Sodium and Calcium Movements in Dog Red Blood Cells

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ABSTRACT Determinants of \(^{45}\text{Ca}\) influx, \(^{45}\text{Ca}\) efflux, and \(^{22}\text{Na}\) efflux were examined in dog red blood cells. \(^{45}\text{Ca}\) influx is strongly influenced by the Na concentration on either side of the membrane, being stimulated by intracellular Na and inhibited by extracellular Na. A saturation curve is obtained when Ca influx is plotted as a function of medium Ca concentration. The maximum Ca influx is a function of pH (increasing with greater alkalinity) and cell volume (increasing with cell swelling). Quinidine strongly inhibits Ca influx. Efflux of \(^{45}\text{Ca}\) is stimulated by increasing concentrations of extracellular Na. \(^{22}\text{Na}\) efflux is stimulated by either Ca or Na in the medium, and the effects of the two ions are mutually exclusive rather than additive. Quinidine inhibits Ca-activated \(^{22}\text{Na}\) efflux. The results are considered in terms of a model for Ca-Na exchange, and it is concluded that the system shows many features of such a coupled ion transport system. However, the stoichiometric ratio between Ca influx and Ca-dependent Na efflux is highly variable under different experimental conditions. Because the Ca fluxes may reflect a combination of ATP-dependent, outward transport and Na-linked passive movements, the true stoichiometry of an exchanger may not be ascertainable in the absence of a specific Ca pump inhibitor. The meaning of these observations for Ca-dependent volume regulation by dog red blood cells is discussed.

INTRODUCTION

Dog red blood cells are unusual among animal cells in that they lack a Na-K pump and a Na-K ATPase. Their cytoplasmic Na concentration is about equal to that of plasma. The mechanism by which they protect themselves from osmotic swelling appears to be dependent on the availability of metabolic substrate and also upon external Ca (Parker, 1977b).

Omachi et al. (1961) reported studies of \(^{45}\text{Ca}\) movements in dog red blood cells incubated in artificial electrolyte solutions. They found that the radioisotope disappeared from the medium in suspensions where KCl was the principal extracellular solute, but not when NaCl media were used. They interpreted their results in terms of competition between Na and Ca for entry into the cell (Mullins, 1956) and related their work to similar observations by Niedergerke (1959) in perfused frog hearts.

The first part of this paper is an extension and quantitation of the results of Omachi et al. In addition, an effect of external Ca on Na efflux is described.
The two phenomena, Na-inhibited Ca influx and Ca-stimulated Na efflux, are then discussed in terms of their possible relationships to each other. A brief account of this work has been published in abstract form (Parker, 1977a).

**Materials and Methods**

Blood from mongrel dogs was drawn into heparinized syringes and used within 1 h of venipuncture. After centrifugation the plasma anduffy coat were discarded and the red cells were washed in preparation for flux measurements. Unless otherwise noted, all wash and incubation media contained (mM): HEPES 10; KHCO₃ 0.5; and glucose 5. Other constituents are given below and in the figure legends. The pH of all media (unless otherwise noted) was adjusted to 7.40-7.42 at 37°C with Tris-OH. Cell suspension pH was measured with a thermostatted pH microelectrode unit (Radiometer, Copenhagen). Radioisotope purchases included ⁴⁵Ca (as CaCl₂, 10 mCi/mg Ca) from New England Nuclear, Boston, Mass., and ²²Na (as NaCl, 1 Ci/mg Na) from Radiochemical Centre, Amersham, England. ⁴⁵Ca was counted in a liquid scintillation spectrometer (Packard Instruments Co., Downer's Grove, Ill.) with a scintillation mixture described previously (Parker, 1970). ⁴⁵Ca counting efficiency under all conditions described was 60-62%. ²²Na was counted in a gamma scintillation spectrometer (Packard).

