

Cytodifferentiation and Membrane Transport Properties in LK Sheep Red Cells

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ABSTRACT Young cells produced in LK sheep during rapid hematopoiesis after massive hemorrhage contain more K than the cells which are normally released into the circulation. The K content in these new cells falls to that characteristic of mature LK cells after a few days in the circulation. K transport properties in young and old cells before and after massive bleeding were studied. Young and old cells were separated by means of a density gradient centrifugation technique. Evidence showing that younger cells are found in the lower density fractions is presented. Active transport of K in the lightest fraction as measured by strophanthidin-sensitive influx was four to five times greater in red cells drawn 6 days after massive bleeding while the K leak as measured by strophanthidin-insensitive influx was only slightly larger. No change after bleeding was observed in older cells which had been present in the circulation prior to the hemorrhage. It is concluded that the high K content of young cells produced in LK sheep after bleeding is due to temporary retention of membrane K transport properties characteristic of HK cells. Thus, genetically determined modification of membrane transport properties has been shown to occur in nondividing circulating red cells.

INTRODUCTION

The red blood cells of two genetic types of sheep differ widely in their cation composition (Evans, 1954). One type has a high concentration of K and a low concentration of Na (HK) while the other type has a low concentration of K and a high concentration of Na (LK). One important difference between the cation transport systems in these two cell types is that the rate of active transport of K and Na is about four times higher in HK cells than in LK cells (Tosteson and Hoffman, 1960). Recently Blunt and Evans (1963, 1965) reported the observation that when LK sheep were subjected to massive hemorrhage, the young cells entering the circulation during the subsequent phase of increased hematopoiesis contained substantially more K than the cells which are normally released into the circulation. This paper reports an investigation of several possible explanations for the increased K content of these genetically LK cells. For example, the young cells may possess mem-

branes with the K-Na transport properties of mature HK cells. On the other hand, the new cells may have membranes with the K-Na transport properties characteristic of mature LK cells but temporarily retain K acquired from a high K parent cell. In the latter case, the young cells may not have enough time to lose K and accumulate Na to assume a K-Na composition characteristic of mature LK cells. In order to distinguish between these possibilities, K influx was measured in young and old cells of LK sheep before and after massive bleeding. Young and old cells were separated by density since it has been shown by many authors that erythrocytes become denser during their first few weeks in the circulation (Borun, Figueroa, and Perry, 1957; Pranker, 1958; Rigas and Koler, 1961; Garby and Hjelm, 1963; Leif and Vinograd, 1964).

METHODS

Experimental Procedure

Density distribution of erythrocytes was determined on three adult HK sheep and three adult LK sheep. Two male adult LK sheep were bled to promote hematopoiesis and obtain high K young cells. Since results from both of these experiments were similar, data from only one are presented in this paper. About 2.5 liters of blood were removed from each of these sheep by two consecutive venipunctures over a 2 day period. Immediately following the second venipuncture 1.0 mc ^{59}Fe in the form of ferrous citrate was injected intravenously in order to tag the new cells. To supply enough iron for hemoglobin synthesis and to minimize the reutilization of ^{59}Fe in the event of random destruction of the red cell population, nonradioactive iron dextran was given daily beginning on the 4th day after ^{59}Fe injection (150 mg/day). K influx was measured in young and old cells before and 6 days after bleeding.

Young and old cells were separated by means of a density gradient centrifugation technique modified from the one described by Leif and Vinograd (1964). A 10 ml linear density gradient was formed with bovine serum albumin (BSA) solutions of specific gravities 1.107 and 1.085 (4°C). The denser solution was prepared by dissolving 100 g of BSA (fraction V) in 200 g of water containing 50 g of Amberlite MB-3 resin. 1.86 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ was added to every 100 g of the deionized BSA. The pH of the BSA was adjusted to pH 7 with a few drops of 3 N Tris base. Specific gravity of the final dense BSA was found to be 1.107 ± 0.001 . The light BSA was made by diluting the dense preparation with a solution containing MgCl_2 (0.108 M) and Tris Cl (0.017 M, pH 7.0). The final osmolality of both the dense and light BSA was 310 milliosmols per liter as measured by freezing point depression using NaCl standards.

