

# LK Sheep Reticulocytosis

## *Effect of Anti-L on K Influx and In Vitro Maturation*

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**ABSTRACT** After massive hemorrhage, adult sheep with genotypically low potassium (LK) red cells temporarily produce high potassium (HK) cells with ouabain-sensitive  $K^+$  pump fluxes equivalent to mature HK red cells. In light of recent reports of different red cell volume populations accompanying the HK-LK transition also occurring in newborn LK sheep and the unresolved controversy over the effect of anti-L on  $K^+$  transport in these immature red cells, we have reexamined the  $K^+$  transport changes and the effect of anti-L in the newly formed HK cells at various times after anemic stress and under in vitro conditions. We found that ~7 d after bleeding, maximum reticulocytosis occurred in the peripheral blood. After separation by density centrifugation, the top 10% cell fraction contained 100% reticulocytes, with a mean cell volume 2.5 times larger than that of mature erythrocytes. These immature red cells were of HK type, and their  $K^+$  pump and leak fluxes were 30 and 10 times higher, respectively, than those found in mature LK cells. The new cells may possess HK- and LK-type pumps because  $K^+$  pump influx was significantly stimulated by anti-L. When separated by density centrifugation on days 9, 17, and 23 after bleeding, some of the cells apparently maintained their large size while gaining higher density. Large cells from day 9, kept in vitro for 22 h, showed anti-L-sensitive  $K^+$  pump and leak fluxes that declined within hours, paralleling the behavior of these cells in vivo, whereas cellular  $K^+$  levels changed much less. It is concluded that the newly formed red cells may belong to a stress-induced macrocytic cell population that does not acquire all of the characteristics of adult LK cells.

### INTRODUCTION

Lee et al. (1966) have reported that adult sheep with genotypically low potassium (LK) red cells, when subjected to massive hemorrhage, temporarily produce immature red cells of high potassium (HK) type. Active  $K^+$  transport was fourfold to fivefold higher in the newly formed cells, whereas the ouabain-insensitive  $K^+$  leak flux was only slightly larger. It was concluded that the

immature cells only temporarily retain their HK characteristics before joining the pool of mature LK red cells. The HK-LK transition was considered to be a result of modification of membrane transport properties in the circulating immature red cells.

Since these pioneering studies, additional information has been gathered with respect to the kinetic and quantitative differences between mature HK and LK sheep red cells (Hoffman and Tosteson, 1971; Joiner and Lauf, 1975 and 1978 *a* and *b*). The discovery of an antibody directed against the L antigen (anti-L) genetically associated with the LK status (Rasmussen, 1969; Ellory and Tucker, 1969) was followed by the finding that anti-L stimulates  $K^+$  pump flux in these cells by reducing the inhibitory action of cellular  $K^+$  at the  $Na^+$  loading site of the pump (Lauf et al., 1970) without changing the number of pumps as measured by ouabain binding (Joiner and Lauf, 1975). Furthermore, a second antibody (anti- $L_L$ ) has been reported to reduce the passive  $K^+$  leak flux (Dunham, 1976; Lauf et al., 1977), apparently acting at a site different from the site for the anti-pump (anti- $L_p$ ) antibody (Lauf et al., 1977). Whether the phlebotomy-induced immature cells of LK sheep are affected by anti-L remains controversial. Whereas Dunham and Blostein (1976) reported a discernible response, Tucker and Ellory (1971) found only little anti-L effect.

In addition to the experimentally induced HK-LK transition in bled LK sheep, the HK to LK transition actually takes place naturally in the newborn lamb. Recently, we have shown that newborn genetically LK lambs, which possess only HK red cells at birth, acquire peripheral LK red cells within the next few months, primarily by cellular replacement (Valet et al., 1978; Lauf and Valet, 1979). These LK cells are comprised of three consecutive cell populations with distinct volume and transport characteristics (Lauf and Valet, 1979, and footnote 1.). In light of these advances, a reinvestigation of the HK-LK transition in acutely anemic LK sheep was warranted. In particular, it was of interest to ascertain whether volume differences occur in the newly produced cells and whether anti-L could affect their  $K^+$  pump and leak fluxes. A preliminary report has been presented elsewhere (Kim et al., 1979).

