

## Volume-responsive Sodium and Proton Movements in Dog Red Blood Cells

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**ABSTRACT** Shrinkage of dog red blood cells (RBC) activates a Na transport pathway that is Cl dependent, amiloride sensitive, and capable of conducting Na-proton counterflow. It is possible to establish transmembrane gradients for either Na or protons and to demonstrate that each cation species can drive reciprocal movements of the other. The nature of the coupling between Na and proton movements was investigated using the fluorescent probe diS-C<sub>3</sub>(5) and also by an indirect method in which K movements through valinomycin channels were used to draw inferences about the membrane potential. No evidence was found to suggest that the Na-proton pathway activated by shrinkage of dog RBC is a conductive one. By exclusion, it is presumed that the coupling between the counterflow of Na and protons is electroneutral. The volume-activated Na-proton fluxes in dog RBC have certain properties that distinguish them from similar transport pathways in other cell types.

### INTRODUCTION

Ever since Davson's (5) observations on the behavior of dog red blood cells (RBC) in solutions of potassium salts, investigators have been aware that these cells have strongly volume-dependent cation permeabilities. Na flux rises sharply when the cells are shrunken; K and Ca movements increase as the cells are swollen (15, 22, 23). Recently, Na fluxes in the nucleated RBC of *Amphiuma* were found to be activated by cell shrinkage and to be amiloride sensitive (26, 27). The features of a Na-proton exchanger in shrunken *Amphiuma* RBC were reported in detail by Cala (3, 4). It appears that dog RBC may have a similar pathway (19). The present experiments were designed to explore the characteristics of the dog RBC system and to compare it with Na-proton countertransporters in other tissues. Some of these results have been presented in abstract form (17, 20).

It should be borne in mind that mature, circulating dog RBC are unusual among all animal cells in that they lack a Na-K pump. They have no digitalis-sensitive Na or K fluxes, and they lack Na,K-ATPase. Their cytoplasm has the

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same concentration of Na (per kilogram of cell water) as does dog plasma. Despite their lack of a Na-K pump, the cells can extrude Na by means of a transport pathway that involves external Ca and has some properties of a Ca-Na exchanger (14, 15). The cells have a very sensitive Ca-activated K (Gardos) pathway (18, 24). They are subject to hemolysis when alkalinized even slightly beyond physiologic pH levels (28). Extracellular ATP causes dog RBC to become greatly and reversibly permeable to Na and K via conductive pathways (21), a phenomenon that is useful in preparing cells with differing Na, K, and water contents for experimental purposes.

#### MATERIALS AND METHODS

Sigma Chemical Co. (St. Louis, MO) was the source of dipyridamole, acetazolamide, and quinidine sulfate. Amiloride was the kind gift of the Merck Institute for Therapeutic Research (West Point, PA). 3,3'-Dipropylthiadicarbocyanine [DiS-C<sub>3</sub>(5)] was the kind gift of Dr. Alan Waggoner, Amherst College, Amherst, MA.

Blood was drawn from the antecubital veins of mongrel dogs into heparin-rinsed syringes and processed within 20 min for each experiment. At the beginning of each study, the blood was centrifuged at room temperature and the plasma and buffy coat were discarded. Details regarding the washing, preincubation, and incubation of the cells are given in the legends to the figures and tables. Analysis of packed RBC for Na, K, Cl, and water were done by methods previously published by this laboratory (13).

The experiments shown in Figs. 3 and 16 involved the use of a constant-temperature jacketed beaker equipped with continuous pH-monitoring capability. Statting of the pH was done manually, exactly as described in a previous paper (19) and in the legend to Fig. 16.

Several experiments were done to ascertain whether sudden shrinking of dog RBC in a choline medium would cause membrane hyperpolarization. The methods for these studies, adapted from the report of Hoffman and Laris (11), are detailed in a previous publication on dog RBC (21). Procedures used for the experiment in Fig. 4 were as follows. Cells were suspended at a hematocrit of 0.33% at 22°C in a medium containing (mM): sum of choline-Cl plus KCl = 130, 0.5–10.0 KCl, 5.5 glucose, and 10 HEPES (pH 7.4 at 22°C with Tris-OH). The total volume of suspension in the cuvette was 3 ml. DiS-C<sub>3</sub>(5) was present at a concentration of 0.66 µg/ml. After the fluorescence signal stabilized the solution, the osmolarity was raised 100 mosmol by the addition of 0.025 ml/6 M choline-Cl. The new fluorescence level became stable within 15 s. The membrane potential of dog RBC was measured before and after shrinkage by determining the null point for K, i.e., the external K concentration at which there is no change in fluorescence in response to the addition of valinomycin at a final concentration of 1 µM. At the K null point, the membrane potential can be estimated by the Nernst potential for K as described previously (11). Companion experiments were performed with cells suspended in the same choline medium outlined above (minus buffer) at 22°C and a hematocrit of 7.4%. As presented in the next section, the addition of sufficient choline-Cl to raise the osmolarity by 100 mosmol resulted in cell shrinkage and alkalinization of the medium similar to the findings in KCl solutions presented in Fig. 3.

#### RESULTS

##### *Effects of Anion Substitutions and Amiloride*

The events following the exposure of dog RBC to KCl media of various tonicities have been described previously (5, 16, 18). In hypotonic KCl, the cells take up

water immediately and then maintain a stable or gradually increasing volume over hours. In hypertonic KCl solutions, the cells undergo a prompt osmotic water loss, followed by a second phase of cell shrinkage during which solute leaves the cell. The acute cell volume decrease triggers the opening of a passive, Na-selective pathway through which the outward Na gradient is dissipated. Although there is a large, inward K gradient, K permeability remains low, and the cells become depleted of Na, Cl, and water.

Fig. 1 shows that the loss of cell Na in a hypertonic K solution is reduced when the Cl is replaced by NO<sub>3</sub> or SCN, two ions that have a higher conductance than Cl through the dog RBC membrane (18). The lower baseline water content of cells suspended in thiocyanate solutions is inexplicable in terms of medium osmolality; this property of SCN solutions has been noted in human RBC by others (8).

The loss of cell Na into a hypertonic K medium is 50% inhibited by 5  $\mu$ M amiloride, as shown in Fig. 2. In a previous study with 150 mM external Na, outward movements of tracer Na were inhibited 50% by 10  $\mu$ M amiloride (19).

#### *Coupling of Na and Proton Counterflow*

Because movements of Na in shrunken dog RBC were found to be inhibited by amiloride, an early attempt was made to demonstrate the presence in these cells of a known amiloride-sensitive pathway (1). Two likely possibilities seemed to be a conductive Na channel like the ones described in some epithelia (12) or an electroneutral Na-proton exchanger of the type described in shrunken *Amphiuma* RBC by Cala (3, 4). The latter alternative was suggested by experiments showing proton movements between cells and unbuffered media (19). Fig. 3 shows an experiment that confirms and extends this idea. Fresh, high-Na cells were suspended in unbuffered KCl media, either hypertonic (210 mM KCl) or hypo-

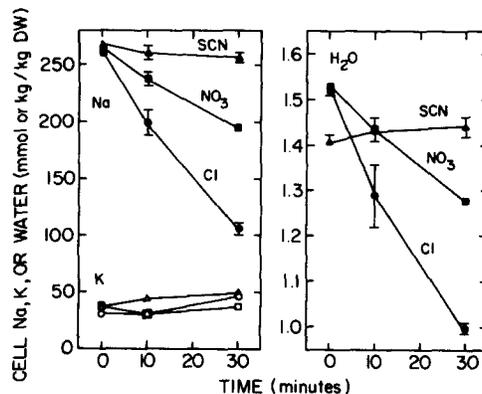


FIGURE 1. Cell Na, K, and water as functions of time. Effects of exposure to hypertonic K media containing Cl (circles), NO<sub>3</sub> (squares), or SCN (triangles) as the major anion. Fresh cells were washed three times in solutions containing (mM): 180 NaCl, NaNO<sub>3</sub>, or NaSCN, 10 HEPES, 5 glucose, 0.2 EDTA (355–360 mosmol, pH 7.4, 37°C). Cells were then incubated at 37°C in media similar to those in which they had been washed, but with 180 mM KCl, KNO<sub>3</sub>, or KSCN replacing the Na salts. Mean and range for two studies.

