Heterogeneity in Dog Red Blood Cells: Sodium and Potassium Transport

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ABSTRACT   After incubation in isotonic KCl, dog red blood cells can be separated by centrifugation into subgroups which assume different cell volumes and possess different transport characteristics. Those red cells which swell in isotonic KCl exhibit a higher permeability to K and possess a greater volume dependence for transport of K than those red cells which shrink. A high Na permeability characterizes cells which shrink in isotonic KCl and these cells exhibit a larger volume-dependent Na flux than those red cells which swell. These two subgroups of red cells do not seem to represent two cell populations of different age. The results indicate that the population of normal cells is evidently heterogeneous in that the volume-dependent changes in Na and K permeability are distributed between different cell types rather than representing a single cell type which reciprocally changes its selectivity to Na and K.

INTRODUCTION

Dog red blood cells, unlike those of humans, are high in Na and low in K (Kerr, 1926). Although these cations are more nearly in Donnan equilibrium in dog red cells than in human, there remains a significant electrochemical driving force favoring the net influx of Na (Parker, 1973a). In human red cells, Na accumulation and the resultant cell swelling are prevented by the active extrusion of Na via a ouabain-sensitive Na:K pump which requires ATP as an energy source (see Tosteson and Hoffman, 1960). This mechanism does not operate in dog red cells since these cells possess no ouabain-sensitive cation movement (Hoffman, 1966) and no Na,K-ATPase has been found in their membranes (Chan et al., 1964).

Davson (1942) and Parker and Hoffman (1965, 1976) have shown that the cation permeability of the dog red blood cell membrane is volume-dependent, i.e., Na permeability increases in shrunken cells and decreases in swollen cells whereas the opposite is true for K permeability. This volume response depends on cell metabolism (Hoffman, 1966; Romualdez et al., 1972). Parker et al. (1973 b, 1975) have proposed a Na-Ca exchange system which is capable of extruding Na and thus returning swollen cells to their normal volume. They have combined this Na extrusion mechanism with the volume dependence of

cation permeability to form a model for volume control in dog red cells. But although a Ca-dependent mechanism for Na extrusion has been identified, it is not clearly ascribable to a Ca-Na exchange process (Parker, 1978). Thus, the mechanism of volume regulation in dog red cells is still obscure.

The reciprocal response of Na and K permeability of dog red cells to volume changes raises an intriguing problem, vis., all the cells may not respond in the same manner. Rather, some cells may exhibit volume dependence of Na permeability while another cell type may possess a volume-dependent K permeability.

Davson (1942) was the first to propose the existence of two distinct classes of dog red cells each possessing different transport characteristics. Supportive evidence for inhomogeneity in dog red cells has been presented by Lange et al. (1970).

The purpose of this investigation was to determine if dog red cells are heterogeneous, and if cells separated on a physical basis exhibit different transport kinetics with particular attention given to the volume dependence of cation permeability. A brief report of some of these results has been presented previously (Castranova and Hoffman, 1977).

METHODS

Cell Preparation

If dog red cells are heterogeneous in transport kinetics, then it might be possible to separate subgroups of cells by incubation in a medium containing a single cation species. When dog red cells are incubated in a Na free-KCl medium, those cells possessing a high K permeability should swell while those with a high Na permeability should shrink. If this is the case, the density difference between cell types can be exploited resulting in a separation of cells on the basis of their transport properties.

Blood from adult dogs was drawn into heparinized containers by venipuncture. The blood was centrifuged at 2°C and 12,000 g. The plasma and buffy coat were removed by suction. The red cells were washed three times by alternate resuspension to a 10% hematocrit and centrifugation at 2°C and 12,000 g in KCl pretreatment medium: (in millimolar) KCl, 160; NaHepes (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid), 5; glucose, 5; and adenosine, 2 (pH 7.4 at 25°C). The red cells were incubated at a 20% hematocrit in this medium for 1 h at 37°C in a shaker bath. After this time, the cell suspension was centrifuged at 2,750 g for 30 min at 2°C using a swinging bucket rotor (Sorvall HB-4, DuPont Instruments-Sorvall, Wilmington, Del.) and 100 × 15-mm centrifuge tubes. As described by Davson (1942) this procedure results in two layers of red cells distinguishable by color, i.e., a top layer of dark red cells and a lower layer of cells bright red in color. The two subgroups of dog red cells were separated. Each group was then washed once and incubated in a shaker bath at 37°C for 3 h at a 10% hematocrit in the appropriate flux medium (described below). This incubation time was used to return both cell subgroups to the same volume and ion content (Table II), and to load the cells with isotope.

