

# Dog Red Blood Cells

## *Adjustment of salt and water content in vitro*

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**ABSTRACT** Dog red blood cells (RBC) lack a ouabain-sensitive sodium pump, and yet they are capable of volume regulation in vivo. The present study was designed to find in vitro conditions under which dog RBC could transport sodium outward, against an electrochemical gradient. Cells were first loaded with sodium chloride and water by preincubation in hypertonic saline. They were then incubated at 37°C in media containing physiologic concentrations of sodium, potassium, chloride, bicarbonate, glucose, and calcium. The cells returned to a normal salt and water content in 16–20 h. Without calcium in the medium the cells continued slowly to accumulate sodium. Removal of glucose caused rapid swelling and lysis, whether or not calcium was present. The net efflux of sodium showed a close relationship to medium calcium over a concentration range from 0 to 5 mM. Extrusion of salt and water was also demonstrated in fresh RBC (no hypertonic preincubation) when calcium levels in the media were sufficiently raised. The ion and water movements in these experiments were not influenced by ouabain or by removal of extracellular potassium. Magnesium could not substitute for calcium. It is concluded that dog RBC have an energy-dependent mechanism for extruding sodium chloride which requires external calcium and is quite distinct from the sodium-potassium exchange pump.

### INTRODUCTION

Mature red blood cells (RBC) from adult dogs lack a ouabain-sensitive sodium efflux pathway, and their plasma membranes are deficient in sodium-potassium-ATPase (6, 10, 13, 14, 18, 24, 25). Compared with most other mammals, dog RBC cytoplasm is high in sodium and low in potassium, although these ions are not in electrochemical equilibrium with plasma (3, 24, 25, 30). The permeability of dog RBC to sodium and potassium is greatly influenced by their volume: Osmotically shrunken cells show an increase in sodium and a decrease in potassium flux. Osmotic swelling has just the opposite effect on sodium and potassium movements (7, 27, 28).

The influence of volume change on sodium and potassium permeability is readily reversible (10). It is therefore possible to alter the ionic composition of dog RBC with minimum damage by incubation in hypertonic or hypotonic solutions. In the present study dog RBC were loaded with sodium chloride by preincubation in hypertonic saline. An investigation was then made to discover conditions under which such salt-loaded cells could return to a normal ion content. The results suggest that net extrusion of sodium chloride by dog RBC depends on an energy source (glucose) and also on the presence of physiologic concentrations of extracellular calcium. Some of the findings have been reported in abstract form (26).

#### METHODS

All studies were begun within 10 min of drawing blood from healthy, unmedicated mongrel dogs. Incubation and wash media are given in Table I. The procedure

TABLE I  
INCUBATION AND WASH SOLUTIONS

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Solution A (wash): NaCl 140, glycylglycine 5, pH 7.5
Solution B (hypertonic preincubation medium): NaCl 230, KCl 5, glycylglycine 10, glucose 10, pH 7.5
Solution C (incubation medium): NaCl 105, NaHCO <sub>3</sub> 25, KCl 5, glucose 10, CaCl <sub>2</sub> variable from 0 to 10, bovine serum albumin 1 g/100 ml, gas with 95% O <sub>2</sub> -5% CO <sub>2</sub>

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All concentrations except albumin and gases are in millimoles per liter.

outlined in Table II applies to all the experiments reported except for one series (Fig. 6) in which hypertonic preincubation (Table II, step 3) was omitted.

Analytical procedures for RBC are detailed in previous publications from this laboratory. They include centrifugation of cells in special Lucite tubes (25), measurement of [<sup>125</sup>I]albumin extracellular space (23, 25), cell water determination by drying to constant weight (23, 25, 28), flame photometry for sodium and potassium (22), conductimetric titration of chloride (22), and enzymatic analysis for ATP using glucose-6-phosphate dehydrogenase and hexokinase with fluorometric determination of NADPH (22).

