Heterogeneity Among Dog Red Blood Cells

JOHN C. PARKER

From the Department of Medicine, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

ABSTRACT A phthalate density-separation technique has been used to study the heterogeneity of dog red blood cells that becomes manifest when they are suspended in KCl media. It is demonstrated that the proportions of cells that separate into light and dense fractions can be varied by altering the tonicity of the KCl medium. This results from the fact that the Na and K permeabilities of each cell are continuous functions of cell volume. It was found that quinidine inhibits selectively the volume dependence of Na permeability. In the presence of this drug, the heterogeneity demonstrated by KCl incubation disappears. The notion that dog red blood cells are heterogeneous in their permeabilities to Na and K is thus upheld, but the heterogeneity is not an abruptly discontinuous one, as has been claimed. A sample of dog blood does not contain two discrete populations of red cells.

INTRODUCTION

The cation composition of dog red blood cells is similar to that of extracellular fluid, being high in Na and low in K. When dog red blood cells are incubated in an isotonic KCl medium, gradients are established that favor a net influx of K and a net efflux of Na. Davson (3) reported that in this circumstance, about one-half the cells undergo a net accumulation of cations: K gain exceeds Na loss, and the cells swell. The other one-half of the cell population sustains a net loss of cations: Na loss exceeds K gain, and the cells shrink. Thus, after a period of time in isotonic KCl solution, the cells resolve into two discrete and discontinuous fractions that differ in cell-water content and therefore in density (4). Davson explained this observation by postulating that dog red blood cells are heterogeneous with respect to $P_K$ and $P_{Na}$, their respective K and Na permeabilities. The cells that swell in isotonic KCl have a higher $P_K/P_{Na}$ ratio than the cells that shrink.

Davson recognized a second feature of dog red blood cells that subsequent workers have confirmed and quantitated (6, 7, 9), namely, that $P_K$ and $P_{Na}$ are both influenced by experimental perturbations of cell volume. Cells become more permeable to K as they swell and more permeable to Na as they shrink.

Recently, Castranova and Hoffman (1) reinvestigated these phenomena. Dog red blood cells were first resolved into light and dense populations by incubation in isotonic KCl. The two cell fractions were separated, washed,
and incubated under conditions that restored their original Na, K, and water contents. Various transport characteristics of the two groups of cells were then studied. The cells that had gained cations and water in isotonic KCl were found to have a higher $P_K$ and a greater response of $P_K$ to cell swelling than the cells that had resolved into the dense fraction during the KCl separation. The latter group of cells had a higher $P_{Na}$ and a greater increase in $P_{Na}$ on cell shrinkage. The authors concluded, in agreement with Davson (3), that in any sample of dog blood there are two discrete populations of red cells that manifest an abrupt discontinuity in their Na and K permeabilities.

The present paper reports evidence that confirms the heterogeneity of dog red blood cells. However, the data indicate that this heterogeneity is gradual rather than abruptly discontinuous. Because $P_K$ and $P_{Na}$ are influenced in opposite directions by changes in cell volume, the relative ratios of these permeabilities can be varied by altering the tonicity of the KCl medium in which the cells are suspended. The proportion of cells which, upon incubation, segregate into the dense and light fractions can thus be varied. At an upper level of KCl tonicity, virtually the entire population of cells will resolve into the dense fraction, because of net Na loss. At a lower level of KCl tonicity, all the cells remain in the lighter fraction. Thus, in any sample of dog red blood cells there is a continuous range of permeability properties that, because of the peculiar influence of cell volume on $P_K$ and $P_{Na}$, allows one to separate the cells into discrete subgroups by incubation in KCl. The number of cells in each subgroup can be controlled by varying the volume that the cells assume when they are first exposed to the KCl medium.

A second point made in this report is that the resolution of cells into two populations can be completely blocked by quinidine, an agent that prevents the cells from increasing their Na permeability when shrunken.