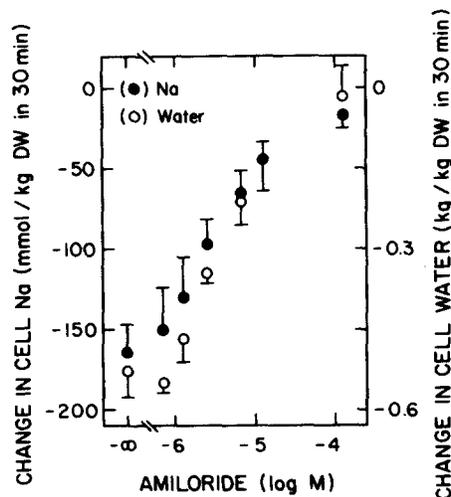


FIGURE 2. Change in cell Na (left ordinate, solid circles) and water content (right ordinate, open circles) over a 30-min incubation period as a function of amiloride concentration in  $\log_{10}$  molar. Wash and incubation procedures were the same as for the 180 mM KCl incubations detailed in the legend to Fig. 1. Mean  $\pm$  SD for three studies.

tonic (110 mM KCl). As found previously (19), the shrunken cells alkalinized the medium progressively with time, although the swollen cells did not do so. Application to the cells of dipyridamole, an agent that in human RBC inhibits both Cl conductance and Cl-CI exchange by 90% (10), increases the rate at which dog RBC alkalinize a hypertonic KCl medium; the drug does nothing to the medium pH in swollen cells.

It might be argued that the proton movements in hypertonic KCl media occur in response to a hyperpolarization of the cell. If the Na pathway that is stimulated by cell shrinkage were conductive, then the creation of a large, outward Na gradient might cause the cell interior to become increasingly negatively charged with respect to the medium, thus driving protons inward. The influence of the Na gradient on the membrane potential would have to be substantial, because the inward proton movements are easily seen when Cl conductance is unmodified by drugs. The action of dipyridamole could be explained in terms of the reduction that the drug causes in  $P_{Cl}$ , rendering the Cl gradient a weaker determinant of the membrane potential and strengthening the relative influence of the large Na concentration difference on the inward driving force for protons.

The hypothesis that a large, selective Na conductance is triggered by shrinking dog RBC was tested using the fluorescent probe diS-C<sub>3</sub>(5) to monitor the membrane potential under conditions that were shown to result in inward proton movements. The fluorescence results (Fig. 4) show that when the cells are shrunken in a buffered, Na-free medium, the membrane is clearly not hyperpolarized, as would be predicted if the Na permeability pathway that opens up under these circumstances were conductive. It can be calculated, given a constant  $P_{Cl}$  of  $2 \times 10^{-8}$  cm/s (21), that if the >10-fold increase in Na flux with cell

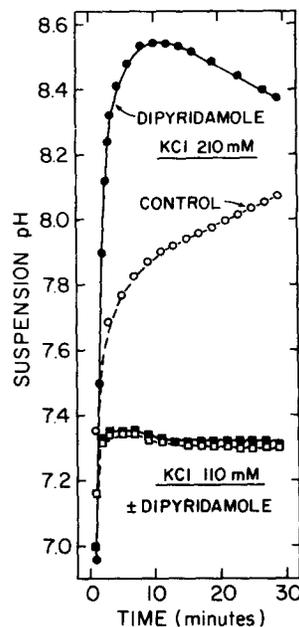


FIGURE 3. Extracellular pH of unbuffered suspensions of dog RBC in hypo- and hypertonic KCl media in the presence (solid symbols, solid lines) or absence (open symbols, dashed lines) of dipyrindamole. Fresh cells were washed three times with a solution containing (mM): 150 NaCl, 10 HEPES, 0.1 KHCO<sub>3</sub> (pH 7.4, 37°C). Cells were then washed three more times with unbuffered media containing (mM): 150 NaCl, 5 glucose, 0.1 KHCO<sub>3</sub>, 0.1 EDTA. 2-ml aliquots of cells were then transferred to 50 ml pre-warmed (37°C) incubation media containing (mM): 210 or 110 KCl, 5 glucose, 0.1 KHCO<sub>3</sub>, 0.1 EDTA, plus or minus 0.05 dipyrindamole. In this and subsequent experiments employing dipyrindamole, all incubation media contained DMSO 0.4 volumes percent, because this agent was necessary to solubilize the dipyrindamole. Suspension pH was monitored continuously and recorded every 30 s. Single experiment representative of four others.

shrinkage (19) represented a (conductive) increase in  $P_{Na}$ , the membrane potential under the conditions outlined in Fig. 4 would become twice as negative after the medium is made hypertonic. Instead, a slight depolarization is observed. In three experiments, the membrane potential before making the suspension hypertonic (cell water  $68 \pm 0.9\%$  wet weight) was  $-6.4 \pm 0.4$  mV. 3 min after sufficient choline-Cl was added to increase the osmolality by 100 mosmol, the cell water was  $62.2 \pm 1.5\%$  and the membrane potential was  $-3.3 \pm 2.2$  mV (mean  $\pm$  SEM).

Several studies were done to validate the conclusions drawn from the fluorescent probe measurements in Fig. 4. First, we showed that alkalinization of an unbuffered medium in response to an outward Na gradient in shrunken dog RBC occurs at 22°C when the major external cation is choline and when no inhibitor of Cl movement is added (see Materials and Methods for details): under these circumstances, the medium pH increased by  $0.18 \pm 0.01$  unit in the first

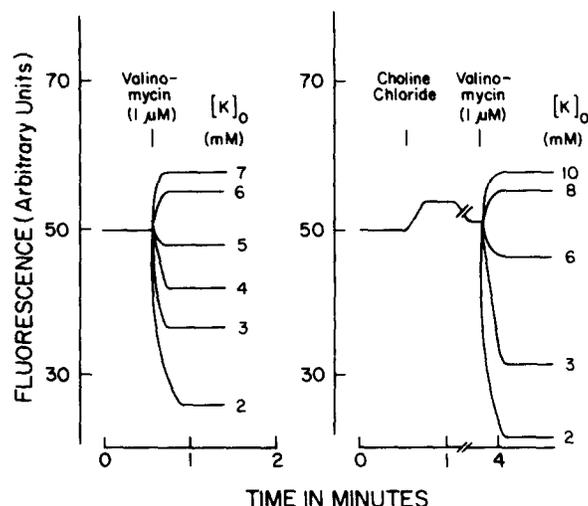


FIGURE 4. Fluorescence (arbitrary units) as a function of time in minutes in HEPES-buffered suspensions of dog RBC containing diS-C<sub>3</sub>(5). The external medium contained mainly choline-Cl with various concentrations of KCl as noted on the graph (see Materials and Methods for precise details). This figure presents tracings of recordings obtained in a single experiment, normalized to the same steady level of fluorescence before the addition of valinomycin (11). The left panel indicates that cells suspended in 130 mM choline-Cl have a null point for external K of 5.2 mM, which together with an internal K of 6.7 mmol/liter cell water gives a Nernst potential for K (equals the membrane potential) of  $-6.4$  mV. The right panel shows that after adding sufficient hypertonic choline-Cl to raise the osmolality by 100 mosmol, the null point for external K changes to 7.3 mM. Cell K under these conditions is 8.4 mmol/kg cell water, giving a membrane potential of  $-3.3$  mV. See text for mean values of three experiments.

minute and  $0.3 \pm 0.05$  unit in the third minute after the medium was made hypertonic (mean  $\pm$  SEM;  $n = 3$ ). Second, in an incubation done under the same conditions used in the fluorometer—in the presence of  $0.66 \mu\text{g/ml}$  diS-C<sub>3</sub>(5) and with 10 mM HEPES buffer (pH 7.40 at 22°C)—an amiloride-sensitive net Na efflux was observed that was unaccompanied by a demonstrable pH change over 10 min and was not different from the Na flux in identical circumstances minus diS-C<sub>3</sub>(5). Thus, the fluorescent probe does not inhibit the Na pathway that is induced by cell shrinkage and the pH changes are effectively clamped by 10 mM HEPES.

