes in normal or shrunken cells. We therefore conclude that the hydrolysis of 2,3-diphosphoglycerate does not in itself play a rate limiting role in the volume effect.

The effect of bisulfite provides further information about metabolic control of the flux changes associated with cell shrinkage. Though bisulfite increases the apparent Na^+ influx in isosmolal conditions, the volume effect disappears as shown in Table III (Line 4). The left hand section of Fig. 4 indicates that the time course of cell $^{24}Na^+$ uptake is virtually independent of cell volume, the right hand section of Fig. 4 shows that the action of bisulfite is at least partially reversible as, when cells were washed in bisulfite free medium, the Na^+ influx was lowered in cells of normal volume to a value below that of untreated cells and a significant increase in the Na^+ flux of shrunken cells was also restored.

The reversibility of the bisulfite action is consistent with our conclusion that the volume effect is not regulated by the hydrolysis of 2,3-diphosphoglycerate. Parker [21] has shown that 10 mM bisulfite causes 2,3-diphosphoglycerate concentration in human red cells to fall by about 50 % in 2 h despite the presence of glucose. We have found a similar effect on 2,3-diphosphoglycerate concentration in substrate depleted dog red cells as shown in Table II. Thus the partial restoration of the volume

effect after the removal of bisulfite indicates that the effect is not dependent on the maintenance of cell 2,3-diphosphoglycerate at or near the normal level.

Gerlach et al. [20] have shown that bisulfite is a strong activator of 2,3-diphosphoglycerate phosphatase. This activation by bisulfite should produce increased levels of 3-phosphoglycerate in the cell particularly since 2,3-diphosphoglycerate is present in such large amounts in both normal and substrate depleted dog cells. Accumulation of 3-phosphoglycerate should cause product inhibition of the reaction catalysed by phosphoglycerate kinase. This explanation is consistent with the inhibition of the volume effect by fluoride which may also produce an increased level of 3-phosphoglycerate. It is unlikely that the effects of fluoride and bisulfite can be attributed to an effect on phosphoglyceromutase since the two anions will tend to drive the 3-phosphoglycerate to 2-phosphoglycerate reaction in opposite directions.

Parker [21] attributes the effect of bisulfite on human red cell cation permeability to the reducing power of this anion. However, Table III shows that fluoride, which is not a reducing agent, can also inhibit the volume effect in the dog red cell. Thus, it does not appear that the action of bisulfite in the dog red cell can be accounted for in terms of a generalized reducing ability, but rather in terms of its action on the glycolytic cycle. This conclusion is consistent with the requirement of a glycolytic substrate for the increased volume effect shown by Hoffman [9] and Romualdez et al. [5].

Because pyruvate rapidly permeates the cell membrane and is a product of the reaction involving pyruvate kinase, it might be expected that a high pyruvate con- would inhibit this reaction or even drive it in reverse. Table III (Line 5) shows that centration pyruvate did not influence the volume effect; this means that the enhanced flux brought about by shrinking the cells is not mediated by pyruvate kinase.

Both the influx and efflux of Na^+ are changed by about the same extent in shrunken cells so that the cells remained shrunken, and even shrank further as some Na^+ leaked out of the cells as shown in Fig. 2. This state of high Na^+ permeability persisted for several hours at 38 °C, even in substrate-depleted cells in the absence of glucose (unpublished observations). The half-time for the $^{24}Na^+$ uptake in non-depleted, shrunken dog red cells was 12.8±0.6 min and this process had essentially come to equilibrium in 2 h, as shown in Fig. 1. The mean influx over this 2-h period was 273 mmoles per l cell per h and yet, as Fig. 2 shows, cell Na^+ fell only by about 15 mmoles/l cell in 2 h (Fig. 2). Hence, this massive flux can only be the result of an almost 1 : 1 exchange of Na^+ across the membrane. The process is specific for Na^+ since it has been shown [5, 8] that the increase in Na^+ flux is accompanied by a decrease in K^+ flux. Thus the volume effect has opened a Na^+ specific channel, through which these cations can exchange freely. Such exchanges require little or no expenditure of energy, which accords well with the persistence of the increased Na^+ flux in substrate depleted cells in the absence of glucose, as well as with the observation

* Rose [17] has shown that 3-phosphoglycerate is a cofactor for diphosphoglycerate mutase so that the velocity of this reaction will also depend upon the concentration of 3-phosphoglycerate. However, Rose also points out that the inhibition of the diphosphoglycerate mutase reaction by 2,3-diphosphoglycerate is important in setting the rate of this reaction under normal metabolic conditions in human red cells. The partial reversal of the volume effect when bisulfite is washed out after having effected the substantial decrease in 2,3-diphosphoglycerate concentration shown in Table II indicates that the diphosphoglycerate mutase reaction is not the rate limiting step.