#### MATERIALS AND METHODS

##### *Anemic Stress*

Two adult sheep (LK genotype, homozygous for the L antigen) weighing 150–200 lbs., housed at Duke University animal facility, were bled during the span of 3 d of a total volume of 2 liters. Blood samples of ~100 ml were drawn into heparin at frequent intervals after phlebotomy.

##### *Cell Separation According to Density*

The cells were fractionated according to their density, which corresponded to their age, by the procedure of Murphy (1973) and segregated from plasma by centrifugation at 5,000 rpm for 15 min in a Sorvall RC-2B centrifuge (DuPont Instruments-Sorvall,

<sup>1</sup> Lauf, P. K., and G. Valet. Cation transport in different volume populations of genetically low  $K^+$  lamb red cells. *J. Cell. Physiol.* In press.

DuPont Co., Newtown, Conn.). Cells were resuspended at a hematocrit of 90% in a centrifuge tube (2.7 × 10.5 cm) and centrifuged for 1 h at 30°C. To achieve a horizontal cell meniscus, the tubes were once again centrifuged in a swinging rotor bucket for a few minutes. The tightly packed white cells were gently separated from the red cells with a spatula and discarded by aspiration. Six to seven arbitrary fractions were made from top to bottom of the cell column, and the volume fraction of each layer relative to total cell mass was determined by hemoglobin measurements. The reticulocytes were stained with new methylene blue dye (Sigma Chemical Co., St. Louis, Mo.) as described elsewhere (Kim and Luthra, 1977).

#### *In Vitro Maturation of Reticulocytes*

Reticulocyte-rich fractions (~75% reticulocytes on day 9 of the experiment), collected under standard sterile conditions, were allowed to mature in an Ecollagen tissue culture apparatus (New Brunswick Scientific Co., Inc., Edison, N. J.) featuring two chambers separated by a Millipore partition (Millipore Corp., Bedford, Mass.). The sterile medium, composed of 5 mM KCl, 5 mM glucose, 20 mM phosphate buffer, pH 7.4, and 120 mM NaCl, was placed into both chambers. Reticulocytes and sheep plasma were introduced into one chamber to give a hematocrit of <5% and a 20% plasma suspension. The other, serving as the diffusion chamber, permitted easy access to the medium, which was frequently replaced with fresh solution. Another advantage of the diffusion chamber was that it allowed continuous gassing of the medium with 95% O<sub>2</sub> and 5% CO<sub>2</sub> without disturbing the cells.

#### *Hematological Parameters*

The cell volume was calculated from the measurements of cell counts determined by Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) and hematocrit. Determination of mean corpuscular hemoglobin (MCH; 10<sup>-12</sup> g of hemoglobin per cell) and mean corpuscular hemoglobin concentration (MCHC; kilograms of hemoglobin per liter of cells) were based on measurements of the hemoglobin absorbance at 527 nm, using an extinction coefficient for hemoglobin of 532 (kilograms per liter)<sup>-1</sup>. Ion fluxes (h<sup>-1</sup>) and cation composition were expressed in millimoles per liter of cells as based on the actual MCHC measured for the cell fraction analyzed. These numbers can easily be interconverted into contents per cell or fluxes per cell × h using the data of Table I.