**METHODS**

Within an hour before the start of each experiment, blood was drawn from the leg veins of healthy, mongrel dogs using syringes rinsed with heparin. After centrifugation, the plasma and buffy coat were discarded. The red blood cells were washed three times at $4^\circ$C by centrifugation at 5,000 $g$. The wash solution contained LiCl at the same concentration as the KCl in the medium in which the cells were ultimately to be incubated plus 5 mM Hepes buffer adjusted with Tris-OH to pH 7.4 at $37^\circ$C. After carefully removing the supernate from the final wash, the cells were resuspended in one to two vol of incubation medium containing KCl at concentrations indicated in the Results section plus 5 mM glucose and 10 mM Hepes adjusted with Tris-OH to pH 7.4 at $37^\circ$C. The cell suspensions were mixed and placed in a $37^\circ$C water bath-shaker oscillating at 100 cycles/min over a traverse of 3 cm. Immediately, and at hourly intervals thereafter, samples were removed from the cell suspension and drawn up into a series of microhematocrit tubes, each containing a drop of phthalate oil (2) of known density, as detailed in Table I. Triplicate samples were done at each phthalate density. The microhematocrit tubes containing cell samples plus phthalate were then sealed at one end by flame and centrifuged at 11,500 rpm (13,000 $g$) for 30 min at room temperature in a microhematocrit centrifuge (model MB; International Equipment Co., Needham Heights, Mass.). The tubes were then removed from the centrifuge, and the lengths of the cell columns above and below the phthalate oil
were measured using a spiral microhematocrit tube reader. There was variation from animal to animal in the amount of hemolysis that occurred under the conditions of these experiments. Some specimens showed as much as 10% lysis. All of the results shown had <3% lysis.

The results were calculated as follows: the volume percent of cells on top equals the length of the cell column on top of the phthalate × 100/sum of lengths of the cell columns on top of and beneath the phthalate. Because the densest cells had a lower volume per cell than the lightest cells, this mode of expression of the results gives greater emphasis to the lighter cells as compared with the heavier population. This consideration is not of concern for the purposes of the argument.

A study of net ion and water movements was done to demonstrate the specificity of quinidine as an inhibitor of volume-responsive Na permeability (Table II). Fresh dog red blood cells were washed three times at room temperature in a solution containing 150 mM LiCl and 5 mM Hepes, pH 7.4, at 37°C. The cells were then preincubated for 45 min at 37°C in nine volumes of a medium containing: NaCl (60 mM), KCl (90 mM), Hepes (10 mM), ATP (1 mM), pH 7.4 at 37°C. The preincubation with external ATP (8) was done to change the cell cation composition to roughly one-half Na and one-half K. The cells were then washed in the buffered 150 mM LiCl solution and incubated for 2 h at 37°C in 20 volumes of hypo- or hypertonic choline chloride medium1 which contained (mM): choline chloride (80 or 205 mM), Hepes (10 mM), glucose (5 mM), pH 7.4 at 37°C, with or without 0.1 mM quinidine. At the beginning and end of the choline incubation, samples of the suspension were centrifuged in special Lucite tubes for the determination of cell Na, K, and water content (8).

**RESULTS**

Fig. 1 shows how the results are presented and depicts a clean separation between two cell populations after 3 h incubation at 150 mM (isotonic) KCl.

1 Haas, M., and T. J. McManus. Volume-sensitive ion transport in duck red blood cells using a zero trans approach. Manuscript submitted for publication.