(unpublished results) that ouabain does not inhibit the volume effect. The present studies also support the view that the changed transport properties are a permanent feature of the shrunken state but revert to the original specificity when the volume is restored to the initial state.

In human red cells, Parker and Hoffman [22] concluded that phosphoglycerate kinase is the point at which the cation transport system can influence the metabolic rate. In dog red cells, Romualdez et al. [5] have proposed that the volume effect depends upon a reaction in the glycolytic sequence at the level of triose phosphate or below. Both of these findings are consonant with the present evidence for phosphoglycerate kinase as the mediator of the volume effect on Na^+ transport in dog red cells. A possible mechanism would be the following: shrinking the cells causes a conformational change in phosphoglycerate kinase, which can only take place if the concentration of 3-phosphoglycerate is in the normal range. This conformational change, in turn, affects the conformation of the Na^+ transport site and endows it with the properties of a Na^+ channel.

Since this study was completed, Ingram* has demonstrated that bisulfite causes a dramatic increase in 3-phosphoglycerate in 2-h depleted dog red cells, which provides strong support for the present hypothesis. In view of this evidence it seems desirable to examine the physical and chemical properties of phosphoglycerate kinase in further detail. It is a Mg^{2+}-stimulated enzyme which catalyses the reaction

$$MgADP + 1,3\text{-diphosphoglycerate} \rightleftharpoons MgATP + 3\text{-phosphoglycerate}.$$

The reaction is reversible and is sensitive to the ADP/ATP ratio [23]. It can be inhibited by 3-phosphoglycerate. There appear to be three adjacent binding sites, one occupied by 3-phosphoglycerate. Recent X-ray diffraction studies of the conformation of yeast phosphoglycerate kinase by Wendell et al. [24, 25] have shown this protein to have an unusual shape, consisting of two opposing lobes connected by a narrow waist. The binding site for the MgATP is on the inside of the 20 Å deep wedge-shaped space between the two lobes. In a molecule of such an unusual shape the en-

* Dr C. J. Ingram (personal communication) has kindly measured the 3-phosphoglycerate concentration in dog red cells treated with bisulfite as described under Methods. After addition of bisulfite, the preparation was titrated back to pH 7.5 and 3-phosphoglycerate was measured by the method of Keitt [18] as modified by Dr. B. E. Glader (private communication). The following results were obtained.

EFFECT OF BISULFITE ON 3-PHOSPHOGLYCERATE IN DOG RED CELLS

	3-phosphoglycerate concn (μmoles/l cells)	
	2 h incubation without glucose	2 h incubation with glucose
Control	0	8±5
+sucrose	8±5	8±5
+bisulfite	7±5	7±5
+bisulfite and sucrose	100±15	70±15

Similar results were obtained in other experiments in which the preparation was not titrated back to a pH 7.5 after addition of bisulfite, except that in these experiments bisulfite also increased the 3-phosphoglycerate concentration in the absence of glucose. Changes in pH within the range of pH 6.8–7.8 do not greatly interfere with the volume effect.

vironment of the MgATP and 3-phosphoglycerate sites might well be sensitive to membrane shape changes. There is another interesting aspect of the structure-function relationship. Parker and Hoffman [22] present evidence that the ADP produced by the (Na^+, K^+, Mg^{2+})-activated ATPase in the human red cell cation transport system is compartmentalized and set apart from the remainder of the ADP. The authors further suggest that the compartmented ADP serves as substrate for phosphoglycerate kinase. These conclusions are entirely consistent with the location of the ADP site on the inside of the 20-Å wedge and indicate the phosphoglycerate kinase is closely apposed to the transport ATPase in man.

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