For the ⁴⁵Ca influx studies cells were washed four times in 20 times their volume of the solution in which they were to be incubated and then resuspended at a cell:medium ratio of 1:10 and placed at 37°C in a waterbath-shaker. After 5 min ⁴⁵Ca was introduced into the flask (to give a radioactivity concentration of about 50,000 dpm/ml suspension), and at intervals thereafter samples were removed from the incubation flask and pipetted into 4 times their volume of ice-cold "stopping solution" containing (mM): LiCl 150; HEPES 5; EGTA 5; pH adjusted to 7.6 at room temperature with Tris-OH. The resulting cold suspensions were centrifuged at 0°C, 28,000 g for 10 min in special lucite tubes with a well at the bottom to localize the pellet. Each supernate was carefully removed and the cell pellet transferred to a tared container where it was promptly weighed (usual sample weight 0.25-0.40 g) and then solubilized in 5.0 ml distilled water containing a drop of detergent (Actaionox; Scientific Products, McGaw Park, Ill.). To 4.0 ml of the solubilized cell pellet was added 1.0 ml of 70% perchloric acid, and after thorough mixing the sample was centrifuged. 1 ml of clear supernate was then added to 9 ml of scintillation fluid to give a homogeneous, clear solution for counting. Ca influx was computed from the slope of the time course of ⁴⁵Ca entry and the specific activity of ⁴⁵Ca in the medium, as detailed in the legend to Fig. 2. For the experiments in Fig. 6 the cells were pretreated before the Ca influx incubation so as to alter their internal ion composition. This was done by first washing fresh cells four times with 20 times their volume of Na-free medium (mM): LiCl 120; HEPES 5; KHCO₃ 0.5; pH 7.4 (37°C) with Tris-OH. The cells were then divided into aliquots and resuspended in 10 times their volume of solutions which contained ATP, an agent which rapidly and reversibly alters Na-K permeability (Parker and Snow, 1972). The composition of these media was (mM): (NaCl + KCl) = 150 (range: Na 0, K 150 to Na 150, K 0); HEPES 10; glucose 5; ATP 1; pH 7.5 (room temperature). The suspensions were incubated at 37°C for 45 min, after which the cells were washed free of ATP in the LiCl wash medium detailed above and then transferred to the influx media described in the legend of Fig. 6. ⁴⁵Ca was added, and the procedure for ⁴⁵Ca influx was followed as noted above.

Efflux studies (Fig. 8) were done by first loading the cells with ⁴⁵Ca as in Fig. 1, then washing the cells, and adding them to nonradioactive media. At suitable time points the cell suspensions were processed exactly as for the influx studies, and the cell radioactivity was measured.
The procedure for \( ^{42}\text{Na} \) efflux was as follows. Freshly drawn cells were washed three times in 20 vol of the buffered, hypotonic LiCl wash medium described in the preparation of cells for the study in Fig. 6. The cells were then washed once in 10 times their volume of "loading solution." In all cases except the study in Fig. 9A, where KCl was replaced by 140 mM NaCl, the "loading solution" contained (mM): KCl 140; HEPES 10; glucose 5; ATP 1; pH 7.5 at room temperature with Tris-OH. The cells were resuspended in twice their volume of "loading solution," \( ^{22}\text{Na} \) was added (5 \( \mu \text{Ci} \) per 10 ml suspension), and after 30 min at 37°C the suspension was centrifuged. The cells were freed of external ATP and \( ^{22}\text{Na} \) by four washes in ice-cold, hypotonic LiCl medium and then pipetted into 100 times their volume of efflux solutions (detailed composition in the figure legends) prewarmed to 37°C. Samples of this suspension were poured off at suitable time intervals and centrifuged at 0°C. The radioactivity of the supernate was then compared with the radioactivity of whole suspension in which the cells were lysed by the addition of a drop of Acationox detergent. The results were expressed as noted in the legend to Fig. 9.

Procedures for measuring the ion and water content of cells have been previously described (Parker et al., 1975). The choline used was recrystallized from ethanol and kept in a desiccator.

**RESULTS**

The data are presented under three headings: Ca influx (Figs. 1-7); Ca efflux (Fig. 8); and Na efflux (Figs. 9-15):

**Ca Influx**

Fig. 1 shows the time course of \( ^{45}\text{Ca} \) movement under conditions which favor the entry of this isotope: the cells are swollen in hypotonic, Na-free media at physiologic pH with a relatively low concentration (40 \( \mu \text{M} \)) of total Ca. After

![Figure 1](image-url). Time course of \( ^{45}\text{Ca} \) entry into cells suspended in Na-free (less than 1 mM Na), hypotonic media with LiCl (upper panel) or choline Cl (lower panel) as the principal extracellular solute. The studies were done in the absence (closed circles, solid lines) or presence (open circles, dashed lines) of EGTA. Media composition (mM): Li or choline Cl 105; HEPES 10; KHCO\(_3\) 0.5; glucose 5; CaCl\(_2\) 0.040; plus or minus EGTA 5; pH 7.42 (37°C). Medium radioactivity was 50,000 dpm/ml (upper panel) and 63,000 dpm/ml (lower panel).
the 1st min, $^{45}\text{Ca}$ enters the cell at a constant rate for at least 30 min and rises to concentrations which exceed that of the medium. Li and choline are equally good replacements for extracellular Na, and although Omachi et al. (1961) showed that K could be used as well, we found that this cation was lytic over long incubation times (Davson, 1942). In the presence of 5 mM EGTA virtually no $^{45}\text{Ca}$ enters the cells, an observation which led us to use this chelator as a component of the "stopping solution" described in Materials and Methods.