Efflux Measurements

CATION EFFLUX   Cells were loaded with either ^{22}Na or ^{42}K by incubation at a 10% hematocrit for 3 h at 37°C in cation flux medium: (in millimolar) NaCl, 130; KCl, 5; NaHepes, 5; glucose, 5; adenosine, 2; and sucrose, 50 (pH 7.4 at 25°C). After incubation,

the cell suspension was centrifuged at 2°C and 12,000 g and the radioactive medium was removed by suction. The red cells were washed three times in ice-cold cation flux medium (2°C) as described above. Cells were added to cation flux media of differing osmolarity to obtain a 1% hematocrit. Osmolarity of cation flux medium was varied by alteration of the sucrose concentration between 0 and 100 mM. Samples of the supernate were then taken at various times (t) and the amount of radioactivity (P_t) measured using a scintillation counter. The amount of radioactivity in the medium at time infinity (P_∞) was estimated from samples of the whole suspension.

Lange et al. (1970) have shown that cation efflux in dog red cells is biphasic. It consists of a rapid initial phase lasting ~30 min and a second slow phase which represents some 95% of the total efflux. This major slow phase of cation efflux can be described by the equation for a simple two compartment efflux:

$$\ln (1 - P_t/P_\infty) = {}^{-o}\!kt.$$

Therefore, $\ln (1 - P_t/P_\infty)$ was plotted vs. time and the rate constant for the major phase of cation efflux (${}^o\!k$) was taken as the slope of the line which best fits the efflux data after the first half-hour (Fig. 1). All rate constants were corrected for differences in relative cell volume by multiplying the measured ${}^o\!k$ by the fraction of control volume.
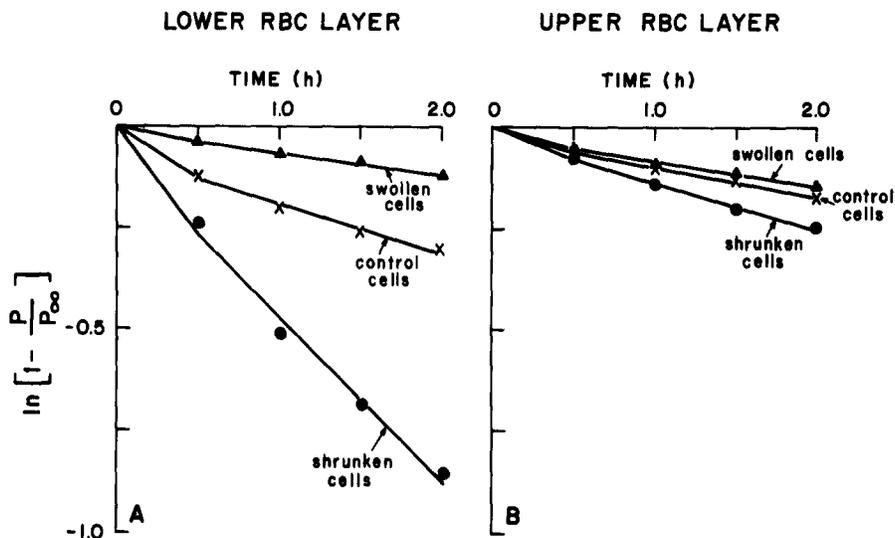

FIGURE 1. Time-course of Na efflux from the two subgroups of dog red cells as a function of cell volume. (A) Data from cells harvested from the lower layer after KCl pretreatment (Na permeable cells); (B) data from the upper red cell layer (K permeable cells). Na efflux was measured with dog red cells incubated at a 1% hematocrit at 37°C in medium of the desired osmolarity: (in millimolar) NaCl, 130; KCl, 5; NaHepes, 5; glucose, 5; adenosine, 2; sucrose, 0, 50, or 100 (pH = 7.4 at 25°C). See Methods for details.