Density gradient separations were performed as follows. Red cells obtained from fresh venous blood drawn into heparin were washed three times in MgCl_2 (0.12 M) with careful removal of the buffy coat after each washing. Washed packed cells were blended into the dense BSA used to form the density gradient column (0.5 ml packed cells in 4.5 ml BSA). Centrifugation was performed in a Sorvall HS swinging bucket

rotor at $20,000 \times g$ for 1 hr. It was found that this procedure was sufficient to bring the cells to density equilibrium in the gradient. Twelve fractions were collected sequentially by inserting a cut 18 gauge needle into the bottom of the tube. Specific gravities of the fractions were determined by weighing the volume of water and of each fraction contained in the same 500 μ l Carlsberg pipette. Since centrifugation was performed at 4°C and fractions were kept at 4°C before and during weighing, specific gravities are given as those at 4°C . Cell concentration in each fraction was determined with a Coulter Counter. K and Na were analyzed by flame photometry and hemoglobin was measured spectrophotometrically at 540 $m\mu$. ^{59}Fe radioactivity in each fraction was determined with an automatic well-type crystal scintillation counter (Packard).

Measurement of K Influx

K influx was measured in cells of six samples obtained from the density gradient separation. These samples were formed by pooling adjacent density fractions (e.g., 1 and 2, 3 and 4, 5 and 6, etc.). Each sample contained the number of cells of a given density obtained from 1.5 ml packed sheep red cells. The cells in each of these pooled samples were washed three times with incubation medium of the following composition [Na (165 mM), K (5.0 mM), Cl (150 mM), HPO_4^- (9.35 mM), H_2PO_4^- (1.65 mM), pH = 7.4), glucose (200 mg %), and chloromycetin (1 mg %)] to remove the serum albumin. These washed cells were then suspended in 20 ml incubation medium. One ml of this suspension was used for hemoglobin analysis and 0.1 ml for cell counting. The remainder of the suspension was divided into two equal portions. Strophanthidin dissolved in ethanol was added to one of these portions to give a final concentration of 10^{-4} M while an equal volume of ethanol was added to the other. After 15 min of incubation at 37°C to allow temperature equilibration, ^{42}K was added. Aliquots (4 ml) were removed from the incubating suspension 30 min and 2 hr 30 min after tracer addition. These were centrifuged and a sample of supernatant was removed for analysis. The packed cells were washed four times with ice cold MgCl_2 (0.12 M) and counted for ^{42}K radioactivity. After counting, the contents in the counting tubes were quantitatively transferred to volumetric flasks for analysis of K and hemoglobin. When ^{59}Fe was present in the blood, its concentration was estimated by recounting the cells at least 1 wk later when the ^{42}K had decayed. When both isotopes were present, ^{42}K activity was calculated from the difference between initial and final counts after having corrected the latter for ^{59}Fe decay. The K influx was calculated according to the following formula (Sheppard and Martin, 1950):

$${}^iM_{\text{K}} = \frac{(dX_c/dt) \cdot (\text{K})_c}{X_m - X_c}$$

where X_c is the specific activity of cells in counts/(min \times mmole).

X_m is the specific activity of the medium in counts/(min) \times (mmole).

$(\text{K})_c$ is the K content in cells in mmole/cell.

dX_c/dt is the change in cell specific activity/hr.

${}^iM_{\text{K}}$ is K influx in mmole/cell/hr.

Determination of ^{59}Fe Uptake

Blood samples were collected at intervals after ^{59}Fe injection. Measurements of ^{59}Fe radioactivity were made on 0.5 ml aliquots of packed cells. A ^{59}Fe standard was prepared at the beginning of the experiment and retained for the purpose of correcting decay of tracer activity in later samples.

RESULTS

Density Distribution of Normal HK and LK Sheep Red Cells

Fig. 1 A shows the density distribution of the red cell population in three HK sheep and three LK sheep. The distributions are approximately normal. Differences in peak height of the curves are mainly due to variations in the sensitivity of the separation with variations in the slope of the density gradient. There is no significant difference between HK and LK cells (Fig. 1 B) although slight variations are seen from one individual sheep to another. All distributions show their peak cell concentrations at a specific gravity of about 1.100. Evidence showing that the younger cells are found in the lower density fractions is presented in the following sections.

