Dog Red Blood Cells

Adjustment of density in vivo

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ABSTRACT Red blood cells from mature dogs contain less Na and more K than would be the case if they were in Donnan equilibrium with plasma. They have no ouabain-sensitive Na pump, and their membranes are deficient in Na, K-ATPase. Experiments are reported in which dog red cells were first loaded with supranormal quantities of Na and water and then reinjected into the dog. Over the course of 26–40 h the Na- and water-loaded cells returned to a normal state of hydration as judged by their density. It is concluded that dog red cells possess some means of correcting their swollen status in vivo, despite their lack of a ouabain-sensitive cation transport apparatus.

INTRODUCTION

The cation content of dog erythrocytes varies with the age of the cell and the age of the dog. Newborn puppies have red cells whose K content is appreciably higher than that of plasma (10), and the same is true for reticulocytes in mature dogs (5, 14). Mature red cells in mature dogs, however, are nearly in equilibrium with plasma with respect to their Na and K concentrations (1, 14, 19). Previous studies have suggested that they do not possess the classical ATP-dependent Na-K pump seen in the red cells of most other mammals (6, 11, 12), and that their membranes are lacking in Na, K-ATPase (2). Despite these deficits they survive in the circulation for 100 days (22). Since they are much more permeable to Na than most other mammalian red cells, a question arises concerning the mechanism by which they protect themselves from Donnan swelling.

This report describes experiments which show that dog red cells are quite capable of regulating their volume in vivo. The means by which they accomplish this remain obscure. A brief account of these findings has been reported (14).

METHODS

Venous blood from healthy mongrel dogs or normal human volunteers was drawn into syringes rinsed with heparin (1000 U/ml). Measurements of ions and water

TABLE I

COMPOSITION OF PHTHALATE MIXTURES

Solution	Percent dibutyl phthalate by volume (25°C)	Specific gravity (25°C)
1	65.0	1.0946
2	63.8	1.0962
3	62.6	1.0980
4	61.4	1.0996
5	60.3	1.1012
6	59.1	1.1029
7	57.9	1.1046
8	56.7	1.1062
9	55.5	1.1079
10	54.4	1.1094
11	53.2	1.1111
12	52.0	1.1128

Dimethyl and dibutyl phthalate were mixed in the proportions shown. See text for other details.

were done after centrifuging cell suspensions at 25,000 *g* (25°C) for 15 min in specially designed Lucite tubes equipped with a narrow, 1 ml well at the bottom for collection of the cell button. The contents of these tubes were used to determine [^{125}I] albumin extracellular space and the ion and water contents of cells and supernatant. The details of these methods are published elsewhere (13, 18). With the exception of the studies reported in Table II, all cells were washed four times in a standard solution containing NaCl 140 mM, glycylglycine 5 mM (pH 7.5) before measuring their contents.

^{24}Na efflux was measured by a published method (18) in which whole blood was preincubated for 2 h with ^{24}Na (chloride salt) 20 μCi/ml at 37°C. The external isotope was removed by washing with standard wash solution at 4°C. Samples of the labeled, washed cells were pipetted into 100 times their volume of incubation solution at 37°C and incubated in a shaker bath. At intervals thereafter portions of the suspension were centrifuged at 4°C, and the radioactivity of the supernatant medium was compared with that of a hemolysate of the suspension. The results are expressed in terms of the percentage of counts released into the medium over a given time interval. No hemolysis correction was necessary.

The methods for ATPase assay and ghost preparation have been described previously (15, 17).

Details of the in vivo studies are reported along with the results. The technique of separating cells by density using mixtures of dimethyl and dibutyl phthalate is a modification of the method described by Danon and Marikovsky (3). The composition of the phthalate solutions[1] is shown in Table I. The specific gravity of solutions 1 and 12 was measured by pycnometry at 25°C. The other mixtures, made up of various proportions of solutions 1 and 12, were calculated to have the specific gravities listed.

[1] Eastman Kodak Co., Rochester, N. Y.