Preincubation and incubations (Table II, steps 3-5) were done with a cell/medium volume ratio of  $\frac{1}{10}$  to  $\frac{1}{12}$  in a 37°C shaker-bath in sealed plastic flasks. In the prolonged incubations (Table II, steps 4 and 5) penicillin and streptomycin (10 mg each per 100 ml suspension) were added to all flasks, and all were gassed with humidified 95% oxygen-5% carbon dioxide for 10 min before sealing or resealing. At each sample time the pH of the suspension was measured at 37°C and was always found to be in the range from 7.3 to 7.4.

#### RESULTS

Over the course of the 3 h preincubation in hypertonic sodium chloride buffer (Table II, step 3) the cells accumulated 40-50 meq of sodium/kg

dry weight. When the preincubated cells were washed in isotonic buffer (step 4) they showed a gain in water of 300–350 g/kg dry weight as compared with fresh RBC.

Table III shows the results of an experiment in which preincubated cells were placed in four test media to demonstrate the conditions under which the excess salt and water could be extruded. In the presence of calcium (5 mM) and glucose (10 mM) the cells returned to a normal (fresh) sodium and water content in about 16 h. With calcium absent but glucose present the cells remained in the swollen state but did not hemolyze. With no glucose

TABLE II  
EXPERIMENTAL PROCEDURE

- (1) Collect venous blood in a heparinized syringe, discard plasma.
- (2) Wash\* a portion of the fresh cells with isotonic solution A. Analyze the cells.
- (3) Wash\* the remainder of the fresh cells with hypertonic solution B. Preincubate the cells with solution B at 37°C for 3 h. This causes the cells to accumulate NaCl.
- (4) Wash\* the preincubated cells with solution A, distribute the cells among the various isotonic incubation media (see solution C), and incubate at 37°C.
- (5) At various time points remove samples of cells from the incubation media and wash\* them in solution A before analysis.

\* Each lot of cells was washed five times in 10 vol of solution at 25°C by alternate suspension and centrifugation.

TABLE III  
NET SODIUM AND WATER MOVEMENTS IN DOG RED BLOOD CELLS

Calcium (mM) Glucose (mM)	Cell water (g/kg dry wt)				Cell sodium (meq/kg dry wt)				Percent hemolysis			
	5	5	0	0	5	5	0	0	5	5	0	0
Experiment 1												
Fresh RBC			—1,880—				—269—					—0—
0 h	2,245	2,189	2,243	2,251	324	322	326	326	0	0	0	0
8 h	2,028	2,358	2,273	2,317	289	328	335	336	1	70	1	4
16 h	1,884	—	2,315	—	261	—	337	—	2	100	1	100
24 h	1,819	—	2,315	—	253	—	338	—	3	100	3	100
Experiment 2												
Fresh RBC			—1,865—				—278—					—0—
0 h	2,236	2,226	2,257	2,257	329	329	327	326	0	0	0	0
8 h	2,058	2,257	2,322	2,344	288	—	336	336	2	73	1	5
16 h	1,882	—	2,300	3,049	264	—	333	413	3	100	2	81
24 h	1,817	—	2,367	—	252	—	340	—	4	100	4	100

Procedure outlined in Table II. Samples were removed for analysis at times noted in column 1. The "0 h" sample was taken immediately after the preincubated cells had been washed and distributed among the four incubation media (all modifications of solution C, Table I) containing various combinations of Ca and glucose.

the cells swelled and lysed, the lysis proceeding most rapidly in the presence of calcium. In separate studies (not shown) it was found that replacement of glucose by either 2-deoxyglucose or 3-O-methylglucose failed to protect the cells from hemolysis.

Figs. 1-5 show the results of experiments in which preincubated cells were placed in glucose-containing solutions with differing amounts of calcium. Samples were taken at 0.5 and 20 h. Cell water (Fig. 1), sodium (Fig. 2), and chloride (Fig. 3) all returned to normal levels at a calcium of 3-4 mM. Higher calcium concentrations resulted in further cell shrinkage, although