Density Distribution of LK Sheep Red Cells and ^{59}Fe after Massive Bleeding

The appearance of injected ^{59}Fe in the circulating red cell population of a LK sheep after massive bleeding is shown in Fig. 2. In 4 to 5 days the ^{59}Fe content of the red cells had already reached 80% of the maximal value. Uptake was completed by the 6th day after ^{59}Fe injection. Thereafter during the period of observation the curve shows a plateau. Fig. 3 shows the density distribution of ^{59}Fe at different times after injection of the tracer. On day 1, the small amount of ^{59}Fe which appeared in the circulating blood was located exclusively in the lightest fractions. In a few days, the ^{59}Fe was present in cells with a wider range of densities and the peak ^{59}Fe concentration moved steadily toward a denser region. This indicates that the youngest cells were indeed in the lightest fractions and that they became more dense during the first few days of life in the circulation. The slight spread of ^{59}Fe through the density gradient suggests that some of the young cells did not become denser at the same rate as did the majority of the young population. Indeed, about 3 months after bleeding the density distribution of ^{59}Fe approximates the density distribution of cells before bleeding (Figs. 1, 4 A, and 3).

The appearance of a new population of lighter cells in response to massive bleeding is shown in Fig. 4 A to D. The normal density distribution of the red cells of a LK sheep is shown in Fig. 4 A. Six days after bleeding the density distribution of the red cell population was relatively unchanged (Fig. 4 B). The ^{59}Fe activity was mainly located in the light fractions. The fact that the density distribution remained relatively unchanged suggests that the young

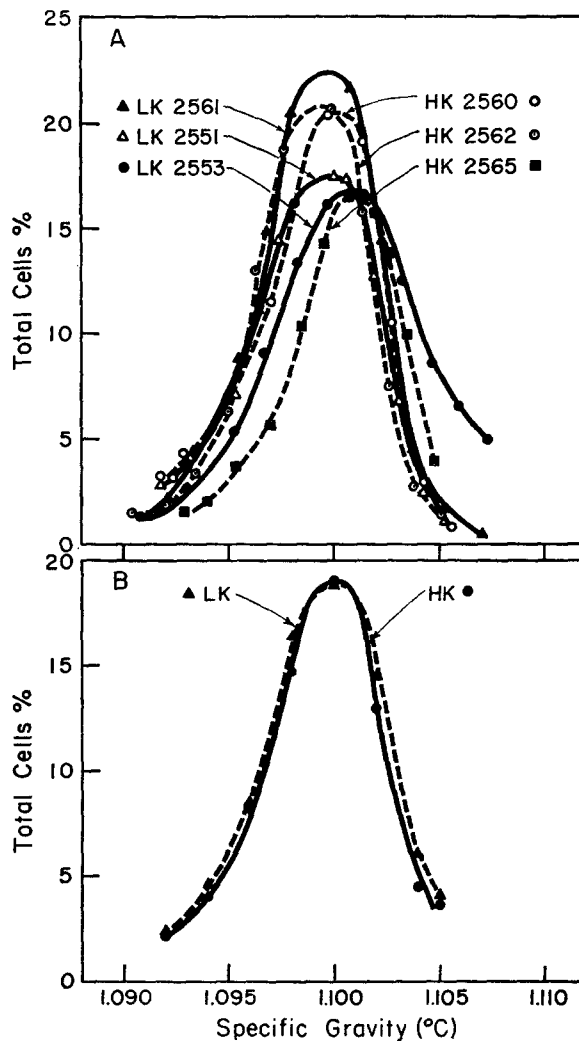


FIGURE 1 (A) Density distribution of red cells from three HK and three LK sheep before bleeding. (B) Composite density distribution curves derived from the data shown in (A).

cells produced at this time represented a small fraction of the total population. As time passed hematopoiesis continued and the young cells became more and more abundant. In 14 days a new cell concentration peak became very apparent in the lighter region (Fig. 4 C). The density distribution became bimodal and the new cell peak was observed to move steadily towards the denser regions with time (Fig. 4 D) until it finally merged with the original cell peak. This observation provides further evidence that young cells are lighter and that denser fractions contain older cells.