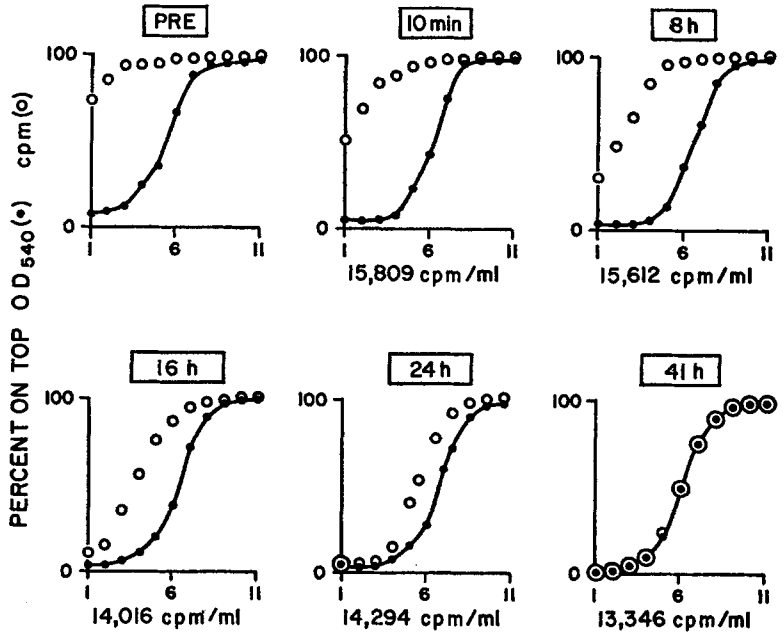

FIGURE 1. Density profile of labeled and unlabeled cells. See text for details. The numbers on the abscissa represent the phthalate solutions shown in Table I. The time interval after reinjection of labeled cells is indicated in the box above each graph. Pre, preinjection sample, explained in the text. Whole blood radioactivity is shown under each graph.

The density distribution of dog red cells shown in Fig. 1 agrees closely with the values found by this method in human cells (3).

3 ml of each phthalate mixture was pipetted into a centrifuge tube to which was added 1 ml of blood. The contents of the tube were thoroughly mixed and then centrifuged at 28,000 *g* (25°C) for 45 min. A Sorvall RC-2B centrifuge[2] was used, with the tubes placed in the outermost holes of the SM-24 rotor. The cells which floated on top of the phthalate were lysed with 2–3 ml water containing a drop of Acationox[3] detergent and quantitatively removed. The phthalate was then discarded, and the bottom layer of cells was recovered. The amount of hemoglobin (OD 540 nm) and radioactivity in lysates of both upper and lower cell layers was measured.

Autoradiography of blood smears was done by the method of Ronai (20).

RESULTS

Ionic Equilibria

Table II presents the Na, K, and Cl content of mature dog red cells and plasma. If Cl is assumed to be at equilibrium across the membrane, and if

[2] Ivan Sorvall, Inc., Norwalk, Conn.

[3] Biological Research, Inc., Evanston, Ill.

all ions are completely in solution, then it can be stated from the cell/plasma ratios that the system is not in Donnan equilibrium. By this reasoning the cells contain less Na and more K than they would if these ions were passively distributed.

Effect of Ouabain on Na Efflux

The cation permeability of dog red cells is strongly influenced by their state of hydration (4, 16). Na flux is most rapid in shrunken cells and slows progressively with sublytic swelling. These changes are independent of the Na content or tonicity of the medium; they can be demonstrated by following isotope movements in either direction across the membrane or by measurements of net ion fluxes (6).