Fig. 2 shows a plot of Ca influx as a function of medium Ca concentration. A saturation curve is obtained with a half-maximum value at about 0.1 mM Ca.

Extracellular Na inhibits Ca influx (Fig. 3). Above 20 mM Na there is almost

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**Figure 2.** Ca influx as a function of extracellular Ca. LiCl (closed circles) or choline Cl (open circles) was the principal extracellular solute. Media composition (mM): LiCl or choline Cl 105; HEPES 10; KHCO$_3$ 0.5; glucose 5; CaCl$_2$ as noted on abscissa; pH 7.42 (37°C). Ca influx was computed by dividing the slope of the $^{45}\text{Ca}$ influx curve (dpm/kg cells·h) by the specific activity of the medium Ca (dpm/mmol Ca).

**Figure 3.** Ca influx as a function of external Na in hypotonic LiCl (closed circles, solid line) or choline Cl (open circles) solutions. Media composition (mM): sum of LiCl or choline Cl + NaCl = 110; HEPES 10; KHCO$_3$ 0.5; glucose 5; CaCl$_2$ 0.040; pH 7.42 (37°C). All solutions were isosmotic with one another. Mean ± SEM for three LiCl studies; a single choline Cl study is shown.
no measurable accumulation of $^{45}$Ca in the cells. A strong determinant of Ca influx is the pH of the cell suspension (Fig. 4). This finding is in good quantitative agreement with the curves published by Omachi et al. (1961). The right-hand panel of Fig. 4 suggests that inhibition of Ca accumulation at low pH reflects a decrease in the maximum rate rather than a change in the affinity of the influx mechanism. Cell volume exerts a profound effect on Ca influx, as shown in Fig. 5. The cells accumulate more Ca as they are swollen. This result

![Figure 4](image1.png)

**Figure 4.** Ca influx as a function of pH at a single extracellular Ca level (left) and as a function of medium Ca at two suspension pH values (right). Media composition (mM): LiCl 105; HEPES 10; KHCO$_3$ 0.5; glucose 5; CaCl$_2$ as noted in graphs; pH (37°C) adjusted over range indicated by titration with Tris-OH. Pooled data from four experiments.

![Figure 5](image2.png)

**Figure 5.** Ca influx as a function of cell water content at a single extracellular Ca level (left) and as a function of medium Ca at three values for cell water (right). Media composition (mM): LiCl 105-200 mM; HEPES 10; KHCO$_3$ 0.5; glucose 5; CaCl$_2$ as noted in graphs; pH 7.42 (37°C). Normal water content of freshly drawn dog red blood cells in plasma (Parker, 1973a) indicated by arrow. Pooled data from four experiments.

indicates that the pH effect shown in Fig. 4 cannot be explained on the basis of pH-associated volume changes, since one would expect the cells to shrink with increasing alkalinity. The result in Fig. 5 is not an ionic strength effect, since addition of sucrose to media of constant LiCl concentration has the same influence as adding more salt (Parker et al., 1975). Fig. 5 shows a close resemblance to a previously published graph displaying K influx as a function of cell water (Parker and Hoffman, 1976). In each case the major change in flux occurs as the cells rise above a normal water content.

Fig. 6 demonstrates that the entry of Ca into dog red blood cells is inhibited
by lowering their normally high Na concentration. The cation composition was altered by preincubating the cells in media containing ATP as described in Materials and Methods. Although K was used as a replacement for Na in the studies shown, similar results were obtained with Li which, like K, exchanges rapidly with Na in the presence of external ATP (Brown and Obaid, 1976).

Quinidine strongly inhibits Ca influx (Fig. 7), and quinine is equally effective (result not shown). These alkaloids were tried because of reports that they block Ca movements in subcellular preparations from rabbit heart (Harrow and Dhalla, 1976) and that they inhibit the influence of Ca on K flux in human red blood cells (Armando-Hardy et al., 1975).

Ca Efflux

Earlier work had led us to believe that Ca efflux might be stimulated by extracellular Na (Parker et al., 1975). This idea was confirmed by the studies in Fig. 8.