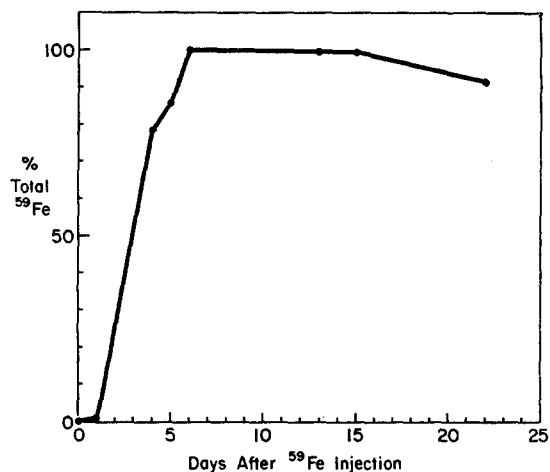


FIGURE 2 Uptake of ^{59}Fe into the circulating red cells of a LK sheep after bleeding. The units of the ordinate are per cent of the maximal ^{59}Fe incorporated into the red cells.

K Content of Cells Produced after Bleeding

The K content of cells in fractions showing peak ^{59}Fe concentration is plotted in Fig. 5 as a function of time after bleeding. The early rising phase probably represents a period of increased entry of new cells into the circulation. The amount of K per cell fell in just a few days from a high value to the low value characteristic of adult LK cells.

Fig. 6 shows the K content of cells in fractions containing the new cell peak as a function of time. Again the K per cell was observed to fall with time

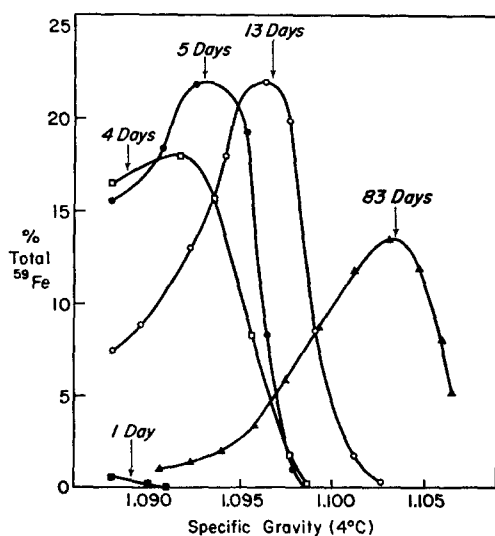


FIGURE 3. Density distribution of ^{59}Fe in LK sheep red cells at different times after injection. 2.5 liters of blood was withdrawn from the sheep on the 2 days prior to the injection. The units of the ordinate are per cent of the maximal ^{59}Fe incorporated into the red cells.

although with a slower time course than in the cells labeled with ^{59}Fe . This difference in the time course of K loss could be due to a difference in the membrane properties between the cells that enter the circulation during the early phase of response to hemorrhage and the cells that are produced later and appear as a new cell peak shown in Fig. 4 C and D. The more rapid loss of K from cells in the ^{59}Fe peak could also be due to dilution of young ^{59}Fe -