It could be argued that when the cells shrink, the proton conductance rises to such a level that it dominates all other conductances, including that of Na, and that the failure to see a hyperpolarization in Fig. 4 is due to clamping of the membrane potential at the proton equilibrium potential. Given the nanomolar concentrations of protons on either side of the membrane, such an explanation would require a vast increase in proton permeability. One would expect that the introduction of a large new conductance incident to cell shrinkage might seriously influence the calibration curve (change in fluorescence vs. external K concentra-

tion) in the presence of valinomycin, and while the calibration curves for normal and shrunken cells in Fig. 4 are not identical, they are of the same general magnitude. This objection to the fluorescence data still remains a theoretical possibility, but as the next series of experiments will show, we think it is unlikely that dog RBC shrunken in proton-buffered media have a dominating proton conductance.

Figs. 5–7 present an alternative approach to the question of whether the Na-proton pathway that appears in shrunken dog RBC is conductive. The strategy here is to recapitulate in part the conditions of Fig. 3; that is, to put dog RBC into Na-free, hypertonic media. Instead of following inward proton movements, however, we stabilize the medium pH with HEPES buffer, add valinomycin and a low concentration of K to the medium, and look to see whether K will be drawn into the cells through valinomycin channels in response to a putative change in membrane potential caused by a shrinkage-induced increase in Na conductance.

Fig. 5 shows movements of Na, K, and water in fresh cells suspended in a buffered 200 mM choline-Cl medium with 2.5  $\mu$ M valinomycin plus and minus dipyridamole and plus and minus ATP, an agent known to induce conductive Na (and K) movements in dog RBC (21). The external K concentration is 2 mM. Note that under control circumstances (minus ATP), Na efflux is hardly influenced by dipyridamole, whereas K efflux into the 2 mM K medium is retarded by the drug, as would be expected if valinomycin-induced net K movements are limited by Cl permeability. ATP causes an increase in the rate of outward Na movement and, in the presence of ATP, dipyridamole has a retarding effect on Na efflux. With ATP added, K is actually taken up by the cells against a concentration gradient during the early time points; this effect is amplified by dipyridamole, an agent that reduces Cl conductance and thus brings the membrane potential closer to the Na equilibrium potential. In ATP-treated cells, K is driven inward by hyperpolarization of the membrane incident to the large, outward Na gradient. Evidence for this can be found in an earlier report (21), where we showed with diS-C<sub>3</sub>(5) that movements of K in response to imposed Na gradients in ATP-treated dog RBC were due to changes in membrane potential. Fig. 6 shows an experiment almost identical to that of Fig. 5, but with an external K of 4 mM.

The results in Figs. 5 and 6 bear indirectly on the interpretation of the fluorescence data presented in Fig. 4. The membrane potential of shrunken dog RBC in 10 mM HEPES-buffered RBC suspensions is not so dominated by the proton (or Cl) equilibrium potential as to be uninfluenced by an ATP-induced change in Na permeability that mediates Na movements of the same general magnitude as those triggered by cell shrinkage. Thus, if cell shrinkage activated a conductive Na permeability, the cells in Fig. 4 should have become hyperpolarized when the medium was made hypertonic.

In Fig. 7, a similar series of experiments is reported that examines the effect of amiloride and ATP on K movements through the valinomycin channel in shrunken cells with a large, outward concentration gradient for Na. Extracellular K is 4 mM and dipyridamole is present in all cases. In the absence of ATP

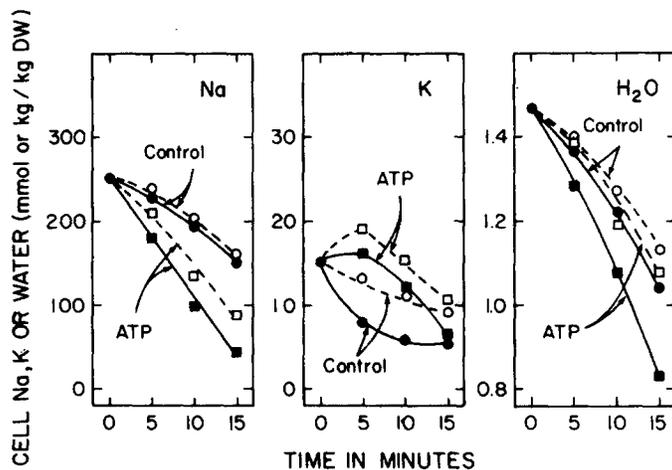


FIGURE 5. Cell Na (left panel), K (center panel), and water (right panel) as functions of time in minutes. Fresh RBC were washed twice with a solution containing (mM): 140 NaCl, 10 HEPES (pH 7.4, 37°C), and once with a solution similar to the first wash but with all the NaCl replaced by 140 mM choline-Cl. One aliquot of the cells was washed one more time with a solution containing (mM): 200 choline-Cl, 10 HEPES (pH 7.40, 37°C) for the zero-time determinations. The remaining cells were divided into four equal portions and resuspended in four solutions, prewarmed to 37°C, containing the following ingredients (mM): 200 choline-Cl, 2 KCl, 10 HEPES, 5 glucose, 0.0025 valinomycin (pH 7.4, 37°C). Additions to this medium together with the graph symbols are as follows (mM): ●, no additions; ○, 0.05 dipyrindamole; ■, 0.125 ATP; □, 0.125 ATP plus 0.05 dipyrindamole. At 5, 10, and 15 min, samples were centrifuged at 2°C for determination of Na, K, and cell water. Single experiment. For a closely similar study, see Fig. 6.

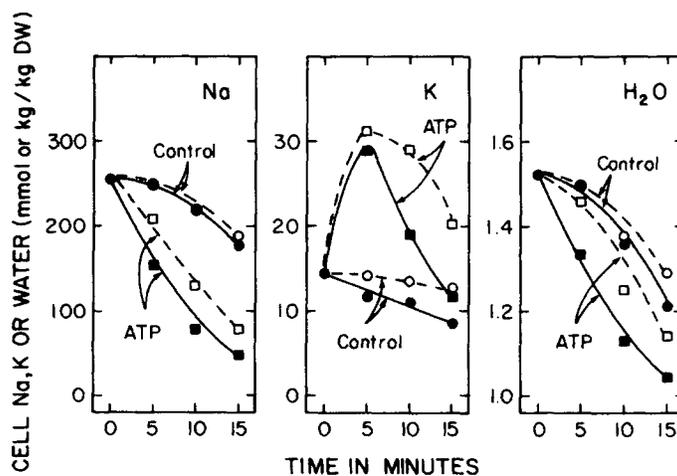


FIGURE 6. Precisely the same protocol and solutions as in Fig. 5, with the exception that extracellular KCl was 4 mM.

(circles), the outward Na movements are clearly inhibited by amiloride. The open squares and triangles on the graph show that in the presence of amiloride, low concentrations of ATP can induce conductive Na fluxes that are smaller than those mediated by the shrinkage pathway. The hyperpolarizing influences of these small concentrations of ATP are such as to pull substantial amounts of K into the cells, whereas larger Na effluxes through the shrinkage-activated pathway cause no K entry.