---

**TABLE I**

**COMPOSITION AND DENSITY OF PHTHALATE SOLUTIONS**

| Percent by volume | Density (25°C) |
|------------------|---------------|
|                  | gm/ml         |
| **Dibutyl phthalate** | **Dimethyl phthalate** |
| 90               | 10            | 1.0596         |
| 80               | 20            | 1.0736         |
| 70               | 30            | 1.0876         |
| 60               | 40            | 1.1016         |
| 55               | 45            | 1.1086         |
| 50               | 50            | 1.1156         |
| 45               | 55            | 1.1226         |
| 40               | 60            | 1.1296         |
| 35               | 65            | 1.1366         |
| 30               | 70            | 1.1436         |
| 25               | 75            | 1.1506         |
| 20               | 80            | 1.1576         |
The left panel of Fig. 1 shows on the ordinate the volume of cells (expressed as a percentage of the total cell column) that lies on top of the phthalate oil mixtures of densities indicated on the abscissa. Thus, after 3 h in 150 mM KCl, 55% of the packed cell column has a density ≤1.13; 100% of the cells have a density <1.145. This mode of presenting the results of the density separation is termed the "integral" plot. On the right panel of Fig. 1, the same data are expressed in a "differential" plot. Here the differences between successive ordinate points on the integral figure are plotted as a function of the specific gravity of the phthalate oils. The differential plot gives the volume percentage of cells with a given density. Thus, after 3 h (Fig. 1 right panel)

![Integral and Differential Plots](image)

**Figure 1.** Separation of cells into two populations after 3 h of incubation in 150 mM KCl. See text for details. Zero-time values in open circles and dashed lines. 3-h values in solid circles and solid lines.

~25% of the cells have a density of 1.10 and 38% of the cells have a density of 1.14. The open circles and dashed lines in Fig. 1 indicate that at the beginning of the KCl incubation (0 h) there was a single population of cells that had a density of ~1.10.

Fig. 2 shows the time-course of separation of cell populations upon incubation in 150 mM KCl at 37°C. The results are shown in the differential mode (closed circles and solid lines) and the integral mode (open circles and dashed lines). Each panel represents a different time point. Although the cells separate into two populations by 3 h, they show a small third population of intermediate density at 2 h.

Experiments with quinidine show that Na movements are largely responsible for the separation of cells into two major populations when suspended in KCl.

In a survey of possible inhibitors, it was found that quinidine, which slows Ca and Na movements in swollen dog red blood cells (5), also blocks the increase in net passive Na flux that occurs when cells are shrunken, as shown
T H A T  E E

| Initial values                  | Na      | K      | Water          |
|--------------------------------|---------|--------|----------------|
| Hypotonic control              | 148 (11) | 161 (6) | 3,119 (84)     |
| Hypotonic control plus quinidine | 146 (9)  | 160 (7) | 3,137 (111)    |
| Hypertonic control             | 144 (7)  | 162 (7) | 1,661 (68)     |
| Hypertonic control plus quinidine | 144 (7)  | 162 (6) | 1,672 (64)     |

| Change (loss) in 2 h            | Na      | K      | Water          |
|--------------------------------|---------|--------|----------------|
| Hypotonic control              | 25 (6)  | 74 (12) | 722 (197)      |
| Hypotonic control plus quinidine | 20 (4)  | 75 (16) | 793 (90)       |
| Hypertonic control             | 104 (3) | 15 (5)  | 434 (36)       |
| Hypertonic control plus quinidine | 22 (2)  | 14 (1)  | 136 (21)       |

* Mean ± SD (in parentheses) for four studies.

In Table II. In this experiment, the cells are preincubated briefly in the presence of external ATP to alter their internal cation composition so that it becomes about one-half Na and one-half K. The ATP is then washed off, and the cells are incubated for 2 h at 37°C in hypotonic or hypertonic choline media that contain no Na or K. The values for cell Na, K, and water content immediately after the cells are placed in hypotonic and hypertonic choline are shown in the upper portion of Table II. As expected, the water content of
the cells is markedly affected by the medium tonicity whereas the cation content is not. With time, all the cells lose cations and water, as shown in the lower part of Table II. The cells incubated in the hypotonic solution lose more K than Na, whereas the hypertonically shrunken cells (control) lose more Na than K. These results are entirely consistent with previous demonstrations that swollen cells have a selective increase in $P_K$, whereas shrunken cells undergo a selective increase in $P_{Na}$ (1, 3, 7). Quinidine (0.1 mM) inhibits the loss of Na from the cells in hypertonic medium, and also inhibits their water loss. Quinidine has no effect on K movements, either in hypotonic or hyper-