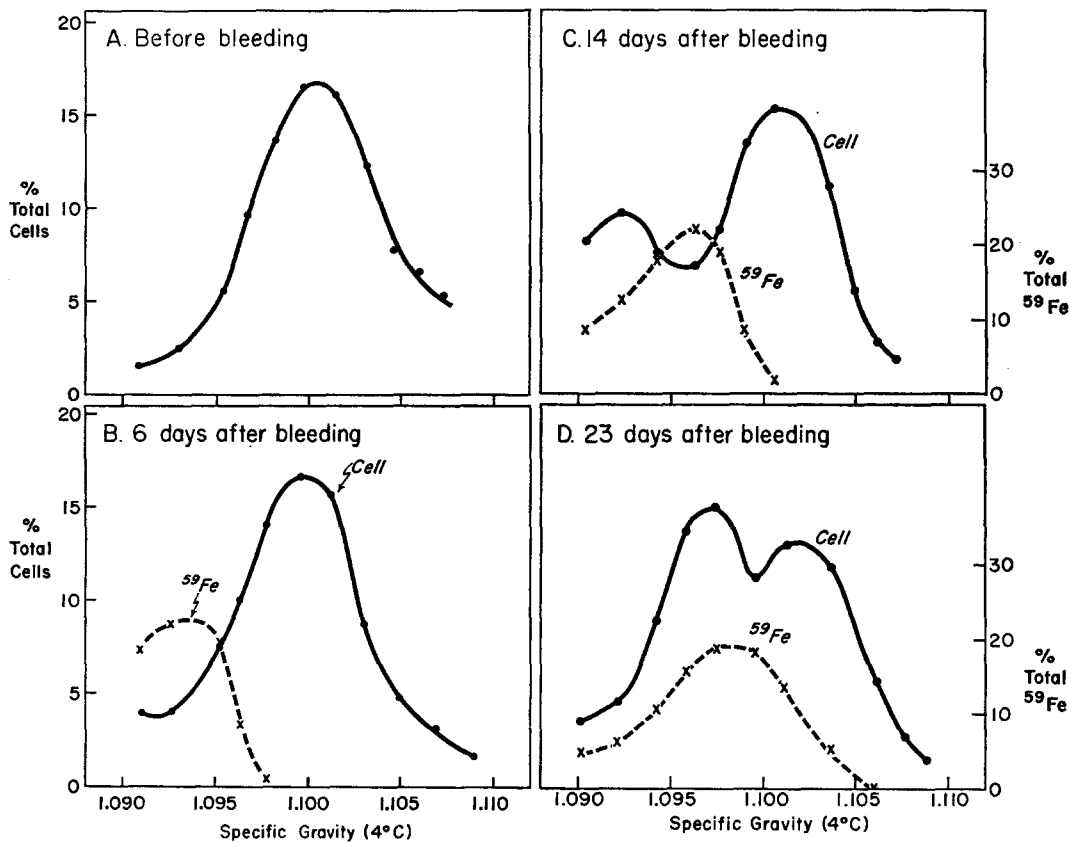


FIGURE 4. Density distribution of LK sheep red cells and ^{59}Fe at different times after bleeding.

labeled, high K cells with older, nonlabeled, low K cells as the young cells move to denser fractions of the gradient (Fig. 5). This effect would not be observed in the cells at the light density peak shown in Figs. 4 C-D and 6. The decrease in cell water content (Fig. 6) correlates well with the increase in density of the lighter peak from the 14th to the 23rd day after bleeding. The cell water content was computed by assuming that the sum of Na and K contents represents one-half of the total cell osmoles and equality of cellular and extracellular osmolality.

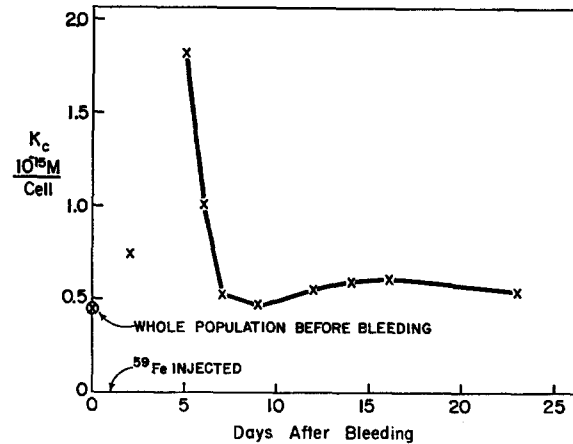


FIGURE 5. K content (K_c) of LK sheep red cells in density fractions containing the ^{59}Fe peak at different times after bleeding.

The transitory nature of the high K content of the new cells produced after bleeding is further shown in Fig. 7. This figure presents the K content per cell in different density fractions. It is interesting to note that 6 days after bleeding the K content in the lightest fraction had doubled while the majority of the population remained unchanged. Twenty-three days after bleeding the K content in the lightest fraction had fallen back to close to the original value. Fig. 8 shows that young high K cell produced after bleeding can contain a normal or an increased amount of hemoglobin.

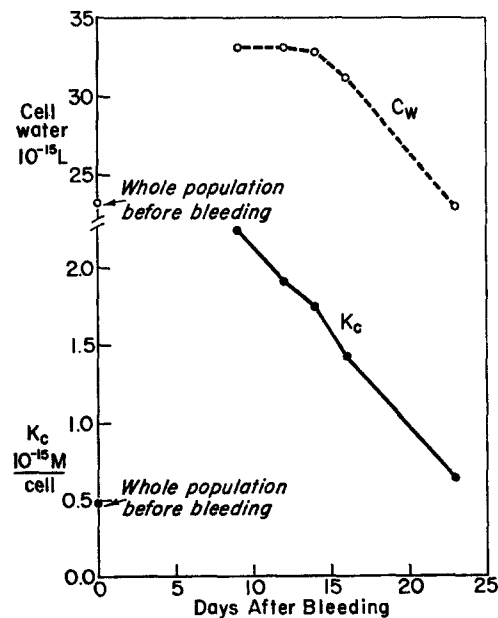


FIGURE 6. K and water content (K_c and C_w) of cells in the lighter density peak (Fig. 4 C-D) at different times after bleeding.