Na efflux

The method used here for loading cells with $^{22}$Na makes use of the action of extracellular ATP, which causes a reversible increase in monovalent cation permeability in dog red blood cells. Depending on the composition of the loading medium the cells can be made to contain any combination of Na and K.
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(see Fig. 6). In preliminary work with cells loaded in high-Na media so as to preserve their normal ion content, we found the kinetics of $^{22}\text{Na}$ efflux under some of the conditions we wished to examine to be complicated (Fig. 9A). When cells were made low in Na at the time of $^{22}\text{Na}$ loading, however, the subsequent efflux plots could be described with a single rate constant (Fig. 9B, C). Accordingly, all Na efflux movements with the exception of those in Fig. 9A were measured in cells with Na content ranging from 24 to 34 mmol/liter cells. Because of variations in cell Na from day to day the results are expressed in terms of rate constants rather than fluxes. Values for Na flux are presented in the discussion (Table III) where the stoichiometry between Ca and Na movements is considered.

Fig. 9B demonstrates that extracellular Ca stimulates the exit of $^{22}\text{Na}$ from cells suspended in a hypotonic, Na-free medium. Mg ion had no effect. Fig. 9C shows that the release of $^{22}\text{Na}$ is likewise increased when Na is substituted for choline in the medium. The effect of Na was greater at a concentration of 18 mM than at 110 mM.

Fig. 10A shows that the stimulation of $^{22}\text{Na}$ efflux by Ca is maximal at Ca concentrations as low as 0.1 mM (EDTA 0.01 mM was present in the medium). In Fig. 10B are displayed the results of studies using 0.01 mM EDTA media to which smaller concentrations of Ca were added so as to give levels of ionized Ca from $10^{-7}$ to $10^{-4}$ mM (Portzehl et al., 1964). The great sensitivity of Na efflux to extracellular Ca (half-saturation at $10^{-6}$-$10^{-5}$ M Ca) led us to include EDTA in all media so as to obtain a reliable value for the "zero Ca" point, inasmuch as we found up to 10 mM contamination of our nominally Ca-free media with Ca. As will be pointed out, this consideration applies also to studies of Na-stimulated $^{22}\text{Na}$ efflux.

Fig. 11 shows the concentration dependence of $^{22}\text{Na}$ efflux on extracellular
Na. Optimum stimulation occurs at a medium Na concentration of 15-30 mM.

The combined effects of Ca and Na on $^{22}$Na release are shown in Fig. 12. At zero Na the stimulatory effect of Ca is readily seen, but as Na is increased, Ca ceases to affect $^{22}$Na efflux. Conversely, Na stimulation of $^{22}$Na efflux is readily seen at zero Ca (with EDTA added), but when Ca is present no stimulatory effect of Na can be observed. Thus the effects of Ca and Na are of about the same magnitude, and each eclipses the other. It is likely that our failure in the past to show Na stimulation of Na efflux in dog red cells (unpublished observations) was due to micromolar contamination of the media by Ca.

![Figure 8](image_url)

**FIGURE 8.** $^{45}$Ca efflux as a function of time (left panel) and medium Na (right panel). Freshly drawn dog red blood cells were loaded with $^{45}$Ca by incubation for 30 min at 37°C under the conditions described in Fig. 1, i.e. in a medium containing (mM): LiCl 105; HEPES 10; KHCO$_3$ 0.5; glucose 5; CaCl$_2$ 0.040; pH 7.42 (37°C). The medium contained $^{45}$Ca 50,000 dpm/ml. The cells were then washed at 4°C with the same medium (minus $^{45}$Ca) so as to free them from external radioactivity. At the beginning of the efflux period the cells were resuspended at 37°C in media containing (mM): sum of LiCl + NaCl = 110; HEPES 10; KHCO$_3$ 0.5; glucose 5; pH 7.42 (37°C). At intervals thereafter cells were centrifuged out of the suspension and prepared for counting as noted in Materials and Methods. The cell radioactivity is expressed as a percentage of that present at the beginning of the efflux period. Left panel shows efflux at media Na of less than 1 mM (closed circles, solid lines) and 100 mM (open circles, dashed lines). Right panel shows values for 30 min samples with various Na concentrations as indicated on the abscissa. Two separate experiments.

Fig. 13 shows the pH dependence of $^{22}$Na efflux in the presence and absence of external Ca and Na. The pH curve for Ca- or Na-stimulated Na efflux is similar to that for Ca influx (Fig. 4) in that both show inhibition under acid conditions; but the slope of the curve for Na efflux is not as great as that for Ca influx.