Table III shows Na efflux measurements in dog red cells incubated in a

TABLE II

CONSTITUENTS OF DOG RED CELLS AND PLASMA

	Na	K	Cl	Water
		meq/kg water		*percent wet wt*
Cells	161.9±2.3	7.8±0.5	80.1±5.2	63.6±0.5
Plasma	165.3±2.1	4.6±0.2	122.7±3.9	91.7±0.2

Concentration ratios

$Na_{plasma}/Na_{cells} = 1.02 \pm 0.02$

$K_{plasma}/K_{cells} = 0.59 \pm 0.04$

$Cl_{cells}/Cl_{plasma} = 0.65 \pm 0.04$

Heparinized venous blood (pH 7.32–7.35) was centrifuged under oil within 10 min of collection. Specific gravity of plasma was 1.014 and of cells was 1.104. Mean ± SD for five samples from four dogs.

TABLE III

Na OUTFLUX FROM DOG AND HUMAN RED CELLS

Cells	Medium	Medium Na	n	^{24}Na released in 1 h	
				Control	Δ Ouabain
		meq/liter		%	
Dog	Synth.	225	6	78.4±8.1	+0.2±2.0
		130–150	10	12.3±9.7	−0.5±0.7
		75	6	9.3±0.7	+0.4±0.9
	Plasma	152	6	16.7±6.1	−0.7±2.1
Human	Synth.	135	6	35.9±2.4	−29.0±1.9

All synthetic media (Synth.) contained KCl 5 mM, glycylglycine 10 mM, glucose 10 mM (pH 7.5). Plasma was gassed with 95% O_2-5% CO_2. Ouabain was added in a concentration of 0.1 mM. The ouabain effect is expressed as the average of the differences between control and ouabain-containing media. A negative sign indicates ouabain inhibition. Mean ± SD.

series of buffered media with varying NaCl concentrations. In all cases the external KCl concentration is 5 mM. Na efflux is also shown for dog red cells incubated in their own plasma. Values for human red cells subjected to the same procedure and incubated with the 135 mM NaCl buffer are also shown. The reason for expressing the results in terms of isotope released is that the kinetics of Na movements in dog red cells are so complex that a single value for milliequivalents per liter cells per unit time is not meaningful (9).

It is evident that as the dog red cells are placed in progressively dilute solutions of NaCl their Na efflux falls, in accordance with the volume effect noted effect noted above. The difference in efflux with ouabain present is negligible in dog red cells under all circumstances. In human red cells ouabain causes an 80% drop in the rate of isotope release. In separate experiments scillaren and digoxin were also shown to be without effect on dog red cells.

Membrane ATPase

ATP hydrolysis was measured in the presence of ghosts prepared from dog and human red cells. Preliminary experiments showed that magnesium concentrations of 2.5–5 mM gave maximum rates of phosphate release in the assay system used. The lack of effect of Na, K, and ouabain on dog red cell ghosts is shown in Table IV. Values for ghosts prepared from human red cells are included for comparison.

Loading of Cells with Na and Water

Table V shows the results of an experiment designed to raise the Na and water content of dog red cells. The procedure takes advantage of the high permeability of these cells to Na when they are dehydrated, as by incubation in a hypertonic medium. Freshly drawn cells were divided into two portions, one of

TABLE IV

ATPase ACTIVITY OF RED CELL GHOSTS

		Na			
		0	0	100	100
Cations added to assay media, *meq/liter*		K			
		0	5	0	5
Dog	Control	38.0±6.1	39.3±5.5	31.7±5.8	36.3±2.5
	Δ Ouabain	+1.0±5.8	−0.3±5.8	−2.0±3.0	−1.3±2.9
Human	Control	65.2*	—	—	157.7±17.6
	Δ Ouabain	—	—	—	−84.0±13.0

ATPase activity is expressed in nanomoles of inorganic phosphate released per hour per mg dry ghost weight. All assay media contained (millimoles/liter) Tris, 10; ATP (Tris salt), 2; EDTA, 0.25; $MgCl_2$, 5.0 (pH 7.5, 37°C). The ouabain effect is expressed as in Table II. Mean ± SD for four studies except for * mean of two studies.

which was promptly washed four times in isotonic NaCl buffer (see Methods) in preparation for the measurement of cell Na, K, and water. The second portion was washed four times in hypertonic buffer (NaCl 225 mM, KCl 5 mM, glycylglycine 10 mM, glucose 10 mM, pH 7.5) and incubated in 10 vol of this solution for 3 h at 37°C. The incubated cells were then washed five times with the isotonic NaCl buffer before the determination of their ion and water content. Table V shows that the incubation resulted in a net gain of about 40 meq Na and 400 g water per kg dry cell weight.