![Graph](image)

**Figure 3.** Inhibition of cell separation in 160 mM KCl by 0.1 mM quinidine. Integral plot in open circles and dashed lines. Differential plot in solid circles and solid lines.

tonic circumstances. Fig. 3 shows that when fresh dog red blood cells are incubated in 160 mM KCl and then placed in phthalate gradients, quinidine completely blocks the emergence of the very dense cell population.

Fig. 4 shows that when the cells are suspended in hypotonic KCl solution (110 mM) they have a mean density of $\approx 1.075$, less than the initial density of cells suspended in 150 mM KCl. The hypotonic KCl incubation does not result in a separation of the cells into two populations. Rather, the cells become more widely distributed about the mean density with progressive incubation time.

The effect of hypertonic KCl incubation is shown in Fig. 5. Immediately after suspension in 200 mM KCl, the cells have a mean density of $\approx 1.125$. With time, there is a separation of the cells into two populations: a small one (5–6% of the cells) with a density of $\approx 1.105$, and a major one with a peak at a density of 1.140.

Fig. 6 shows the results of six separate experiments on blood from the same
dog. The red blood cells are suspended in a variety of concentrations of KCl media ranging from 120 to 180 mM. Here the results are all plotted in the differential mode with the value for time zero given in open circles and dashed lines and the value after 3 h of incubation given in closed circles and solid lines.
lines. Clearly, the relative size of the two major populations varies depending on the medium tonicity and therefore on the initial cell volume. The percentage of cells separating into the high-density fraction increases with increasing KCl concentration. Conversely, the percentage of cells remaining in the low-density fraction increases as the KCl concentration in the suspending medium is decreased.

**Figure 6.** Variable separation of the cells into light and dense fractions depending on the KCl tonicity. Differential plots. Zero-time values in open circles and dashed lines. 3-h values in solid circles and solid lines.

**Discussion**

The foregoing experiments confirm the observation by Davson (3) that dog red blood cells can be separated into at least two populations by suspension in a KCl medium. The data further indicate that the relative size of the populations is a function of the tonicity of the KCl solution employed. In very hypotonic media (e.g., 110 mM KCl) nearly all the cells remain in a light or overhydrated population, whereas under hypertonic conditions (e.g., 200 mM KCl) most of the cells segregate into a hyperdense population.

These results can be explained by reference to Davson’s original formulations (3). When dog red blood cells are placed in KCl medium, gradients are established that favor the inflow of K and the outflow of Na. The degree to which one of these net fluxes predominates over the other will determine the solute and water content of each cell. The cells in which K uptake equals or exceeds Na loss will maintain their volume or swell; the cells in which Na loss exceeds K gain will start to shrink. Because of the dependence of \( P_K \) and \( P_{Na} \) on cell volume (7), the initial tendency of the cells to diverge will be reinforced by a kind of positive feedback. Cells that initially have a high \( P_{Na}/P_K \) ratio will shrink, undergo a further rise in \( P_{Na} \), and thus shrink faster. Cells that
start with a low \( P_{\text{Na}}/P_{\text{K}} \) ratio will either swell, and thus undergo a further decrease in \( P_{\text{Na}}/P_{\text{K}} \), or they will balance Na losses by \( K \) gains and remain at the same volume. With time, different populations of cells will emerge, and these can be readily separated from each other by density-gradient techniques. The action of quinidine suggests that it is the effect of volume on \( P_{\text{Na}} \) that dominates the separation of cells in KCl. Quinidine inhibits the rise in \( P_{\text{Na}} \) that occurs when cells shrink. In the presence of this drug, the resolution of the cells into different populations is prevented as the hyperdense fraction fails to emerge.