Fig. 14 shows that quinidine has a biphasic effect on $^{22}$Na efflux, causing inhibition at levels of 0-0.5 mM and stimulation at higher concentrations. Above 2 mM the drug is hemolytic. Addition of Ca or Na to the incubation medium raises the base-line $^{22}$Na efflux, as already shown, and the inhibitory effect of quinidine is greater with either of these two ions in the solution (Fig. 14, Table 1).
Fig. 15 shows that the effect of shrinking the cells in hypertonic media is to raise the Na efflux rate constant, an observation which has been made repeatedly on dog red blood cells (Davson, 1942; Parker and Hoffman, 1976). It is notable that neither the exposure to ATP during the isotope load nor the low Na concentration of the cells following the loading phase inhibited the effect of cell shrinkage on Na efflux. Of importance is the observation that the increment in the rate constant for $^{22}$Na efflux with 0.5 mM Ca present is about the same in swollen as in shrunken cells.

In Figs. 5 and 15, movements of Ca and Na in cells of different volumes are compared. A proper interpretation of such data should take account of the fact that when cells are shrunk or swollen there is a change in number of cells.

**Figure 9.** A, B. $^{22}$Na efflux time course in a low (<0.5 mM)-Na medium, with high Na (panel A) and low Na (panel B) cells. The effects of adding CaCl$_2$ 0.5 mM to the media are shown. The quantity $f$ on the ordinate represents the ratio of medium counts to counts in a hemolysate of the suspension. In Materials and Methods the procedure for loading cells with $^{22}$Na and simultaneously altering their Na/K content is given. The basic efflux media for both studies was (mM): choline CI 110; HEPES 10; KHCO$_3$ 0.5; free EDTA 0.1; pH 7.42 (37°C), plus CaCl$_2$ as noted in the graphs. C. $^{22}$Na efflux time course in a zero Ca medium with low-Na cells. The effects of substituting NaCl for choline Cl in the medium are shown. The ordinate notation and $^{22}$Na loading procedure are as noted above (A, B). Efflux media contained (mM): sum of choline CI + NaCl = 110; HEPES 10; KHCO$_3$ 0.5; glucose 5; free EDTA 0.1, pH 7.42 (37°C). Na concentrations in cells and medium are as noted in the graph.
FIGURE 10. Rate constant for $^{22}$Na efflux as a function of extracellular Ca in predominantly LiCl (closed circles, solid lines) or choline Cl (open circles, dashed lines) solutions. Left panel (A) shows a range of Ca from 0 to 1.0 mM; right panel (B) shows levels of ionized Ca from 0 to $10^{-4}$ M in log units, as calculated from Ca and EDTA concentrations by the method of Portzehl et al. (1964). Media composition (mM): LiCl or choline Cl 110; HEPES 10; KHCO$_3$ 0.5; glucose 5; free EDTA 0.010; pH 7.40 (37°C), plus CaCl$_2$ as noted. Cell Na = 24–34 mmol/kg cells. Four separate studies.

FIGURE 11. Rate constant for $^{22}$Na efflux as a function of extracellular Na in predominantly LiCl (left panel) or choline Cl (right panel) media with equimolar substitutions of NaCl in the concentrations indicated. Media composition (mM): sum of LiCl or choline Cl + NaCl = 110; HEPES 10; KHCO$_3$ 0.5; glucose 5; free EDTA 0.1; pH 7.42 (37°C). Cell Na = 24–34 mmol/kg cells. Left, two separate studies plotted in open and closed circles. Right, mean ± SEM for three studies.

(and therefore of total cell-surface area, if one assumes that surface area per cell is constant) per volume of cells. A way of expressing the data, recognizing this, is to compute "apparent permeabilities" as is done in Table II. These calculations show in the case of the Ca fluxes that the effect of cell swelling on
Figure 12. Rate constant for $^{22}$Na efflux as a function of extracellular Na in a predominantly choline Cl medium in the presence (open circles, dashed lines) and absence (closed circles, solid lines) of CaCl$_2$ 0.5 mM. Media composition (mM): choline Cl + NaCl = 110; HEPES 10; KHCO$_3$ 0.5; free EDTA 0.1; pH 7.42 (37°C) plus or minus CaCl$_2$ as indicated in the graphs. Cell Na = 24-34 mmol/kg cells. Two separate studies are shown.

Figure 13. Rate constant for $^{22}$Na efflux as a function of suspension pH in a 110 mM choline medium with no added Ca or Na (closed circles), with Ca 0.5 mM (open circles), or with equimolar substitution of NaCl 18 mM for choline (crosses). Media composition (mM): control (closed circles)—choline Cl 110, HEPES 10, KHCO$_3$ 0.5, glucose 5, free EDTA 0.1; Ca (open circles)—choline Cl 110, HEPES 10, KHCO$_3$ 0.5, glucose 5, free EDTA 0.1, CaCl$_2$ 0.5; Na (crosses)—choline Cl 92, NaCl 18, HEPES 10, KHCO$_3$ 0.5, free EDTA 0.1. pH was adjusted to indicated values (37°C) with Tris-OH. Cell Na = 24-34 mmol/kg cells. Three separate studies are shown, one for each medium.