TABLE V

LOADING OF DOG RED CELLS WITH SALT AND WATER

	Cell contents		
	Na	K	Water
	meq/kg dry cell wt		*g/kg dry cell wt*
Fresh cells	328.3±2.1	15.0±1.0	2002±46
Incubated cells	370.3±4.5	14.0±2.0	2403±17

Mean ± SD for four studies. See text for details of incubation.

In Vivo Volume Regulation

The ability of Na- and water-loaded cells to correct their volume in vivo was assessed by making use of the fact that they have a relatively low density. By labeling the cells with ^{51}Cr and then reinjecting them into the dog their survival in the circulation could be followed, and their density in relation to the remainder of the dog's cells could be monitored.

The cells from 50 ml of fresh dog blood were treated by hypertonic incubation as noted above, so as to raise their Na and water content. $Na_2{}^{51}CrO_4$ 0.2 mCi (1.8 μg) was added to the hypertonic medium. After 3 h at 37°C the cells were washed with isotope-free isotonic NaCl buffer and reinfused into the antecubital vein of the dog, who received no medication and was fully ambulatory. Immediately before the injection a small portion of the treated cells was mixed with 20 vol of fresh, unlabeled dog blood as a "preinjection sample."

At intervals after infusion of the labeled cells 15 ml of blood was withdrawn from the dog and processed immediately. Each blood sample was distributed among 11 tubes containing phthalate solutions 1 through 11 (Table I). After mixing and centrifugation the red cells layer out above or below the immiscible phthalate oil according to their density. In Figs. 1–4 the points along the abscissa correspond to the numbers of the phthalate mixtures presented in Table I. The least dense mixture is on the left. The ordinate shows the percentage of hemoglobin and radioactivity in each blood sample which floats on top of the phthalate. The figures immediately under the graphs show the whole blood radioactivity for each time point. The experiments presented in Figs. 1

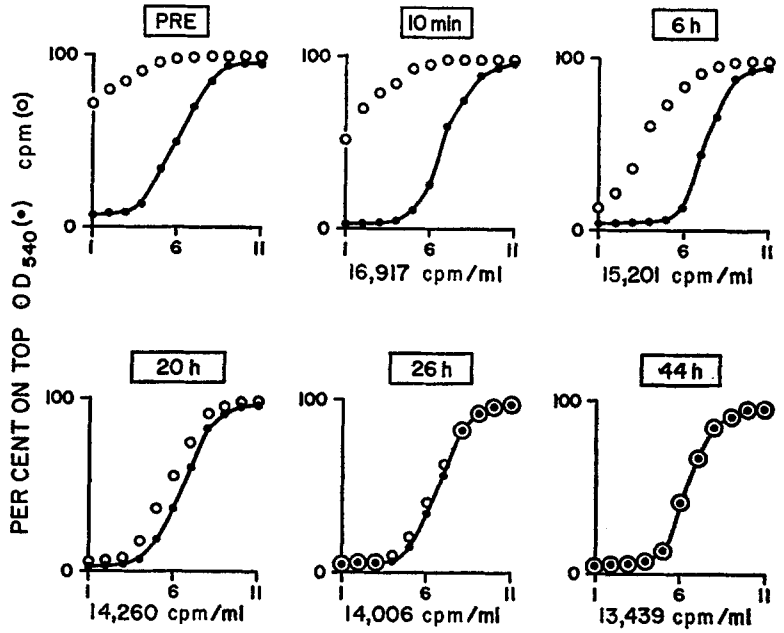

FIGURE 2. Same experiment as Fig. 1, except that ouabain (0.1 mM) was added during the hypertonic preincubation of the labeled cells.