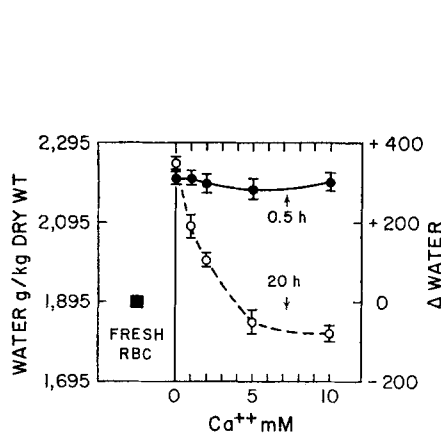


FIGURE 1

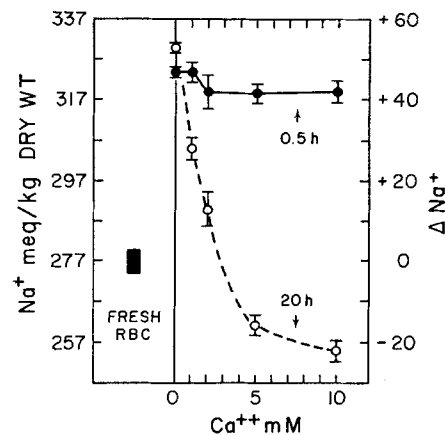


FIGURE 2

FIGURE 1. Cell water content as a function of medium calcium concentration. Water content of freshly drawn RBC is given in the left-hand panel. Cells were preincubated in hypertonic sodium chloride buffer, washed, and distributed among incubation media of varying calcium concentrations. Samples were taken after 0.5 h (closed circles, solid lines) and 20 h (open circles, dashed lines). Left ordinate shows absolute values; right ordinate shows changes. Mean  $\pm$  SEM for four experiments.

FIGURE 2. Cell sodium content as a function of medium calcium concentration. See legend to Fig. 1 for procedure and notation.

this effect seems to be maximal above a calcium of 5 mM. Comparison of Figs. 2 and 3 shows that cell chloride did not rise to the same extent as cell sodium in the preincubation phase (compare 0.5 h samples). A possible explanation of this is that in swollen cells the hemoglobin molecules become more negatively charged (9).

Fig. 4 shows that potassium movements are minimally influenced by calcium and that cell ATP, which falls in the preincubation phase, is stable with time and independent of calcium.

That the net sodium efflux in Fig. 2 was against an electrochemical gradient is suggested by the data in Fig. 5 in which the ratios of sodium and chloride between intracellular and extracellular water are given. If the chloride ion

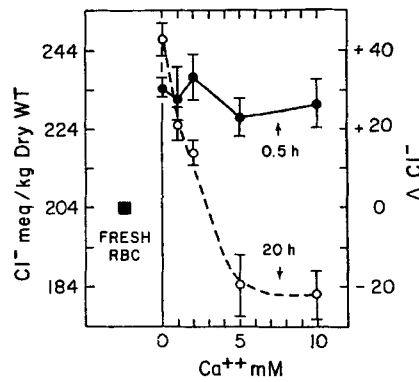


FIGURE 3. Cell chloride content as a function of medium calcium concentration. See legend to Fig. 1 for procedure and notation.

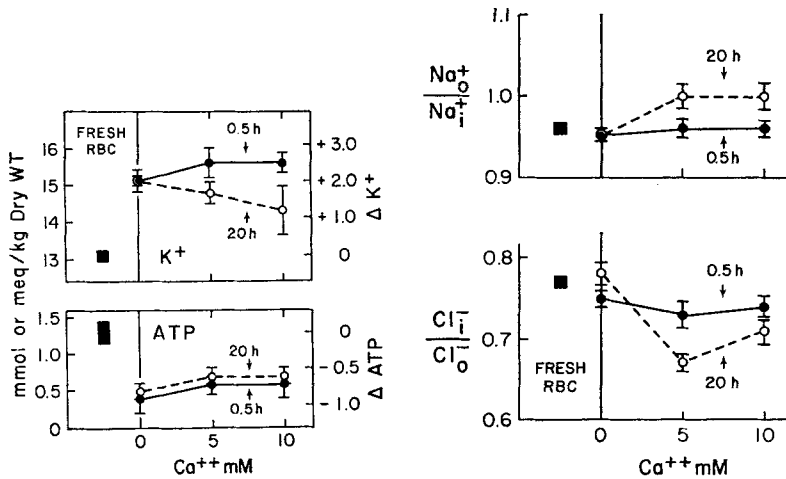


FIGURE 4

FIGURE 5

FIGURE 4. Cell potassium and ATP content as a function of medium calcium concentration. See legend to Fig. 1 for procedure and notation.