SULFATE EFFLUX The rate constant for SO_4 efflux was determined using a modification of the method described by Gardos et al. (1969). For loading with ${}^{35}SO_4$, red cells were incubated at a 10% hematocrit and 37°C in sulfate flux medium:(in millimolar) NaCl, 126; KCl, 5; Na_2SO_4, 10; NaHepes, 5; glucose, 5; adenosine, 2; sucrose, 50 (pH 7.4

at 25°C). After loading, the cell suspension was centrifuged at 2°C and 12,000 g and the radioactive medium was removed. The red cells were washed three times at a 10% hematocrit in ice-cold sulfate flux medium (2°C) and then added to this medium at a 0.5% hematocrit to initiate the efflux which was run at 37°C. Samples were taken as described above for cation efflux.

Sulfate efflux was found to behave as a simple two-compartment system. Therefore, the plot of ln $(1 - P_t/P_\infty)$ vs. time is linear and the slope of this line is the rate constant for SO_4 efflux under equilibrium self-exchange conditions.

Cell Volume Measurement

Samples from various cell suspensions were drawn into microcapillary tubes, spun for 10 min using a micro-hematocrit centrifuge, and the hematocrit measured. Samples from the same cell suspensions were used for cell counting with a Coulter Counter (model Z_{BI}, Coulter Electronics Inc., Hialeah, Fla.). Mean cell volume (MCV) was calculated by dividing the hematocrit by the cell count.

Measurement of Cell Contents

SODIUM AND POTASSIUM CONCENTRATIONS   Cell suspensions were centrifuged at 2°C and 12,000 g and the supernate was removed. The red cells were washed three times at a 10% hematocrit in an ice-cold medium (2°C) of the following composition: (in millimolar) $MgCl_2$, 110 and LiCl, 15. Cells were then resuspended in this medium to a 10% hematocrit and the suspension was centrifuged using a Sorvall HB-4 rotor at 2°C and 27,000 g for 10 min in special lucite tubes with a narrow well at the bottom for collection of cell pellets. The supernate was removed and an aliquot of packed cells was added to a known volume of medium. Intracellular Na and K content were measured by flame photometry using lithium as an internal standard. Cell water content was obtained using a second aliquot of packed cells weighed before and after drying at 80°C for 24 h. Intracellular Na and K concentration were determined by dividing the cation content of a given sample of cells by the cell water content of that sample.

CHLORIDE DISTRIBUTION   The procedure used was similar to that described by Hoffman and Laris (1974). Cells were incubated at a 10% hematocrit in an appropriate medium containing [36]Cl for 1 h at 37°C. Cell suspensions were then transferred into the lucite tubes and centrifuged using a Sorvall HB-4 rotor at 27,000 g for 10 min and the radioactivity of samples of supernate and packed cells was measured. The water content of a second aliquot of packed cells was measured as described above. The chloride ratio (r_{Cl}) was calculated as:

$$r_{Cl} = \frac{\text{(counts per minute in cell sample)}/\text{(cell water content)}}{\text{(counts per minute in supernate sample)}}.$$

The membrane potential (E_m) was calculated as follows:

$$E_m = 61 \log_{10} (r_{Cl}).$$

RESULTS

Davson (1942) has reported that dog red blood cells can be separated into two distinct groups by centrifugation of cells which have been incubated in isotonic KCl. We have repeated this separation (Table I). Such a procedure results in two layers of packed red cells distinguishable by a sharp change in color. The upper layer of less dense cells is dark in color. This layer comprises about 60%

of the total packed cell volume as estimated visually from the packed cell column in 100 × 15-mm centrifuge tubes. Volume measurements indicate that these cells are swollen compared to normal. The more dense lower layer comprises the remaining 40% of the packed cell volume. These cells appear bright red and are shrunken compared to normal dog red cells (Table I).

Davson (1942) and Parker and Hoffman (1965, 1976) have shown that cation permeability of dog red blood cells is dependent on cell volume. For this reason, before studies of cation transport are made, it is essential to demonstrate that dog red cells harvested from the two layers after KCl treatment can be returned to the same cell volume. Table II shows that after incubation in a high Na-low K medium cells from the two subgroups have returned to approximately the same

TABLE I

SEPARATION OF DOG RED CELLS AFTER KCl TREATMENT

Cell layer	Appearance	MCV
		μm^3
Upper	Dark	80.1±1.6
Lower	Bright	63.8±2.4

Mean cell volumes (MCV) are means of five experiments ± SEM. Normal cell volume is ~72 μm^3.