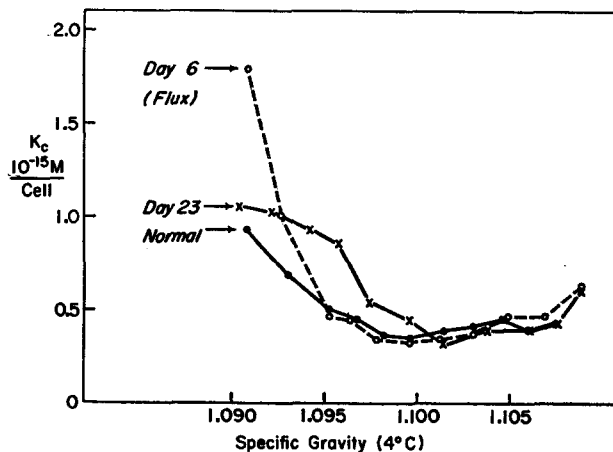


FIGURE 7. K content (K_c) of LK sheep red cells in different density fractions at different times after bleeding. The word "flux" indicates that measurements of K influx made on the 6th day after bleeding.

Measurements of K Influx

Table I shows the results of K influx measurements. Comparative values before and 6 days after bleeding are shown for the whole population and three different density fractions; the lightest, the second lightest, and the fraction containing the peak cell concentration. The K concentration in the lightest fraction increased about threefold 6 days after bleeding. There was no appre-

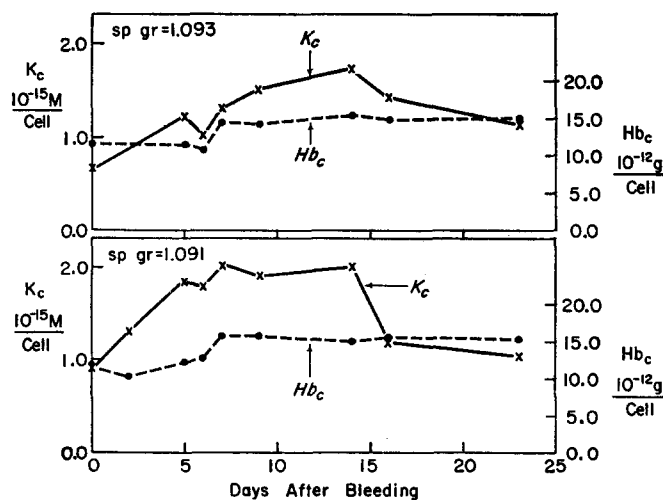


FIGURE 8. K and hemoglobin content (K_c and Hb_c) of LK sheep red cells in the lightest (sp gr 1.093) and second lightest (sp gr 1.091) fraction at different times after bleeding.

ciable difference in the volume of water in the lightest cells before and 6 days after bleeding. The amount of hemoglobin per cell changed only very slightly. K influx in the presence of strophanthidin (10^{-4} M) was taken as a measure of passive diffusion or leak (${}^iM_K^L$) while the strophanthidin-sensitive portion of the flux was taken as a measure of active transport or pump (${}^iM_K^P$). Six days after massive bleeding, cells found in the lightest fraction had a slightly greater K leak but a four- to fivefold greater pump influx. Similar but less impressive changes occurred in the second lightest fraction and in the whole

TABLE I
MEASUREMENTS OF K TRANSPORT AND RELATED PARAMETERS
IN LK SHEEP RED CELLS OF DIFFERENT DENSITIES

V_c and V_w are total cell volume and cell water volume respectively. Hb_c is the cell content of hemoglobin, while $(K)_c$ and $(Na)_c$ are cell concentrations of these ions. ${}^iM_K^L$ and ${}^iM_K^P$ are K influxes by leak and pump respectively. β is the ratio of ${}^iM_K^P$ to ${}^iM_K^L$; i.e., ${}^iM_K^P/{}^iM_K^L$. For details, see text.