FIGURE 5. Ratios of concentrations of sodium and chloride in intracellular and extracellular water as a function of medium calcium concentration. See legend to Fig. 1 for procedure and notation. Subscripts *o* and *i* refer to medium and cells, respectively.

distribution reflects the membrane potential, and if both sodium and chloride are completely in solution, then the electrochemical gradient in all circumstances of this experiment is such as to favor sodium entry into the cell.

Table IV shows that net sodium and water extrusion from preincubated cells is not influenced by ouabain or by removal of potassium from the medium. Magnesium will not take the place of calcium in potentiating the salt and water extrusion. Ethacrynic acid (11) was lytic in 8 h, and furosemide (8, 32) had no effect when included in the complete system.

TABLE IV  
NET SODIUM AND WATER MOVEMENTS UNDER VARIOUS  
CONDITIONS

Modifications of incubation medium (solution C, Table I)	<i>n</i>	Change in cell water (g/kg dry wt)	Change in cell sodium (meq/kg dry wt)
Complete system	4	-367 ± 21	-59.2 ± 2.8
Omit Ca	4	+52 ± 8	+17.1 ± 1.8
Omit Ca, add Mg 5 mM	4	+31 ± 4	+16.2 ± 3.0
Add ouabain 0.1 mM	8	-378 ± 11	-61.9 ± 4.0
Omit K	8	-384 ± 13	-60.9 ± 3.1

Experimental procedure as in Table II. Values show the net change in cell water and Na over a period beginning at 0.5 h and ending at 20 h after re-suspension of preincubated cells in incubation media. "Complete system" refers to solution C, Table I with  $\text{CaCl}_2$  5 mM. Mean values ± SEM.

The effect of various calcium concentrations on fresh cells which had not been preincubated in hypertonic sodium chloride is shown in Fig. 6. Calcium has a smaller maximal effect on such cells, although the form of the calcium response curve is similar for both fresh and preincubated cells. As shown in Table V, the water extruded from fresh cells is richer in sodium and poorer in chloride than that extruded from preincubated, swollen cells. Again, these data are consistent with the observation that as red cells decrease in volume at physiological pH, hemoglobin becomes less negatively charged (9).

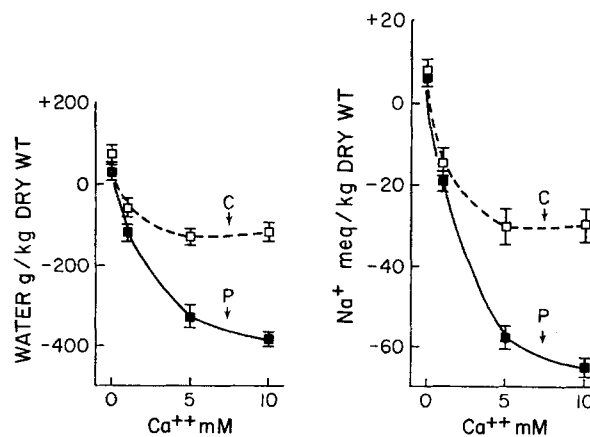


FIGURE 6. Net change in water (left-hand panel) and sodium (right-hand panel) over a period beginning at 0.5 h and ending at 20 h after suspension of cells in incubation media of various calcium concentrations. Values for cells preincubated in hypertonic medium, taken from the data in Figs. 1 and 2, are shown in closed squares and solid lines labeled P. The results with fresh cells (no hypertonic preincubation) are shown in open squares and dashed lines labeled C. Mean ± SEM for four experiments.