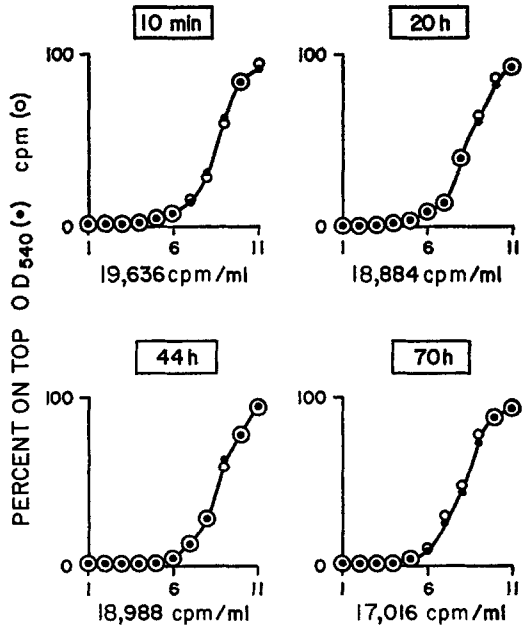

FIGURE 3. Survival and density distribution of unswollen cells. Chromium labeling was accomplished at 37°C in plasma for 30 min. The cells were then washed in isotonic medium and reinjected. Density gradient procedures and graphical conventions as in Fig. 1.

and 2 were carried out in almost identical fashion except that ouabain (0.1 mM) was included in the hypertonic preincubation phase of the latter study.

It is evident that 80–85% of the injected isotope remained in the circulation over a period of 40 h. In the preinjection sample and at the early time points the labeled cells were distinctly lighter than the bulk of the cells in the sample. Later on, however, the density of the labeled cells increased, until at 40 h the curves representing radioactivity and hemoglobin became superimposable. Preincubation with ouabain did not prevent the labeled cells from increasing their density.

Fig. 3 presents the results of injecting chromium-labeled cells which had not been preloaded with salt and water. It is clear that the density of the infused cells remain normal for at least 70 h thus ruling out the possibility that the that the labeling procedure itself predisposes the cells to shrink in vivo.

Fig. 4 shows the result of injecting swollen, labeled cells not into the dog, but into a flask of the dog's blood in vitro. Both labeled and unlabeled cells swell progressively in parallel with each other. No tendency of the preincubated cells to adjust their volume is noted in these circumstances.

One possible explanation for the results of Figs. 1 and 2 would be that somehow the ^{51}Cr was eluted from the injected cells and redistributed

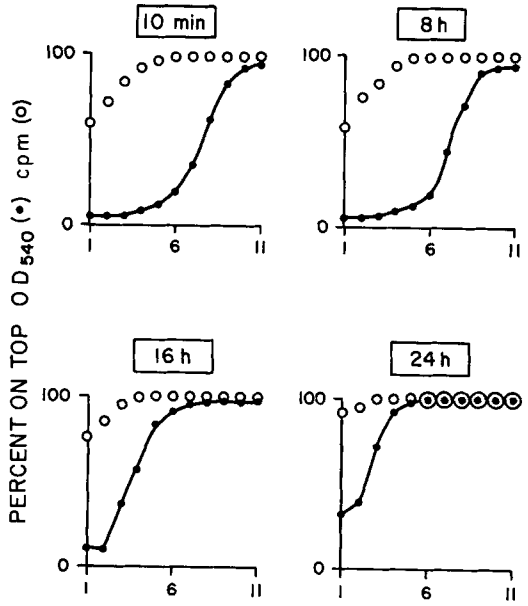

FIGURE 4. In vitro study. 10 ml of cells were loaded with Na and labeled with ^{51}Cr just as for the experiments in Figs. 1 and 2. After isotonic wash the labeled cells were injected into a flask containing 500 ml of freshly drawn blood from the same dog. Additions to the blood included heparin 5000 U, glucose 0.9 g, and penicillin 25 mg. The cell suspension was gassed with 95% O_2, 5% CO_2 after each sample was taken. Radioactivity, 18500 cpm/ml suspension.