In studies preliminary to the experiments presented in Figs. 5–7, it was demonstrated that at the cell/medium ratios used, with Cl the major anion and no inhibitor of Cl conductance present, valinomycin exerts a maximum effect on net K movements at a concentration of 2  $\mu$ M. It was further shown in fresh dog RBC (internal K concentration 7–8 mmol/liter cell water) that the amiloride-sensitive Na pathway that is activated by cell shrinkage is not influenced by valinomycin concentrations as high as 10  $\mu$ M with external K concentrations of 0–10 mM. At higher external K concentrations, the cells swell and the shrinkage-activated Na pathway is progressively turned off. Finally, to dispel the objection that extracellular ATP might cause an effect on K conductance over and above that of 2.5  $\mu$ M valinomycin, the study in Fig. 8 was done. Cells were incubated in hypertonic choline-Cl with 4 mM KCl and dipyrindamole. The effects of 0.080 mM ATP alone and valinomycin alone are compared with the two agents combined. Note that valinomycin alone influences K loss but not Na loss. ATP alone speeds Na loss and leads to K entry, because, as shown previously, ATP

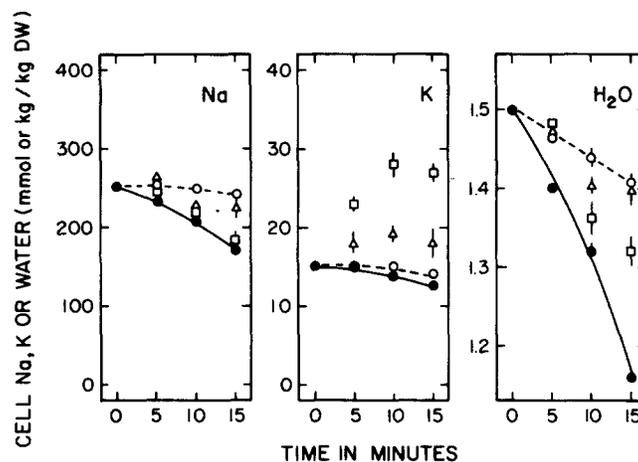


FIGURE 7. Cell Na (left panel), K (center panel), and water (right panel) as functions of time in minutes. Washing and experimental procedures were the same as described in the legend to Fig. 5. Incubation solutions contained the following common ingredients (mM): 200 choline-Cl, 10 HEPES, 5 glucose, 0.0025 valinomycin, 0.05 dipyrindamole (pH 7.0, 37°C). Additions to this medium, together with the graph symbols, are as follows (mM): ●, no additions; ○, 0.4 amiloride; △, 0.4 amiloride plus 0.045 ATP; □, 0.4 amiloride plus 0.090 ATP. Means  $\pm$  SD for five studies. Where there are no error bars, the SD was smaller than the height of the symbol.

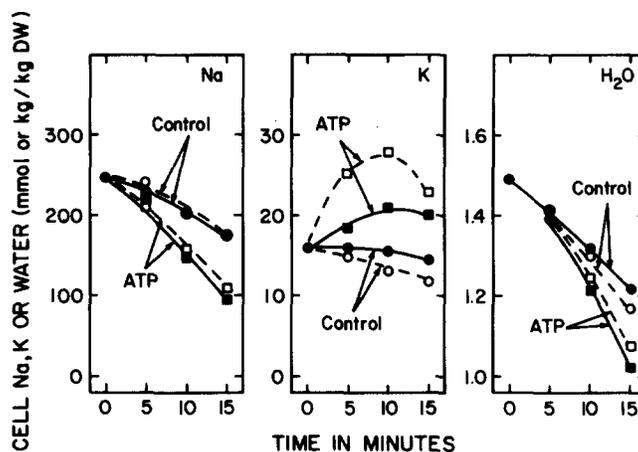


FIGURE 8. Cell Na (left panel), K (center panel), and water (right panel) as functions of time in minutes. Washing and experimental procedures were the same as described in the legend to Fig. 5. Incubation solutions contained the following common ingredients (mM): 200 choline-Cl, 4 KCl, 10 HEPES, 5 glucose, 0.05 dipyrindamole (pH 7.0, 37°C). Additions to this medium, together with the graph symbols, are as follows (mM): ●, no additions; ○, 0.0025 valinomycin; ■, 0.080 ATP; □, 0.0025 valinomycin plus 0.080 ATP. Single experiment representative of four others.

affects both Na and K permeability about equally (21). Valinomycin in the presence of ATP accelerates the inward K movements in response to the ATP-induced increase in Na conductance. Thus, under the conditions of Figs. 5–7, the fact that ATP increases the K conductance in addition to that of Na is irrelevant, because valinomycin was present in all cases in a concentration that dominates the effect of ATP on K movements. For the purposes of these experiments, the action of external ATP that is of importance is solely its effect on Na conductance.

#### *Na Movements in Response to an Imposed Proton Gradient*

The experiment in Fig. 3 showed how protons can be driven into shrunken dog RBC in response to an imposed Na gradient. Figs. 9–13 present experiments that culminate in a demonstration of proton-driven Na movements. The strategy here is to alter the transmembrane pH by varying the external Cl concentration, using gluconate as a nonpenetrating anion substitute.

Fig. 9 shows that incubation of dog RBC in a low-Cl, hypertonic Na medium causes a net efflux of ions and water. In this case, the outward movement of Cl down its concentration gradient is associated with an outward movement of Na up its concentration gradient. Fig. 10 shows that when this same experiment is repeated in hypotonic media, no such Na movement is observed. When RBC are suspended in media containing an impermeant anion substitute like gluconate,<sup>1</sup> several driving forces are created. First, if nothing happens to alter the dominat-

<sup>1</sup> Citrate is equally effective, although no results with that ion are shown.

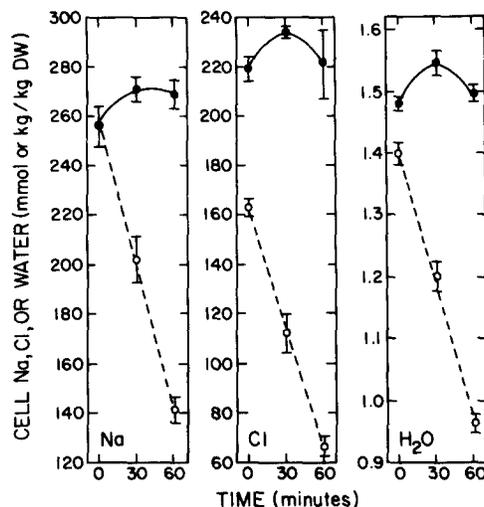


FIGURE 9. Cell Na, Cl, and water content as functions of time. The effects of incubation in two hypertonic Na solutions are compared: one (solid symbols, solid lines) had Cl as the major anion and the other (open symbols, dashed lines) had most of the chloride replaced by gluconate. Fresh cells were washed three times with a solution containing (mM): 140 NaCl, 10 HEPES, 5 glucose, 0.1 EDTA, and 1 NaHCO<sub>3</sub> (pH 7.05, 37°C). Cells were then incubated at 37°C in one of two solutions containing (mM): either 200 NaCl or 25 NaCl plus 175 Na gluconate, 10 HEPES, 5 glucose, 0.1 EDTA, and 1 NaHCO<sub>3</sub> (390 mosmol, pH 7.05, 37°C). Mean and SEM for three studies. Cell K varied between 13.8 and 15.2 mmol/kg dry weight in these studies, showed no relation to external Cl, and is not included on the graph.

ing influence of the Cl gradient on the membrane potential, the cell polarity should become reversed and the cytoplasm will be positive with respect to the medium. Second, a large, inward proton gradient is created because of the following relationship, which is independent of the membrane potential and mediated via the electroneutral anion exchanger (29):

$$Cl_o/Cl_i = HCO_3_o/HCO_3_i = OH_o/OH_i = H_i/H_o.$$

A third possible driving force might be the Cl gradient itself, presuming some sort of coupled NaCl cotransport system such as has been discussed in ascites tumor cells (9). All these influences would tend to drive Na from the cell, either via a conductance pathway (membrane potential reversal), a coupled Na-H pathway (inward proton gradient), or a symport coupling with Cl.