TABLE II

VOLUME AND ION CONTENT OF SUBGROUPS OF DOG RED CELLS AFTER INCUBATION IN A HIGH Na-LOW K MEDIUM

Subgroup cell type	MCV	Na_i	K_i	E_m
	μm^3	*meq/liter cell* H_2O		mV
Upper	77.8	105.6	33.4	−4.5
	(77–78.7)	(104.9–106.4)	(33.2–33.7)	
Lower	79.8	105.0	29.4	−2.2
	(78.9–81)	(104.0–106.0)	(28.4–30.3)	

Pretreated cells separated by centrifugation into upper and lower subgroups. The experimental values given are means of two experiments with the range given in parentheses. The sign of membrane potential, E_m, refers to inside negative with respect to outside of the cell. Approximate normal values are as follows: mean cell volume (MCV) is 72 μm^3, Na_i and K_i are 144 and 8 meq/liter cell H_2O, respectively, and E_m is −8 mV.

volume. These data also indicate that the intracellular concentrations of Na, K, and Cl are about the same in both cell subgroups. It should be mentioned that both cell types possess an internal Na concentration which is lower than normal and an internal K concentration above normal.

Once the red cells in each subgroup have been returned to the same volume and ionic content, measurements of various transport properties can be made. Table III lists the rate constants for efflux of Na, K, and SO_4. Cells obtained from the upper layer (K-permeable cells) exhibit a higher rate constant for K efflux (^0k_K) than those from the lower layer. The cells with the high ^0k_K are those which swell in isotonic KCl. The cells harvested from the lower layer (Na-

permeable cells) are those which shrink in isotonic KCl. These red cells exhibit the higher rate constants for Na efflux ($\%k_{Na}$) and sulfate efflux ($\%k_{SO_4}$).

Fig. 1 shows the volume dependence of Na efflux in the two subgroups of dog red blood cells. Control cells are those incubated in isotonic medium. This figure presents results which demonstrate that the rate constant for Na efflux from control red cells harvested from the lower layer (Na-permeable cells) is greater than that of the upper layer (K-permeable cells). These data also

TABLE III

TRANSPORT CHARACTERISTICS IN TWO SUBGROUPS OF DOG RED
BLOOD CELLS

Cell types	$\%k_{Na}$	$\%k_K$	$\%k_{SO_4}$
		h^{-1}	
Upper	0.075	0.068	1.61
	(0.070–0.080)	(0.065–0.070)	(1.52–1.70)
Lower	0.140	0.040	1.95
	(0.120–0.160)	(0.037–0.042)	(1.88–2.02)

All flux measurements were made at 37°C. Values are means of two determinations with the range given in parentheses.

TABLE IV

VOLUME DEPENDENCE OF SODIUM EFFLUX IN TWO SUBGROUPS OF DOG
RED BLOOD CELLS

Subgroup cell type	MCV	$\%k_{Na}$	Fraction of control $\%k_{Na}$
	μm^3	h^{-1}	
	84.6	0.073	0.97
	(84.4–84.8)	(0.067–0.078)	
Upper	75.7	0.075	1.00
	(75.5–76.0)	(0.070–0.080)	
	69.6	0.106	1.41
	(69.4–69.8)	(0.101–0.110)	
	83.7	0.065	0.46
	(83.0–84.5)	(0.064–0.066)	
Lower	77.7	0.140	1.00
	(77.4–77.9)	(0.120–0.160)	
	67.4	0.344	2.46
	(67.1–67.6)	(0.331–0.357)	

Values are means of two determinations with the range given in parentheses. MCV, mean cell volume. $\%k_{Na}$ is the outward rate constant for Na in reciprocal hours.

indicate that red cells taken from the lower layer (Na-permeable cells) exhibit far greater volume dependence for sodium transport, i.e., the increase in $\%k_{Na}$ of shrunken cells and the decrease in $\%k_{Na}$ with cell swelling is more apparent in the lower subgroup of cells (Na-permeable cells). Table IV summarizes these and other data on volume-dependent Na efflux in the two cell types. Note that even though cell volume is varied over the same range in both cell types, the

volume dependence of Na transport seems to be mainly a property of red cells harvested from the lower layer (Na-permeable cells).