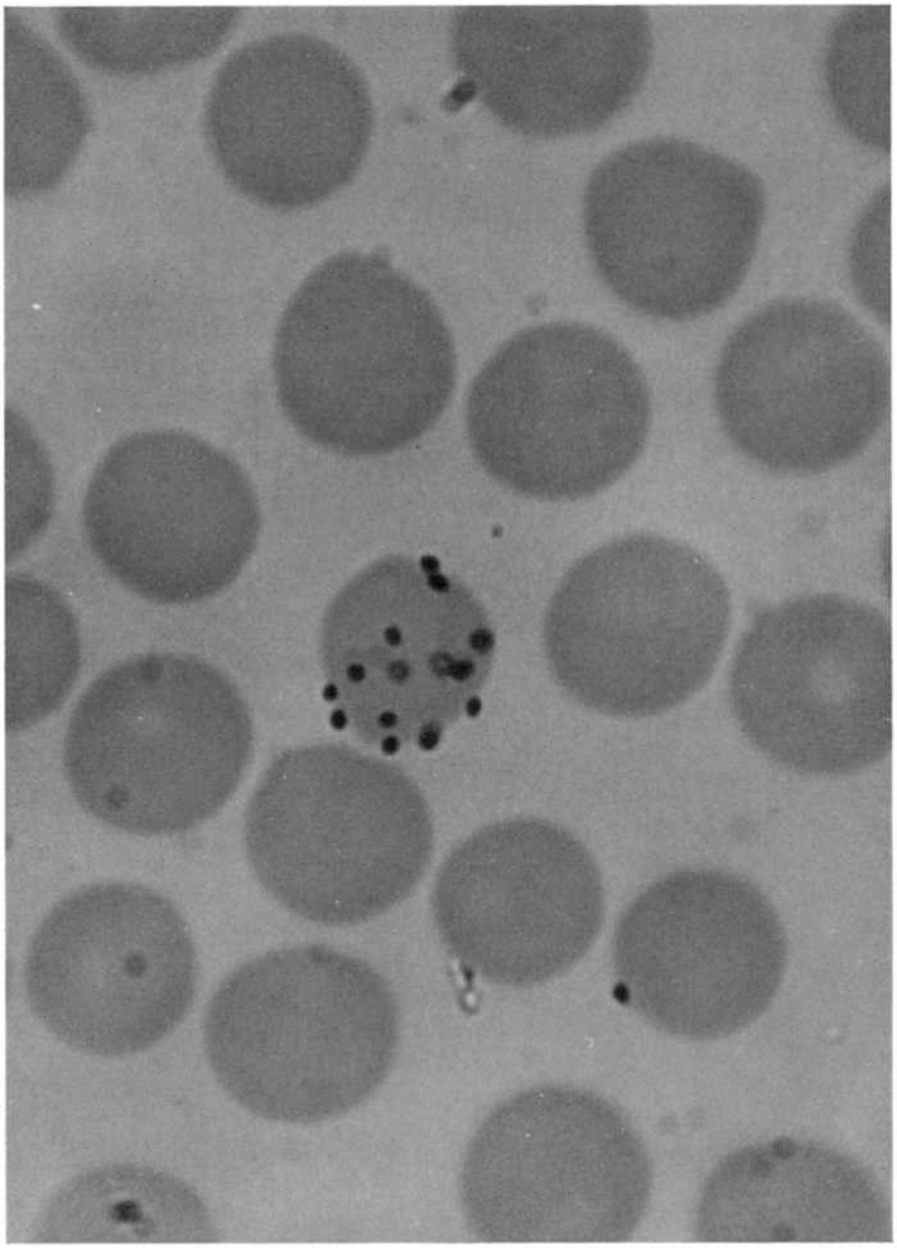

FIGURE 5. Autoradiograph of a blood smear obtained 48 h after injection of ^{51}Cr-labeled cells.

homogeneously among all the cells in the circulation. Evidence against this possibility was obtained by autoradiography of blood smears made 48 h after reinjection of the labeled, swollen cells (Fig. 5). This showed that the isotope was present in only a small fraction of the cells, which were incidentally noted to be quite normal in size and shape. The distribution of the photographic grains is shown in Fig. 6. The great bulk of the cells contained 0–1 grains (background), while only a few cells had a large number of grains.