Table I summarizes the influence of certain agents on the outward Na movements that occur in response to lowering external Cl in shrunken cells. The Na efflux is inhibited by amiloride and quinidine. Thiocyanate, a highly conductive anion that also participates in band 3-mediated anion-anion exchange (6), will not substitute for Cl. Dipyridamole, a drug that inhibits both net and exchange anion movements in human RBC by 90% (11), has very little inhibitory effect on the outward Na flux induced by lowering the external Cl in shrunken

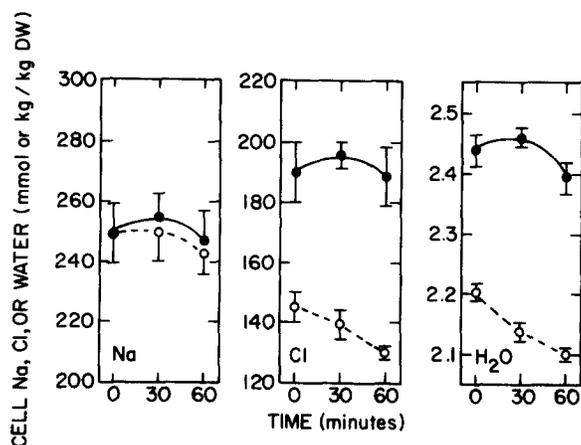


FIGURE 10. Cell Na, Cl, and water content as functions of time. The effects of incubation in two hypotonic Na solutions are compared: one (solid symbols, solid lines) had Cl as the major anion and the other (open symbols, dashed lines) had most of the chloride replaced by gluconate. Procedure and solutions were the same as in the legend to Fig. 9 except that the incubation solutions were hypotonic (218 mosmol) and contained either NaCl 95 mM or NaCl 5 mM plus Na gluconate 90 mM. Mean and SEM for 3 studies.

TABLE I  
Net Ion and Water Movements in 1 h (mmol or kg/kg dry weight)

Media	n	Na	Cl	Water
<b>Basic medium</b>				
Control	7	-113 (SD 10)	-97 (SD 16)	-0.46 (SD 0.05)
+ amiloride	3	-8 (SD 4)	-43 (SD 9)	-0.11 (SD 0.02)
+ quinidine	2	-14, -12	-31, -40	-0.07, -0.11
SCN medium	3	-13 (SD 2)	-15 (SD 5)	-0.09 (SD 0.03)
<b>DMSO medium</b>				
Control	10	-98 (SD 13)	-92 (SD 22)	-0.40 (SD 0.03)
+ DPM (- bicarbonate)	3	-106 (SD 8)	-99 (SD 13)	-0.43 (SD 0.01)
+ ACZ (- bicarbonate)	4	-81 (SD 16)	-106 (SD 16)	-0.39 (SD 0.05)
+ DPM and ACZ, (- bicarbonate)	3	-60 (SD 7)	-62 (SD 10)	-0.25 (SD 0.02)

Basic medium contains (mM): 25 NaCl, 175 Na gluconate, 10 HEPES, 5 glucose, 0.1 EDTA, 1 Na bicarbonate (pH 7.0 at 37°C). SCN medium is identical except that NaCl is replaced by NaSCN. DMSO medium is identical to the basic medium, but with DMSO (400  $\mu$ l/100 ml) added. Where noted, amiloride and quinidine were present at 0.2 mM, dipyridamole (DPM) was 0.05 mM, and acetazolamide (ACZ) was 1 mM.

cells. A combination of dipyridamole and the carbonic anhydrase inhibitor acetazolamide has a 35–40% inhibitory effect. The combined effect of the last two drugs permitted us to design some experiments that may bear on the mechanism by which an imposed, outward Cl gradient drives Na from the cell.

Figs. 11–13 present the results of experiments in which the strategy was to set the internal pH by exposing cells to buffered media with various Cl concentrations. Acetazolamide and dipyridamole were then applied to the cells in order to retard the dissipation of the transmembrane proton gradient when the Cl gradient was subsequently changed. In this way, we hoped to dissociate proton-mediated driving forces from those relating to the Cl gradient. The object was to see whether an induced pH difference across the membrane could drive Na movements against a concentration gradient under circumstances in which the Cl ratio that had been used to pre-set intracellular pH was different from the Cl ratio imposed during the period of flux measurement.

The experiment in Fig. 11 represents a preliminary approach to this goal in which the cell pH was initially fixed at a low value ( $<7.0$ ) and the effects of acetazolamide and dipyridamole on Na efflux in response to a subsequently imposed outward Cl gradient were examined. Cells were pretreated in hypotonic NaCl at an external pH of 7.0 for 15 min. Half the cells were then centrifuged and resuspended in the same medium minus  $\text{HCO}_3^-$  and plus acetazolamide and dipyridamole for 30 min. Apparently, acetazolamide takes  $\sim 30$  min to equilibrate with cells and exert its effect as a carbonic anhydrase inhibitor (29). Both groups of cells (controls and those exposed to drugs) were then transferred to a hypertonic medium with 25 mM NaCl plus 175 mM Na gluconate. The exposure to acetazolamide and dipyridamole was continued for the cells that had been pretreated with those drugs. Compared with controls, the drug-exposed cells had a retarded efflux of Na, Cl, and water.

Our interpretation of the findings in Fig. 11 is as follows. During the preincubation in hypotonic NaCl at pH 7.0, the cell interior becomes acidic. When the control (drug-free) cells are transferred to the low-Cl solution, their interior becomes alkaline with respect to the medium: an inward proton gradient is created. Furthermore, because the low-Cl medium is hypertonic, the cells shrink and a transporter is activated that can trade protons for Na. Thus, Na is extruded from the cells as the proton gradient collapses. The action of dipyridamole and acetazolamide is to slow the process by which a Cl gradient is translated (by the anion exchanger) into a proton gradient: hence, the retarding effect of the drugs.

This same strategy was used for the experiments in Figs. 12 and 13, but the sequence of exposure of the cells to low external Cl was the reverse of that shown in the experiment of Fig. 11. The hypotonic preincubation was carried out in a low-Cl medium to alkalinize the cell interior. No Na movements took place during this phase, despite the large proton gradient, because the cells were swollen and the putative Na-H pathway was not turned on (see Fig. 10). As in the previous experiment, half the cells were treated with dipyridamole, acetazolamide, and bicarbonate removal. Both the control and the drug-treated cells were then transferred to a 200 mM NaCl solution at pH 7.0. At this point, the anion exchanger of the undrugged cells rapidly translates the newly established inward Cl gradient into a reversal of the cytoplasmic alkalinity, and Na is accumulated by the cells. The drug-treated cells, on the other hand, experience a persistence of their internal alkalinity: dipyridamole inhibits the process by