Table V lists data on the volume dependence of K efflux in the two red cell subgroups. The upper layer of red cells (K-permeable cells) exhibits the greater volume dependence of K efflux, i.e., $\%k_K$ increases with cell swelling to a far greater extent in these cells compared with cells from the lower layer (Na-permeable cells). These cells also have the higher control rate constant for K efflux.

Studies concerning the distribution of ^{59}Fe-tagged red cells after centrifugation indicate that human and rabbit red cells increase their density with age (Borun et al., 1957; Hoffman, 1958). This characteristic seems to hold for dog red cells as well, in that Lee and Miles (1972) have shown in puppies that

TABLE V

VOLUME DEPENDENCE OF POTASSIUM EFFLUX IN TWO SUBGROUPS OF
DOG RED BLOOD CELLS

Subgroup cell type	Fraction of control volume	$\%k_K$	Fraction of control $\%k_K$
		h^{-1}	
Upper layer	1.00	0.068	1.00
		(0.065–0.070)	
	1.11	0.180	2.65
		(0.173–0.185)	
Lower layer	1.00	0.039	1.00
		(0.038–0.039)	
	1.11	0.044	1.14
		(0.041–0.047)	

Fraction of control volume calculated from the Ponder equation:

$$\text{Relative volume} = 1 + 0.72 \left(\frac{\text{control osmolarity}}{\text{test osmolarity}} - 1 \right).$$

(See Ponder, 1948.) $\%k_K$ refers to the outward rate constant for K in units of reciprocal hours. Experimental values are means of two determinations with the range given in parentheses.

neonatal cells which are of the high K-low Na type become more dense with age. The question arises whether the two cell layers harvested after incubation in isotonic KCl represent a separation of dog red cells by age. To test this possibility, normal dog red cells which had not been treated in isotonic KCl were suspended in an isotonic high Na-low K medium and then centrifuged and separated into layers in the same manner as KCl treated cells. Sodium efflux was then measured using red cells from the various layers. Table VI lists $\%k_{Na}$ from dog red cells separated in this fashion. Note that $\%k_{NA}$ for cells taken from the upper layer (young cells) is about the same as $\%k_{Na}$ for the lower cells (old cells). The volume dependence of Na efflux is also very similar in the upper and lower layer of dog red cells separated in this manner (data not shown).

Therefore, the differences in transport characteristics of the two subgroups of dog red blood cells reported in this investigation do not seem to be attributable to differences in red cell age of the two cell layers.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis studies were performed using solubilized membranes prepared from the two red cell layers obtained after incubation in isotonic KCl. Gel scans indicate that there are no discernable differences obtained in the positions or relative magnitudes of the various Coomassie Blue-staining protein bands when the membrane preparations from the two cell subgroups are compared with each other or with the normal parent cell population (data not shown).

TABLE VI

SODIUM EFFLUX AS A FUNCTION OF CELL AGE IN DOG
RED BLOOD CELLS

Cell layer	Cell age	$^k{}_{Na}$
		h^{-1}
Upper	Young	0.067
Middle	—	0.069
Lower	Old	0.064

DISCUSSION

Evidence from the present study suggests that dog red cells are heterogeneous in their transport properties. This heterogeneity becomes apparent after incubation of dog erythrocytes in isotonic KCl, i.e., centrifugation of KCl-treated cells results in two distinct layers of cells. Our data support the hypothesis proposed by Davson (1942) that the top layer of swollen red cells do exhibit a high K permeability and a low Na permeability whereas the lower cells shrink in KCl as a result of their low K permeability and high Na permeability. These results are in contrast to those of Lange et al. (1970) which suggest that Na permeability is highest in red cells obtained from the upper layer after KCl treatment. The discrepancy between these data might result from differences in the methods used in treating the cells as well as in the kinetic characterization of the Na permeability in the two studies.

It should be kept in mind that each population of cells separated into layers by density differences after incubation in KCl still displays heterogeneity in the unidirectional movements of both Na and K, e.g., Fig. 1. This is so regardless of the medium tonicity (and hence cell volume) on which the determinations are made. This kinetic heterogeneity, while second order compared to the differences in subgroup permeabilities, probably means that there are more than two types of subgroups of cells, but the possibility that there may be more than one compartment of Na and K present in each cell cannot be excluded.