The apparent Ca permeability is actually greater than suggested by the dimensions used in Fig. 5. The rise in $^{22}$Na efflux rate constant with cell shrinkage (Fig. 15) reflects an increase in apparent permeability, although the magnitude of the effect is somewhat less than the rate constants per se would indicate. These
computations also bring out the point that Na permeability does not rise appreciably until the cell volume drops below a normal value.

**DISCUSSION**

The foregoing experiments were designed to examine Ca and Na movements in dog red blood cells as functions of several variables: medium pH; cell volume; cell and medium Na; medium Ca; and quinidine. The hypothesis under consideration is whether there is any linkage between Na and Ca transport. The conclusion will be that since Na-inhibitable Ca influx responds to some of the imposed conditions differently from Ca-stimulated Na efflux, the relationship between the two fluxes is not straightforward. Before discussing these matters in detail, several features of these experiments should be pointed out.

![Figure 14](image-url)

**TABLE 1**

| Media conditions (mM) | Media composition (mM): Ca0, Na 0—LiCl or choline Cl 110, HEPES 10, KHCO3 0.5, glucose 5, free EDTA 0.1; Ca 0.5, Na 0—LiCl or choline Cl 110, HEPES 10, KHCO3 0.5, glucose 5, free EDTA 0.1, CaCl2 0.5; Ca 0, Na 18—LiCl or choline Cl 92, NaCl 18, HEPES 10, KHCO3 0.5, glucose 5, free EDTA 0.1. All were at pH 7.40-7.42 (37°C). The quinidine concentration is that at which maximum inhibition was seen and ranged from 0.2 to 0.5 mM (see Fig. 14). Mean ± SEM for five studies with each medium.

| Na efflux rate constant (h⁻¹) in absence of quinidine | Media conditions (mM) | Na efflux rate constant (h⁻¹) in absence of quinidine | Maximum inhibition by quinidine (%) |
|-------------------------------------------------------|-----------------------|-------------------------------------------------------|---------------------------------|
| Na efflux rate constant (h⁻¹) in absence of quinidine | Na efflux rate constant (h⁻¹) in absence of quinidine | Maximum inhibition by quinidine (%) |
| 0.040±0.003                                           | 0.097±0.002           | 0.101±0.006                                           |
| Ca 0, Na 0                                           | Ca 0.5, Na 0          | Ca 0, Na 18                                           |

The hypothesis under consideration is whether there is any linkage between Na and Ca transport. The conclusion will be that since Na-inhibitable Ca influx responds to some of the imposed conditions differently from Ca-stimulated Na efflux, the relationship between the two fluxes is not straightforward. Before discussing these matters in detail, several features of these experiments should be pointed out.
Except for the studies of Na efflux into Na-containing media, the radioisotopes in these experiments are moving into compartments where the initial concentration of their unlabeled chemical counterparts is very low (dog red cell Ca is about 0.020 mmol/liter cells; the nominally Na-free media contain less than 0.1 mM Na). The measurements do not represent "steady-state" fluxes in any sense. The tracer movements are indicative of net ion movements (except in the case of the Na efflux studies mentioned). The radioisotopes were used because they are easier to measure in the small concentrations dealt with in these experiments. All the conditions which favor the movements of $^{45}$Ca are the same as those which in a previous report were shown to stimulate chemical

![Figure 15. Rate constant for $^{22}$Na efflux as a function of cell water content in the presence (clear symbols) and absence (solid symbols) of CaCl$_2$ 0.5 mM. Efflux media consisted principally of choline Cl (square symbols) or LiCl (round symbols). Media composition (mM): LiCl or choline Cl 110-210 mM; HEPES 10; KHCO$_3$ 0.5; glucose 5; free EDTA 0.1, plus or minus CaCl$_2$ as noted. pH (37°C) was 7.42. Normal water content of freshly drawn dog red blood cells in plasma (Parker, 1973a) indicated by arrow. Cell Na ranged from 34 mmol/kg cells at 71% cell water to 44 mmol/kg cells at 58% cell water. Pooled data from four experiments.](image)

Ca flux (Parker et al., 1975). Lack of information about the physical state of Ca in cells, however, prevents a quantitative statement about the electrochemical potential gradient for this ion. Thus, the finding that $^{45}$Ca concentrations in the cell may rise to levels which exceed that of the medium (Fig. 1) is difficult to interpret in terms of active versus passive transport.