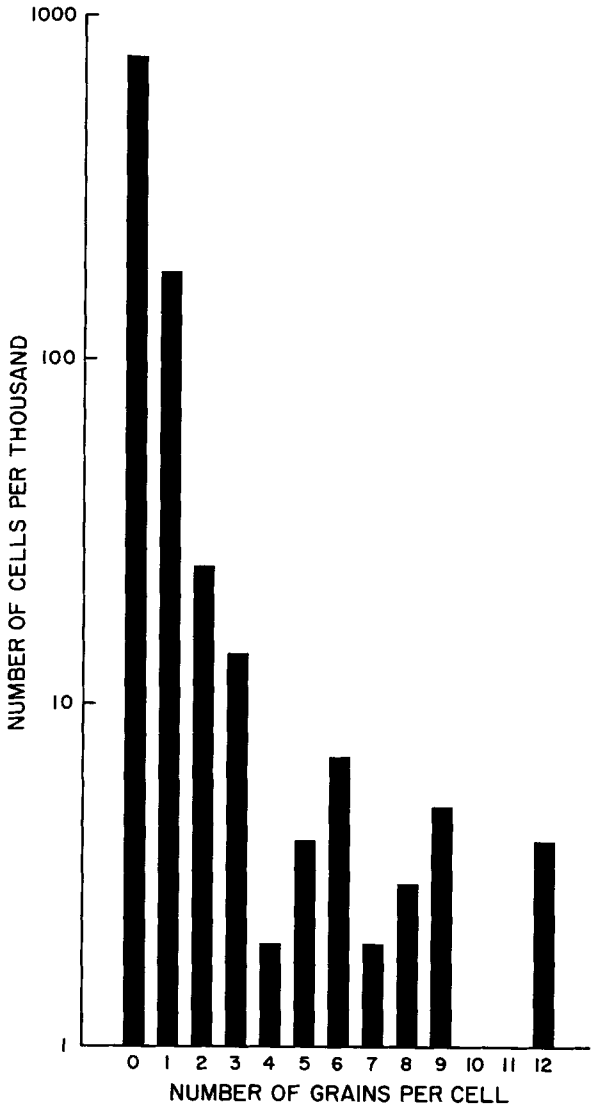

FIGURE 6. Histogram showing the number of cells (ordinate, log scale) containing the number of grains shown on the abscissa. Total cells counted, 1000.

DISCUSSION

The data in Tables III and IV confirm previous reports that mature dog red cells lack both a ouabain-sensitive Na efflux pathway and an Na, K-activated membrane ATPase (2, 6, 11, 12). In this respect dog red cells differ from the red cells of other species which depend on the Na-K pump for volume homeostasis (23). Lee and Miles (11) have recently demonstrated that ouabain will slow the Na efflux from a small, rapidly exchanging compartment of newborn puppy red cells. They find no such effect in adult dog red cells—an observation which we have confirmed in more detailed kinetic studies than are presented here.

The ionic distribution ratios across the membrane of adult dog red cells (Table II) (19) are nevertheless suggestive that active Na and K transport may be taking place. This possibility is supported by the results shown in Figs. 1 and 2: swollen cells are able to correct their density in vivo, presumably by extruding the Na and water with which they were loaded in the preinjection incubation. Quite clearly there are features of the dog's circulation which enable the cell to adjust its density to normal—features which are missing in the in vitro circumstances of Fig. 4.

These experiments raise the possibility that in dog red blood cells there are physiologic mechanisms of volume control which are not dependent on the Na-K pump. Experiments with other cell types (7, 8, 21) are consistent with this view.

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