Data from this investigation also suggest that the two subgroups of dog red cells do not exhibit volume-dependent cation transport to the same degree. K permeability is more sensitive to volume in cells harvested from the upper layer, i.e., those cells which swell in isotonic KCl and exhibit a high rate for K movement. The volume dependence of Na movement is mainly a characteristic

of cells from the lower layer, i.e., cells in which $^o\!k_{Na}$ is high. Thus, it is evident that the KCl incubation procedure provides a means for dividing the population of dog red cells into at least two different subgroups. How unique is this method of separating cells? Would the same types of subgroups emerge if the procedure were varied such that instead of incubating cells in isotonic KCl the tonicity of the KCl medium was changed? From the evidence referred to before (Parker and Hoffman, 1965, 1976 and Hoffman, 1966), cells increase their permeability to Na only when shrunken and increase their permeability to K only when swollen. Net changes in total cellular Na and K content can be brought about by preincubating cells in media of varying tonicity and Na/K compositions. Thus, different sets of cells can be prepared such that they assume different volumes when suspended in the same isotonic solution; in this way it was shown that swollen cells had increased K and that shrunken cells had increased Na permeability (Hoffman, 1966), indicating that the volume response was independent of the medium tonicity, per se. This result implies that, unless the properties of the cells were unique, the Na and K permeability measured on upper and lower groups of harvested cells suspended in the same isotonic medium would be expected to be the same. The fact that they are different (Table III) supports the idea that the subgroup populations are discrete entities as developed in this paper. What can be expected of the properties of the two subgroups if the cells are preincubated in anisotonic KCl media? For cells preincubated in anisotonic KCl media it would be anticipated that although the proportions of the two subgroups would not be much affected, the rate at which they change their density would depend upon the relative permeabilities of the two subgroups to Na and K. For instance, if the KCl preincubation were hypotonic (say, 130 mM KCl, compared to 160 mM KCl for isotonic media) the "upper" cell types would swell faster than the "lower" cell types would shrink, because the "upper" cell types are more permeable to K than Na (the reverse is characteristic of the "lower" cell types), and the hypotonicity would favor the "upper" subgroup by increasing their permeability to K and decreasing their permeability to Na. The "lower" cell types would shrink at a slower rate compared to their rate in isotonic media because hypotonicity has worked to decrease their Na permeability even through they are more permeable to Na than K. When this type of experiment was carried out, not only did the proportion of the two sub-group populations separated after incubation in 130 mM KCl remain approximately the same, but the same relative permeabilities were observed as when the two subgroups were separated after incubation in 160 mM KCl. (Unfortunately, cells incubated in hypertonic KCl medium (190 mM) became sticky upon centrifugation and are therefore intractable with regard to subsequent flux studies.) Thus, using an analogous protocol for the hypotonic (130 mM KCl) separated subgroups as for the isotonic medium (160 mM KCl) separated subgroups described before, the $^o\!k_{Na}$ of "upper" cells (cell volume, 73.3 μm^3) in hypotonic flux medium was 0.09 h^{-1} compared to 0.24 h^{-1} for "lower" cells (cell volume, 71.6 μm^3). As part of the same experiment, the $^o\!k_{Na}$ of cells separated in isotonic KCl (160 mM) was 0.15 h^{-1} for "upper" cells (cell volume, 65.1 μm^3) and 0.22 h^{-1} for "lower" cells (cell volume, 68.7 μm^3). When the change in permeability that accompanies change in cell volume is

taken into account (compare Table IV), it is apparent that the properties of the two subgroups are not appreciably modified by the tonicity of the medium used to separate them.

Thus, it can be concluded that the type of subgroup heterogeneity in the response of membrane permeability to a volume change as described in this paper should be considered when proposals are made regarding mechanisms for volume regulation in dog red blood cells. It would appear that the situation is somewhat simplified in the sense that the changes in permeability associated with changes in cell volume do not require reciprocal changes in a cell's permeability to Na and K. Even so, the basis for the volume response as well as the differential selectivity towards Na and K of cells in the two subgroups is still obscure.

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