TABLE V  
SODIUM AND CHLORIDE CONCENTRATIONS IN FLUID  
GAINED AND LOST BY DOG RED CELLS

Calcium in incubation medium (mM)	Preincubated cells		Fresh cells	
	5	10	5	10
Fluid gained				
Na	-150 ± 6-		--	--
Cl	- 97 ± 6-		--	--
Fluid lost				
Na	164 ± 19	169 ± 5	228 ± 19	251 ± 10
Cl	123 ± 17	125 ± 19	74 ± 14	101 ± 10

Na and Cl concentrations (in meq/liter) of fluid gained by preincubated cells and lost by preincubated and fresh cells in the experiment presented in Fig. 6. Mean Values ± SEM.

#### DISCUSSION

In a previous study (25) cells preincubated and washed as in Table II, steps 3 and 4, were labeled with chromium-51 and reinjected into the bloodstream of the donor dog. Because of their increased water content the treated, labeled cells had a low density in relation to the bulk of the animal's RBC. Over the course of 20-24 h, however, the labeled cells gradually assumed a normal density, presumably by ridding themselves of salt and water. The present studies show that cells which have been swollen by preincubation in hypertonic saline can return to a normal volume *in vitro*, provided calcium and glucose are present in the medium. The time-course for extrusion of the added salt depends on the calcium concentration. At physiologic calcium levels (2-3 mM)<sup>1</sup> the return of cell contents to normal takes about as long as the return of cell density to normal *in vivo*.

Evidence for coupling between sodium and calcium transport has been found in excitable tissues, where it is thought that expulsion of calcium ions from cells may be linked to the inward movement of sodium (1, 2). It is possible that in dog RBC a similar type of counter-transport exists. Human RBC ghost studies suggest that the maintenance of low intracellular calcium involves an ATP-dependent calcium pump (33, 34). If dog RBC also pump out calcium, then it could be speculated that the energy for expulsion of sodium might be derived from passive inward movements of calcium. It may be pertinent in this regard that the uptake of isotopic calcium by dog RBC has been found to be measurably influenced by external sodium (20). An alternative hypothesis is that dog RBC may have a neutral sodium-chloride pump which is in some way dependent on calcium.

<sup>1</sup> The ionized calcium in these studies is probably 10-20% lower than the total calcium concentration because of binding to albumin (19).

Measurements of unidirectional sodium efflux from dog RBC show little or no effect of extracellular calcium (28), but such data are difficult to interpret. Analysis of unidirectional sodium flux in dog RBC gives a complex picture suggesting both multiple sodium compartments in each cell and heterogeneity among cells (12). Furthermore, cell volume influences sodium permeability in such a way that any reduction in volume would be expected to cause an increased rate of isotope movement (27). Finally, the increment in unidirectional flux which would account for the net sodium movements in these studies is a rather small fraction of the total unidirectional sodium flux in the steady state. About two-thirds of the sodium in normal dog RBC exchanges at a rate of about 16 meq/liter of cells or 46 meq/kg of dry cell weight per h. From Fig. 6 it can be seen that fresh RBC in 5 mM calcium have a net loss of 30 meq of sodium per kg of dry cell weight in 20 h. If both unidirectional and net sodium movements were linear with time, and if unidirectional influx were unaffected by calcium, an increase in unidirectional efflux of only 3 percent would account for the calcium-dependent net sodium movements observed in fresh dog RBC.

There are a number of instances in which calcium appears to influence movements of monovalent ions in RBC. Tortoise RBC become highly permeable to sodium and hemolyze in the absence of external calcium (16, 17). Calcium is necessary to restore monovalent cation permeability in human RBC which have been made leaky to sodium and potassium by incubation in solutions of low ionic strength (5). Accumulation of calcium ions within human RBC causes a specific and massive increase in potassium permeability, while sodium movements are scarcely affected (4, 15, 21, 29). The present report describes yet another action of calcium in which the divalent ion is required for net movement of sodium chloride against an electrochemical gradient. The process differs from the classical sodium-potassium pump in that it is ouabain insensitive and does not require extracellular potassium.

It is possible that this particular ion transport mechanism is present in cells other than dog RBC: The idea for this study came from a report by Rorive et al. (31) that isolated renal tubules, in which the sodium-potassium pump is blocked by ouabain, require extracellular calcium for volume homeostasis.

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