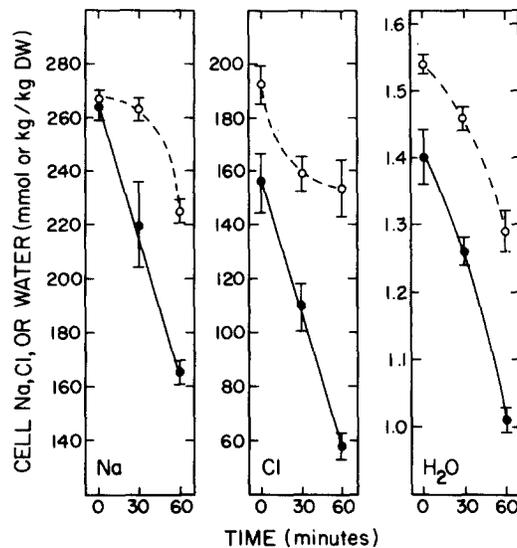


FIGURE 11. Cell Na, Cl, and water content as functions of time. In the controls (solid symbols, solid lines), cells were incubated in a medium containing 200 mM Na, 25 mM Cl, and 175 mM gluconate. The effects of pretreating cells with a combination of dipyridamole, acetazolamide, and bicarbonate removal are shown in the open symbols and dashed lines. Fresh cells were washed and preincubated at 37°C in a hypotonic medium containing (mM): 105 NaCl, 1 NaHCO<sub>3</sub>, 10 HEPES, 5 glucose (pH 7.0, 37°C). After 15 min, half the cells were washed and resuspended at 37°C in the same hypotonic NaCl medium minus bicarbonate and plus 0.05 mM dipyridamole and 1 mM acetazolamide. After a further 30-min preincubation, both lots of cells were centrifuged. The control cells were then transferred to a hypertonic medium that contained (mM): 25 NaCl, 175 Na gluconate, 1 NaHCO<sub>3</sub>, 10 HEPES, 0.1 EDTA, 5 glucose (pH 7.0, 37°C). The cells that had been pretreated with dipyridamole and acetazolamide were transferred to a medium similar to the control but without bicarbonate and with the two drugs at their previous concentrations. Mean and SEM for three studies.

which bicarbonate is reshuffled in accord with the new Cl ratio, and acetazolamide retards the interconversion of hydroxyl and bicarbonate ions. The persistent inward proton gradient in these shrunken, drugged cells is able to drive Na outward by a driving force that is clearly unrelated to the Cl gradient across the membrane. Indeed, as Fig. 13 shows, the net Na efflux occurs simultaneously with a nearly equimolar Cl influx.

Figs. 14 and 15 summarize experiments similar to those detailed in Fig. 9 but at a range of Cl concentrations intermediate between the two extremes (25 and 200 mM) shown in that figure. Two external pH values (pH<sub>o</sub>'s), 7.0 and 7.4, were used, but we found that at the latter pH a great deal of hemolysis occurred when the medium Cl was lowered much below 60 mM. This observation is probably related to the extreme alkali sensitivity of dog RBC (28). All studies presented here had <2% hemolysis. These experiments are predicated on the

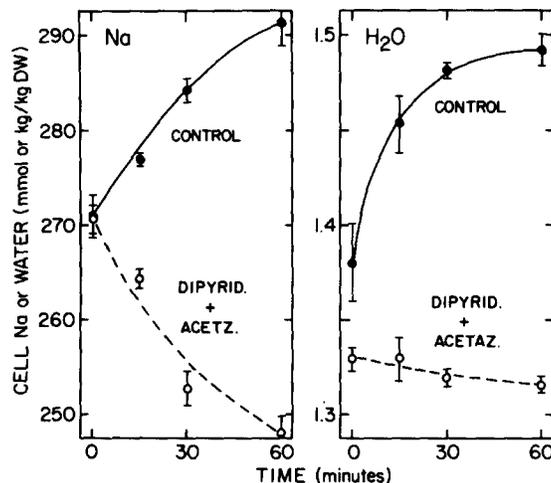


FIGURE 12. Cell Na and water content as functions of time. Both control and experimental cells were pretreated in a hypotonic, low-Cl medium to alkalinize their insides. The control cells (solid symbols, solid lines) were transferred to a hypertonic NaCl medium. The experimental cells (open symbols, dashed lines) were exposed to dipyridamole, acetazolamide, and bicarbonate removal before being placed in the same concentration of NaCl as the controls. Fresh cells were washed and preincubated at 37°C in a hypotonic medium containing (mM): 15 NaCl, 90 Na gluconate, 1 NaHCO<sub>3</sub>, 10 HEPES, 5 glucose (pH 7.0, 37°C). After 15 min, half the cells were washed and resuspended at 37°C in the same hypotonic NaCl medium minus bicarbonate and plus 0.05 mM dipyridamole and 1 mM acetazolamide. After a further 30-min preincubation, both lots of cells were centrifuged. The control cells were transferred to a hypertonic medium that contained (mM): 210 NaCl, 10 HEPES, 0.1 EDTA, 5 glucose (pH 7.0, 37°C). The cells that had been pretreated with dipyridamole and acetazolamide were transferred to a medium similar to the control but with the two drugs at their previous concentrations. Mean and range for two studies.

idea (for which evidence has just been given) that when Na moves outward into a high-Na, low-Cl medium, the driving force is related to the pH gradient imposed. Fig. 14 shows the amount of Na and water (per kilogram dry weight) and the concentration of Cl (per kilogram cell water) in dog RBC after a 1-h incubation at 37°C in a 190 mM Na medium with various Cl concentrations as plotted on the horizontal axes. The left panel of Fig. 14 shows that in high-Cl media, Na is accumulated by the cells, and in low-Cl media, Na is extruded. The experiments at pH<sub>o</sub> 7.0 are shown with solid symbols and those at pH<sub>o</sub> 7.4 are shown with open symbols. At high external Cl concentrations, the cells gain more Na at the higher pH<sub>o</sub>, but at lower Cl<sub>o</sub> concentrations, the medium pH does not affect the amount of Na extruded. The Cl gradient across the membrane differs at the two pH<sub>o</sub>'s, as shown in the center panel of Fig. 14. In media with reduced Cl, the cells suspended at pH<sub>o</sub> 7.0 have a higher internal Cl concentration than

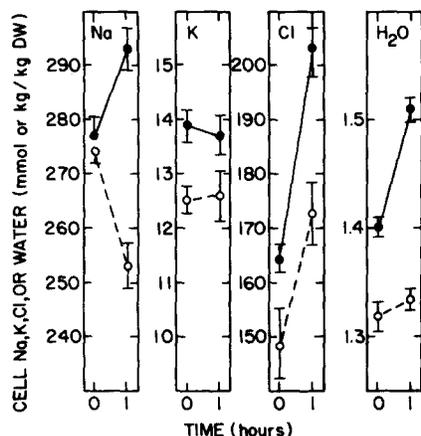


FIGURE 13. Same experiment as Fig. 12 but with only two time points and showing cell K and Cl in addition to Na and water. Mean and SEM for six studies.

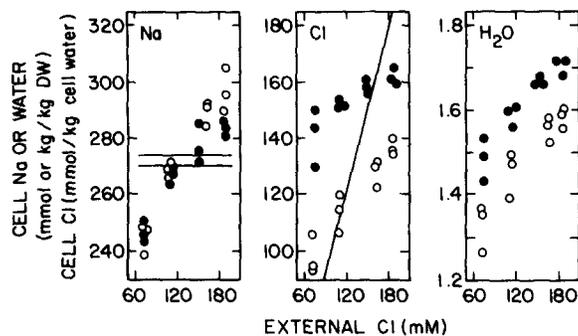


FIGURE 14. Effects of varying extracellular Cl on the ion and water content of dog RBC incubated for 1 h at 37°C in hypertonic Na media with external pH fixed at 7.0 (solid symbols) or 7.4 (open symbols). The procedures and solutions were as noted in the legend to Fig. 9. External Na was 190 mM in all cases, and Cl was varied from 68 to 190 mM using gluconate as a replacement ion. External Cl is shown on all three abscissae. The left panel shows cell Na content after 1 h incubation (the range of values for cell Na at the beginning of the study is shown between the horizontal lines at 269 and 274 mmol/kg dry weight [DW]). The center panel shows the final cell chloride concentration (mmol/liter cell water); the line in this graph is drawn through points where external and internal Cl are equal. The right panel shows the final cell water content. Pooled results of three separate experiments.

do the cells at  $\text{pH}_o$  7.4. Thus, the  $\text{pH}_o$  7.4 cells extruded just as much Na as did the  $\text{pH}_o$  7.0 cells, despite the presence of a smaller transmembrane Cl gradient at  $\text{pH}_o$  7.4.<sup>2</sup>

Knowing the external pH and the Cl ratio, it is possible to calculate the internal

<sup>2</sup> Although the Cl values in the center panel of Fig. 14 are from the end of the experiment (1 h), the same relationships among medium pH, medium Cl, and cell Cl obtain throughout the incubation. For example, at 5 min, cells incubated in a 70 mM Cl solution had internal Cl concentrations (mmol/liter cell water) of 157 at  $\text{pH}_o$  7.0 and 109 at  $\text{pH}_o$  7.4.