Because \( P_{\text{K}} \) and \( P_{\text{Na}} \) are each continuously variable as a function of cell volume (7), the ratio between the two permeabilities is likewise continuously variable as the cells are swollen or shrunken. The ultimate fate of a cell placed in KCl thus depends on its initial volume. In hypotonic media, most of the cells will have a low \( P_{\text{Na}}/P_{\text{K}} \) ratio, and will swell or maintain their volume. In hypertonic media, most of the cells will have a higher \( P_{\text{Na}}/P_{\text{K}} \) ratio, and will therefore sustain a net loss of solute and water. Thus, the separation of dog red blood cells into two populations in KCl does not mean that there are two biologically discrete populations of cells in every dog's blood. Rather, there is a heterogenous population of cells with a continuous range of permeability responses to cell volume perturbation. At any given KCl tonicity, one can postulate that there is a normal distribution of \( P_{\text{Na}}/P_{\text{K}} \) ratios throughout the cell population. There is, furthermore, a \( P_{\text{Na}}/P_{\text{K}} \) ratio above which cells will start to lose more Na than K gain, and therefore shrink. At very low KCl tonicities, none of the cells will have this \( P_{\text{Na}}/P_{\text{K}} \) ratio, and the hyperdense fraction will not emerge. However, as the KCl tonicity is increased a progressively greater fraction of the cells will assume a volume at which their \( P_{\text{Na}}/P_{\text{K}} \) ratio exceeds the critical value above which the "positive feedback" sequence will occur, leading to progressive cell shrinkage, further increase in \( P_{\text{Na}}/P_{\text{K}} \), and further volume loss. Thus, at increasing KCl tonicities, a greater and greater proportion of the cells will segregate into the hyperdense population (Fig. 6).

The findings of Castranova and Hoffman (1) that cells recovered from the light and dense fractions of an isotonic KCl suspension have differing responses of Na and K permeabilities to volume alteration is thus to be expected, but their results do not constitute evidence of an abrupt discontinuity in the heterogeneous cell population. The discontinuity can be made to occur at any dividing point by varying the cell volume, and therefore the relative Na and K permeabilities, at the time the cells are first suspended in KCl.

The technical assistance of Paula S. Glosson is gratefully acknowledged. Supported by grant AM 11357 from the U. S. Public Health Service.

Received for publication 15 December 1980.

REFERENCES

1. CASTRANOVA, V., and J. F. HOFFMAN. 1979. Heterogeneity in dog red blood cells: sodium and potassium transport. J. Gen. Physiol. 73:61–71.
2. Danon, D., and Y. Marikovsky. 1964. Determination of density of distribution of red cell population. *J. Lab. Clin. Med.* 64:668.

3. Davson, H. 1942. The haemolytic action of potassium salts. *J. Physiol. (Lond.)* 101:265–283.

4. Lange, Y., R. V. Lange, and A. K. Solomon. 1970. Cellular inhomogeneity in dog red cells, as revealed by sodium flux. *J. Gen. Physiol.* 56:438–461.

5. Parker, J. C. 1978. Sodium and calcium movements in dog red blood cells. *J. Gen. Physiol.* 71:11–17.

6. Parker, J. C., and J. F. Hoffman. 1965. Interdependence of cation permeability, cell volume, and metabolism in dog red cells. *Fed. Proc.* 24:2527. (Abstr.).

7. Parker, J. C., and J. F. Hoffman. 1976. Influence of cell volume and adrenalectomy on cation flux in dog red blood cells. *Biochim. Biophys. Acta.* 433:405–408.

8. Parker, J. C., and R. L. Snow. 1972. Influence of external ATP on permeability and metabolism of dog red blood cells. *Am. J. Physiol.* 233:888–893.

9. Romualdez, A., R. I. Sha'afi, Y. Lange, and A. K. Solomon. 1972. Cation transport in dog red cells. *J. Gen. Physiol.* 60:46–57.