Many of the present studies were done in Na-free media, and the possibility therefore exists that some of the ion movements were driven by hyperpolarization of the membrane resulting from the large outward Na concentration gradient. We have examined this question in several ways, including the use of a fluorescent dye which monitors membrane potential (Hoffman and Laris, 1974). Dog red blood cells suspended in hypotonic choline solutions do not show measurable hyperpolarization unless agents are included in the system which cause the Na permeability to be increased. Ca is not one of those agents...
(Parker et al., 1977). With Li as the principal extracellular solute no hyperpolarization would be expected, since the Li and Na permeabilities in dog red blood cells are very close (Parker et al., 1975).

The interactions between Ca and Na described in these experiments defy a simple interpretation. Earlier work (Parker et al., 1975) led us to believe that dog red blood cells might have a passive transport pathway, like that seen in

| Table II |

| Solvent volume | Cell water | Surface area | Rate constant | Apparent permeability |
|----------------|------------|--------------|---------------|-----------------------|
| % of wet wt | cm | cm⁻¹ | cm⁻³ |
| Ca influx (Fig. 5) | 65 | 4.3×10⁻⁵ | 3.9×10⁻⁵ | 12×10⁻⁹ |
| 66 | 4.5×10⁻⁵ | 22.2×10⁻⁴ | 69×10⁻⁹ |
| 70 | 5.4×10⁻⁵ | 47.3×10⁻⁴ | 176×10⁻⁹ |
| 58 | 3.2×10⁻⁵ | 1.1×10⁻⁴ | 4.2×10⁻⁹ |
| 60 | 3.5×10⁻⁵ | 0.7×10⁻⁴ | 2.9×10⁻⁹ |
| 64 | 4.1×10⁻⁵ | 0.3×10⁻⁴ | 1.3×10⁻⁹ |
| 66 | 4.5×10⁻⁵ | 0.2×10⁻⁴ | 1.1×10⁻⁹ |
| 70 | 5.4×10⁻⁵ | 0.1×10⁻⁴ | 0.9×10⁻⁹ |

Cell solvent volume:surface area ratios were calculated from the information that under normal conditions dog red blood cells have a volume of 63×10⁻¹⁵ liter/cell, a density of 1.105 kg/liter cells, a water content of 0.636 kg/kg cells, and a surface area of 117×10⁻⁸ cm²/cell. Rate constants were calculated from Fig. 5 at an external Ca of 0.5 mM. Rate constants for Na are from Fig. 15 in the absence of extracellular Ca. Apparent permeabilities were computed according to the following formulas (Sachs et al., 1975):

For Ca influx, \( P = \frac{V}{A} \left( \frac{e^{\frac{-zE}{RT}} - 1}{B e^{\frac{-z}{T}}} \right) \),

For Na efflux, \( P = \frac{V}{A} \left( \frac{e^{\frac{-zE}{RT}} - 1}{B} \right) \).

where \( P \) (cm/s) is the apparent permeability, \( V \) (cm³) is the cell solvent volume, \( A \) (cm²) is the cell surface area (assumed to be constant), and \( k \) (s⁻¹) is the rate constant. The quantity \( B = e^{\frac{zE}{RT}} \) equals \( e^{\frac{zE}{RT}} \), where \( z \) is the valence, \( E \) is the membrane potential (assumed constant at \(-10\) mV), and \( F \), \( R \), and \( T \) (310°K) have their usual meanings.

* Under the conditions of Figs. 5 and 15.

excitable cells, which conducts a coupled exchange of Ca for Na, Na for Na, or Ca for Ca (Reuter and Seitz, 1968; Baker et al., 1969; Blaustein, 1974). The evidence for such a mechanism can be listed as follows: (a) Ca movements in either direction across the membrane are accelerated when the Na concentration is low on the side from which Ca moves and/or high on the side to which Ca moves (Figs. 3, 6, 8); (b) Na efflux is stimulated by external Ca (Figs. 9, 10, 12-15); (c) inhibition of Ca entry by external Na (Fig. 3) occurs at about the same concentration of Na (20 mM) which maximally stimulates "Na efflux (Fig. 11). Thus it appears that Na is competing with Ca for a mechanism which mediates
both Ca entry and Na exit; (d) the effects of external Ca and Na on 22Na efflux are of the same magnitude and are mutually exclusive (Fig. 12), again suggesting competition between the two ion species for some site on the cell surface; (e) quindine inhibits ^4Ca influx (Fig. 7) and Ca-stimulated 22Na efflux (Fig. 14, Table I); (f) the pH dependence curves for Ca influx and Ca-dependent Na efflux are qualitatively similar (Figs. 4, 13).