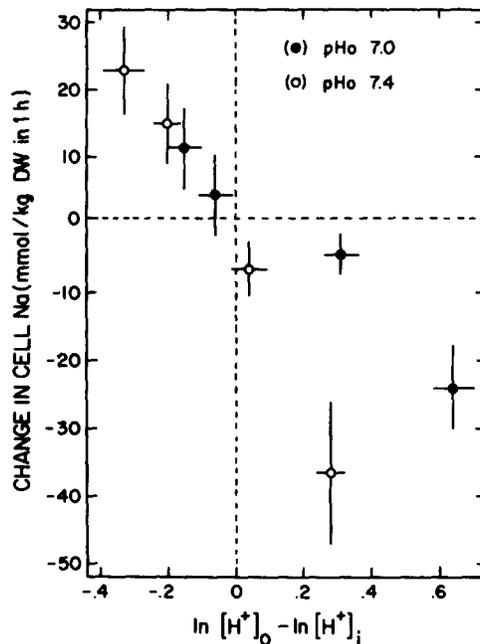


FIGURE 15. Net gain or loss of Na from the cells over 1 h as a function of the transmembrane proton gradient, calculated from the external pH and the Cl ratio, and expressed as the difference between the natural logarithms of the proton concentrations on either side of the membrane. The chemical proton gradient would thus be the number on the abscissa times  $RT$ . The data are taken from studies like those in Fig. 14. Mean  $\pm$  SD for five studies, three shown in Fig. 14 and two others.

pH by the formula:

$$pH_i = pH_o - \log Cl_o/Cl_i.$$

Fig. 15 shows the Na movements from the experiment in Fig. 14 plotted as a function of the natural logarithm of the transmembrane chemical proton gradient as calculated from the cell and medium Cl concentrations. At pH<sub>o</sub> 7.0, a larger transmembrane proton gradient has to be imposed to effect the same net Na efflux as when the external pH is 7.4. This result seems paradoxical: it suggests that a system which extrudes Na ions in exchange for outside protons is somehow more efficient at an external proton concentration of 40 nM (pH 7.4) than at 100 nM (pH 7.0). The paradox may be resolved by reference to the right panel of Fig. 14, where it is shown that for a given external Cl concentration, the cell water content is lower at pH<sub>o</sub> 7.4 than at pH<sub>o</sub> 7.0. Clearly, activation of the Na-proton pathway by shrinking cell volume (19, 23) is a more important determinant of Na efflux than is the external proton concentration.

#### *Proton Movements in Response to an Inward Na Gradient*

Fig. 16 shows that an amiloride-sensitive proton efflux can be driven by the creation of an inward Na gradient. The cells were preincubated to lower their

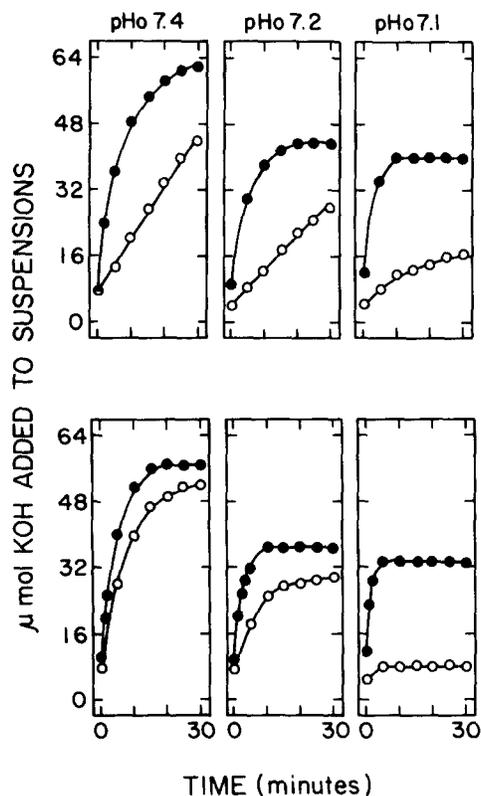


FIGURE 16. Efflux of protons, expressed as micromoles of KOH added to cell suspensions to keep the external pH within 0.05 unit of the value shown above each vertical pair of panels. Time is on the abscissae. 50  $\mu$ M dipyridamole was present in the studies shown in the upper panels; the lower panels show titrations without this drug. The solid symbols represent control runs; the open symbols indicate the addition of 0.2 mM amiloride to the suspension. The cells were pretreated to lower their Na and total solute content as follows. Fresh cells were washed three times in buffered, isotonic NaCl and once in 130 mM KCl. All cells were then incubated for 40 min at 37°C in 100 vol of a medium containing (mM): 100 KCl, 100 sucrose, 1 ATP, 10 HEPES, 5 glucose, 0.2 EDTA (pH 7.4, 37°C, adjusted with Tris-OH). When cells treated in this way are resuspended in isotonic NaCl media, they contain (per kilogram dry weight): 10–20 mmol Na, 170–180 mmol K, and 1.36–1.42 kg water (16). The pretreated cells were then washed three times with an unbuffered, isotonic KCl medium. At the start of each flux period, 2 ml of cells was transferred into 50 ml of a prewarmed (37°C) solution containing (mM): 150 NaCl, 5 glucose, plus or minus 50  $\mu$ M dipyridamole and 0.2 mM amiloride. 20- $\mu$ l aliquots of 40 mM KOH were added to each suspension at 10-s intervals as needed to keep the suspension pH within 0.05 unit of the values given above each pair of panels. Three studies on one donor are shown. A similar group of studies was done on a different donor with qualitatively similar findings. The net efflux of protons is compared with the net change in cell Na content for each of the 30-min studies in the following table.

internal Na concentration and deplete them of total solute so that on resuspension in an unbuffered isotonic NaCl medium they would be shrunken, their Na-H exchanger would be activated, and they would be taking up Na. During the Na uptake phase, the extracellular pH was kept constant (at 7.4 in the left panels, 7.2 in the center panels, and 7.1 in the right panels) by the addition of KOH. The amount of KOH that had to be added to each suspension must have equaled the amount of protons that were extruded from the cells, since there was no extracellular buffer. At each of the three statted pH values, the titrations were done in the presence (upper panels) and absence (lower panels) of dipyrindamole. Under each condition, the effects of amiloride (open symbols) were compared with controls (solid symbols). The amount of Na gained by the cells during the 30-min incubations is given in the legend to Fig. 16. For each condition of drug exposure, the proton output of the cells diminished progressively as the external pH was statted at lower values. With the exception of the studies done at medium pH 7.1, the amiloride-sensitive movements of both protons (graphs) and Na (figure legend) were greater in the presence of dipyrindamole (upper panels) than in the absence of this drug (lower panels), a result explained by dipyrindamole's ability to prevent the dissipation of the pH gradient created by Na-proton counterflow (hence diminishing the effect of cell buffers). The diminished effect of dipyrindamole on amiloride-sensitive proton flow at the lowest external pH may be due to the smaller transmembrane pH gradient at a medium pH of 7.1. The somewhat higher Na uptakes at pH 7.4 may have been due to the lower volume of the cells, as argued in connection with Figs. 14 and 15.

Precise estimates of the stoichiometry of this system will require simultaneous measurements of the two unidirectional counterfluxes. Even in the presence of dipyrindimole, it is likely that some backflux of proton equivalents occurs during the pH stat experiments, rendering the net proton movements smaller than those of Na (see legend to Fig. 15). It is clear, however, that the net transport rates of the counterflowing ions are of the same order of magnitude.