Density fraction	Time after bleeding	V_c	V_w	Hb_c	$(K)_c$	$(Na)_c$	${}^iM_K^L$	${}^iM_K^P$	β	
									Measured	Calculated
<i>sp gr</i> °C	days	10^{-16} liters	10^{-12} g/cell	10^{-12} g/cell	mmoles/liter H_2O	10^{-8} moles/(10^{11} cells) × (hr)				
Lightest (1.089–1.091)	0	28.4	12.1	22	133	0.22	0.41	1.9	2.0	
	6	25.8	13.0	66	89	0.41	2.25	5.4	8.3	
2nd lightest (1.092–1.095)	0	22.7	11.2	23	132	0.28	0.42	1.5	2.2	
	6	20.5	10.9	32	125	0.46	1.48	3.2	3.5	
Cell peak (1.099–1.101)	0	22.1	11.3	17	139	0.42	0.47	1.1	1.3	
	6	20.1	10.7	17	138	0.45	0.41	0.9	1.3	
Whole population	0	33.1	23.2	11.9	19	135	0.27	0.45	1.6	1.7
	6	35.6	25.6	12.8	35	119	0.51	1.16	2.3	4.0

population. No change after bleeding was observed in cells contained in the density fraction with the peak cell concentration. These observations indicate that the K transport properties of the membranes of the cells formed after bleeding are different from those of normal LK cells and that these properties change during the first few days after entry of the cells into the circulation.

DISCUSSION

It is interesting to note that the rate of active K transport is four to five times greater in the cells produced after bleeding than in those normally released into the circulation. It is possible but unlikely that this increase in transport activity is due to changes in intracellular Na or ATP. The concentration of Na required for maximum activation of the pump is probably well below the

concentration present in these young cells (Tosteson and Hoffman, 1960). ATP concentration may be higher in these young cells than in mature red cells. Bernstein (1959) found that glucose utilization was eightfold faster in human reticulocytes than in mature erythrocytes. The ATP concentration decreased in older cells. Hofman and Rapoport (1956) also found that rabbit reticulocytes had two to three times the ATP content of the average circulating erythrocyte. It is possible that most or all the cells in the lightest fraction were reticulocytes and had an increased ATP concentration. The K_m for activation of the pump by ATP has not been well defined but is probably quite low, of the order of 10^{-4} M or less. This is well below the concentration of ATP found in normal LK sheep red cells (about 10^{-3} M). Thus a higher concentration of ATP in the young cells would probably not increase the rate of active K transport. It is also possible but not likely that the increase in pumping activity is due to an increase in surface area of the young cells. No actual measurement of the cell surface was made. Estimates of the cell volume from cell water and hemoglobin content show that the young cells present in the lightest fraction 6 days after bleeding had a normal volume despite a high K content and K pump activity. Even the larger cells that were produced later showed an increase in volume of only about 50% or less. This certainly could not account for the four- to fivefold increase in pump activity per cell observed. It is more likely that the greater rate of active K transport in these young cells is due to a greater maximum pumping capacity of the membrane, e.g., a larger number of active transport sites per cell or a faster effective turnover rate of each site.

It has been shown that the steady-state cation composition of sheep red cells is related to the membrane parameter β (${}^iM_K^P/{}^iM_K^L$, Tosteson and Hoffman, 1960). It is of interest to compare the experimentally measured β with that calculated from the set of equations relating this parameter to the steady-state composition of the cells. Table I shows both these values. The measured value of β is substantially less than that computed for these young cells. This result may indicate that the change of the membrane toward that characteristic of mature LK cells is preceding and is determining the change in cell composition.

The fact that young cells formed after bleeding contain more K and have a larger pump capacity suggests that the LK membrane develops rather late in the maturation process. Data shown in Fig. 8 may have some bearing on this point. Both cell K and hemoglobin contents were greater after bleeding, but the presence of cells containing more K is evident several days before cells with a larger amount of hemoglobin were observed. There is considerable evidence to suggest that the cells produced during rapid hematopoiesis (e.g., after hemorrhage) have failed to undergo the final maturation division (Borsook, 1964). These cells are larger and contain more hemoglobin. The

fact that young high K cells produced after bleeding can contain either a normal or an increased amount of hemoglobin suggests that the K transport properties characteristic of mature LK cells can develop at either of the last two maturation divisions. The results of the K influx measurements indicate that this membrane change does not occur suddenly at the time of division, but gradually during the first few days which the new cell spends in the circulation. Thus, genetically determined modification of membrane cation transport properties occurs in young, nondividing cells. Experiments designed to define more precisely this process of membrane maturation are in progress.

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