Against a simple model for Ca-Na exchange are the data summarized in Table III, which compare flux values for Ca and Na under various conditions of cell volume and Na content. There is no constant ratio between Ca influx (which is completely Na-inhibitable, as shown in Fig. 3) and Ca-stimulated Na

| TABLE III |
|-----------------|
| STOICHIOMETRY BETWEEN Ca INFLUX AND Ca-DEPENDENT Na EFFLUX |

| Osmotically shrunken cells (cell water 60-62% wet wt) | Osmotically swollen cells (cell water 69-71% wet wt) |
|-----------------|-----------------|
| Cell Na (mmol/kg cells) | High Na cells (160-170) | Low Na cells (42-44) | High Na cells (70-80) | Low Na cells (24-34) |
| Ca flux (mmol/kg cells·h) | <0.2* | <0.1* | 7.8± | <0.2* |
| Ca-dependent Na efflux (mmol/kg cells·h) | 9.1± | 2.5 | 3.5 | 1.8 |
| Ratio: Ca influx | <0.03 | <0.04 | 2.2 | <0.02 |
| Ca-dependent Na efflux | | | | |

All studies were done in Na-free media at pH 7.40–7.42 (37°C). * Values obtained from experiments exactly like those in Figs. 5 and 6, but with external Ca raised to 0.5 mM. † Value averaged from Figs. 2, 4, and 5 at 0.5 mM medium Ca. Na effluxes were calculated from Figs. 9 and 15 as follows: (Na efflux rate constant with 0.5 mM external Ca [h⁻¹] – Na efflux rate constant with 0 mM external Ca [h⁻¹]) × cell Na concentration (mmol/kg cells) = Ca-dependent Na efflux (mmol/kg cells·h). In the case of the high-Na cells where Na efflux is not linear with time (Fig. 9A), the flux was calculated from the amount of isotope released at 60 min. § Value obtained from experiments performed exactly as in Fig. 9A (high-Na cells), but in a hypertonic medium consisting of (mM): LiCl 200, HEPES 10, KHCO₃ 0.5, glucose 5, EDTA 0.1; plus or minus CaCl₂ 0.5, pH 7.42 (37°C). This value is the mean of two results: 10.3 and 7.8 mmol/kg cells·h.

As cell volume is reduced Ca influx falls, while Ca-dependent Na efflux is increased. As cell Na is lowered, Ca influx falls more sharply than does Ca-dependent Na efflux. Thus, the stoichiometry of coupling of Ca to Na movements is apparently variable over a wide range and depends on the conditions of the experiment. A further difficulty with a straightforward model for Ca-Na exchange is that the two fluxes respond quantitatively differently to external Ca: Ca-stimulated Na efflux is 10–100-fold more sensitive to external Ca than is Ca influx (Figs. 2 and 10).

Thus, while qualitative evidence for Ca-Na exchange exists in dog red blood cells, considerations of stoichiometry and the activation by external Ca suggest that the process may be complex. Some resolution of the complexity might be possible if one knew more about active Ca extrusion in dog red blood cells. Human red blood cells have a potent, ATP-dependent Ca pump (Schatzmann...
It is likely that dog red cells have a similar mechanism, although the effects of Na on Ca efflux make it difficult to characterize (Parker et al., 1975). The data presented here do not allow one to tell whether a condition which favors Ca entry (cell swelling, low external Na, high internal Na, alkalinity) exerts its effect by raising the inward movement of Ca or by inhibiting the Ca pump. Similarly, when cells are placed in circumstances which do not favor Ca accumulation (cell shrinkage, high external Na, low internal Na, acid conditions, quinidine), the effect could be due to decreased Ca entry or stimulation of the Ca pump. Thus, the dog red blood cell may have a tightly coupled Ca-Na exchanger, but it might not be possible to study its true stoichiometry without selectively inhibiting the Ca extrusion pump, and this has not been possible to date.

These experiments were originally undertaken to clarify the mechanism by which extracellular Ca activates the uphill, outward transport of Na by dog red blood cells during the course of adjustment from a swollen state toward a normal volume (Parker, 1973a, b; Parker et al., 1975). The volume-regulatory studies were all done with cells suspended in high-Na, plasma-like media, a circumstance which should suppress inward Ca flux (Fig. 3). Nevertheless, some net influx of Ca did occur during the cells' adjustment of Na and water content (Parker et al., 1975), and it was on this basis that we postulated a Na pump which derives its energy from the passive, inward movement of Ca through a Ca-Na exchanger. The studies presented here document numerous interactions between Ca and Na movements, but the role of a Ca-Na exchanger in mediating the net salt and water movements which occur in swollen dog red blood cells under physiological circumstances (Parker, 1973a) remains an open question.

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