Statted pH	7.4		7.2		7.1	
	H	Na	H	Na	H	Na
<i>mmol/kg dry cell weight</i>						
Top Panels (+ dipyrindamole)						
Control	77	70	54	55	50	58
+ amiloride	54	18	34	16	21	17
Δ	23	52	20	39	29	41
Bottom Panels (- dipyrindamole)						
Control	71	49	46	44	42	32
+ amiloride	65	13	37	11	10	25
Δ	6	36	9	33	32	27

H and Na refer to net proton efflux and net Na uptake, respectively, over the 30-min incubation periods. Δ is the difference between control and amiloride studies.

## DISCUSSION

The Na permeability pathway that becomes progressively activated as dog RBC shrink below their normal volume has several distinctive features.

First, it is inhibited by replacing Cl with NO<sub>3</sub> or SCN (Fig. 1) (19). The mechanism of this effect cannot be related to the conductance of these two anions, because both are more permeant than Cl through the dog RBC membrane (18). Either the Na-proton pathway is Cl dependent or the substitute anions are inhibitory. Second, the flux of Na through the membrane of shrunken dog RBC is sensitive to amiloride, with 50% inhibition at a drug concentration of 5 μM (external Na nominally zero) (Fig. 2) or 10 μM (external Na 150 mM) (19). Benos (1), in a recent review, points out that amiloride sensitivities like the ones we find in dog RBC (50% inhibition at >1 μM drug) are characteristic of pathways in which Na movements are coupled to protons. Indeed, a third property of the dog RBC shrinkage-activated Na transporter is that it can conduct Na movements in either direction across the membrane in exchange for protons (Figs. 3 and 16). Proton movements can be driven by an imposed Na gradient, and Na movements can be driven by an imposed proton gradient (Figs. 12 and 13).

The nature of the coupling between Na and H movements in dog RBC is addressed in Figs. 4–7. A report (12) that the amiloride-sensitive apical membrane Na conductance channels in toad bladder are selective for H > Li > Na ≫ K suggested that the coupling of Na to H movements in shrunken dog RBC might be electrically mediated. This hypothesis was tested by measurements of membrane potential with the fluorescent probe diS-C<sub>3</sub>(5) and also by assaying movements of K through valinomycin channels in response to outward Na gradients imposed across the membrane of shrunken dog RBC. No evidence could be found that the large Na fluxes triggered by cell shrinkage are conductive.

Thus, the present findings would appear to support the notion that the Na-proton exchange mechanism activated by cell shrinkage is similar to the one described in *Amphiuma* RBC by Cala (3, 4) in being electrically “silent” or electroneutrally coupled. Because we cannot completely inhibit the anion exchanger in dog RBC, we are left with some uncertainties regarding the stoichiometry of the coupling. Fig. 3 shows that the inward movement of protons in exchange for Na stops after ~10 min, whereas we know that Na efflux continues. Fig. 16 shows that proton movements in a pH stat experiment lag behind Na movements. Both of these discrepancies can be explained in terms of shunting of the proton gradient through an incompletely inhibited Cl-bicarbonate exchanger.

One difficulty in studying some quantitative features of the shrinkage-activated Na-proton pathway in dog red cells is the mutual dependence of cell volume and proton concentration. Any change in cytoplasmic pH alters the charge on hemoglobin and cell organic phosphates; Cl ions move to preserve electroneutrality in the cell. The osmotically obliged water shifts change cell volume, which is a powerful determinant of the activity of the Na-proton exchanger (Figs. 14

TABLE II  
*Summary of Features of Two Proton-Na Transport Pathways in Comparison with the System on Dog RBC*

Characteristic to be compared	Na/HCO <sub>3</sub> -Cl/H exchange (invertebrates)	Na-H exchange (mostly vertebrate tissues)	Na-H exchange in dog RBC
Chloride	Required	Not required	Required
Bicarbonate	Required	Not required	Not required
Anion exchange inhibitors, e.g., DIDS, SITS, furosemide, dipyrindamole	Inhibits	No effect	No effect
Amiloride	?	IC <sub>50</sub> = 0.1-1 mM	IC <sub>50</sub> = 1-10 μM
ATP requirement	Present	Absent	Probable
Lithium	?	Inhibits	Inhibits

and 15). More success in studying kinetic features of the pathway in dog red cells might be expected with a resealed ghost preparation in which impermeant, charged species are greatly diluted. To date, however, we have not been able to find conditions for lysing and resealing cells that will preserve their property of volume responsiveness.

Na-proton counterflow in dog RBC has features that distinguish it from similar transporters in other cell types. Table II summarizes some information from a recent review of proton transport by Boron (2) that describes two systems capable of moving Na and protons in opposite directions. The dog RBC Na-proton exchanger has some similarities to both. Unlike the Na-H exchanger in Table II (column 3), the dog RBC transporter has a requirement for Cl (19) (Fig. 1), a characteristic shared with the Na/HCO<sub>3</sub>-Cl/H pathway in excitable tissues from invertebrates. The latter pathway moves Cl and Na in opposite directions, but the dog RBC system can operate with net Cl flows in the same (Fig. 9) or the opposite (Fig. 13) direction to Na movements; thus, the requirement for Cl in the dog Na-H pathway probably does not involve transport of that anion.

Blockers of anion exchange like SITS and DIDS (and presumably dipyrindamole) inhibit the invertebrate pathway but do not affect the Na-H exchanger as described in epithelia or vertebrate muscle. DIDS has been difficult for us to use in dog RBC: several lots of this agent have proved hemolytic in our hands. Dipyrindamole, an agent that shares many of the actions of DIDS (10), does not by itself affect the dog RBC Na-H pathway, but, by helping to prevent equilibration of pH across the membrane, this agent can allow us to do certain experiments that reveal the Na-H exchanger to better advantage. Thus, in Fig. 11, dipyrindamole appears to retard Na movements by preventing the rapid alkalization of the cell interior, while in Figs. 12 and 13, the drug enables the Na-H exchanger to transport Na uphill by preserving a previously imposed proton gradient. In Fig. 16, dipyrindamole amplifies observable movements of Na and protons through the amiloride-sensitive pathway.

Some invertebrate Na/HCO<sub>3</sub>-Cl/H exchangers are blocked by treatments that result in the depletion of ATP and are restored by the addition of this nucleotide to the preparations. No such ATP dependence is described for the pure Na-

proton countertransporters reported to date. An old observation in dog RBC is that their volume-responsive Na flux is diminished by energy depletion (22). At the time these early experiments were done, the mechanism of Na movements in shrunken dog RBC was not suspected to involve reciprocal proton transport. Furthermore, it is not certain in the dog system whether the critical metabolic factor is ATP per se or some other feature of intact glycolysis (7). Since the movements of Na and protons described in all of these transport systems are "downhill" thermodynamically, the role of metabolism or ATP is presumed to be permissive and perhaps to involve phosphorylation of a transport protein (2).

An issue that needs further examination is whether and under what conditions the dog RBC Na-proton exchanger can conduct Na-Na exchange (19), a point that has not been resolved for other Na-proton transporters (2). One feature that the dog system shares with those in vertebrate epithelia is inhibition by Li ions (19).

A final point of comparison is that the volume-responsive Na pathway in dog RBC is perhaps 10 times more sensitive to amiloride than are other Na-H exchangers. In the presence of external Na, the dog system was half-maximally inhibited by 10  $\mu$ M amiloride (19); in the absence of Na in the medium, a twofold increase in sensitivity was seen (Fig. 2). The sensitivities of the dog system to quinidine (16, 19) and phloretin (25) are characteristics that, so far as we are aware, have not been investigated in other preparations.

The technical assistance of Paula S. Glosson is gratefully acknowledged, as are the labors of two anonymous reviewers for *The Journal of General Physiology*. This work was supported by U.S. Public Health Service Grant AM 11357 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

Received for publication 14 November 1983 and in revised form 8 June 1984.

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