#### *Isotopic Flux Measurements*

Because it was important to measure K<sup>+</sup> influxes, particularly at times when the reticulocyte count was high and when the top 10% fraction of separated cells was kept in culture, the <sup>86</sup>Rb, which has a longer half-life, was used instead of <sup>42</sup>K. Previous studies have revealed that K<sup>+</sup> pump and leak fluxes calculated from <sup>86</sup>Rb uptake in 5–8 mM K<sup>+</sup> flux media compare well with K<sup>+</sup> influxes measured with <sup>42</sup>K.<sup>1</sup>

<sup>86</sup>Rb was obtained from New England Nuclear, Boston, Mass., and isotopic fluxes were measured as described elsewhere (Lauf et al., 1977, Joiner and Lauf, 1978 *a*, and *b*; footnote 1). In brief, unseparated cells and the cells from the top fraction of the density separation were suspended at a hematocrit of 10% (vol/vol) in K<sup>+</sup> flux medium composed of (mM): 145 NaCl, 6 KCl, 5 Glucose, and 10 Tris-Cl, pH 7.5, at 37°C, with and without anti-L immunoglobulin (5 mg S42 IgG fraction per milliliter, containing both anti-L<sub>L</sub> and anti-L<sub>p</sub>, dialyzed against K<sup>+</sup> flux medium; Lauf et al., 1977) and in the presence and absence of 10<sup>-4</sup> M ouabain. Suspensions were preincubated for 30 min at 37°C before 25 μCi <sup>86</sup>RbCl (in 0.5 M HCl, neutralized with 0.5 M NaOH and diluted with K<sup>+</sup> flux medium) was added. Tracer influx was

stopped 30 min later and the cells were separated by the dibutylphthalate technique (Kepner and Tosteson, 1972; Joiner and Lauf, 1978 *a*).

Total  $K^+$  uptake was first calculated per kilogram of hemoglobin (Hgb) according to the equation

$$K^+ \text{ uptake} = \frac{{}^{86}\text{Rb}_c}{X_o \cdot t}, \quad (1)$$

where  ${}^{86}\text{Rb}_c$  defines the counts per minute per kilogram of Hgb,  $X_o$  the specific activity of  ${}^{86}\text{Rb}$  in relation to  $[K]_o$ , the extracellular  $K^+$  ion concentration, and  $t$ , the time interval of isotope uptake.  $K^+$  uptake was interconverted into  $K^+$  influx per liter of cells  $\times$  h ( ${}^iM_K^T$ ) by multiplying Eq. 1 by the MCHC value derived for each unseparated and top 10% cell fraction:

$${}^iM_K^T = K^+ \text{ uptake} \times \text{MCHC}. \quad (2)$$

Active or  $K^+$ -pump mediated  ${}^{86}\text{Rb}$  influx ( ${}^iM_K^P$ ) is given by the relation

$${}^iM_K^P = {}^iM_K^T - {}^iM_K^L, \quad (3)$$

where  ${}^iM_K^L$  is the  $K^+$  influx in presence of ouabain. The leak rate coefficient, which provides a comparison of  ${}^iM_K^L$  between the experiments independent of small variations in  $[K^+]_o$ , comes from the relation

$${}^i k_K^L = {}^iM_K^L/[K]_o. \quad (4)$$

#### RESULTS

Fig. 1 depicts the cell volume of the top 10% cells separated according to their density in response to the phlebotomy of LK sheep. Before bleeding, the young cells in the top 10% of the density gradient had a cell volume of  $\sim 30 \mu\text{m}^3$ . A few days after acute anemic stress, the cell volume of the top 10% cells increased dramatically by a maximum of 2.5 times the control counterpart. These large cells were identified as the immature reticulocytes by their methylene blue dye staining characteristics (nucleated red cells were not observed). Both the cell size and the reticulocyte count decreased steadily, reaching the normal level within 3–4 wk after the phlebotomy.

Fig. 2 shows a complete volume profile from the young to the old cells fractionated according to their density after an acute anemic stress. In a normal sheep (control), the young cells were only slightly larger than the older cells. At the time of maximum reticulocytosis, day 7, the largest cells were confined to the uppermost layers of the packed-cell column (with cell size apparently decreasing) approximately half way down the gradient. (On subsequent days, these large cells appeared to gradually migrate to a denser fraction).

The data illustrated in Figs. 1 and 2 suggest that extraordinarily large cells were released into circulation in response to an acute anemic stress, and that these large cells did undergo some volume shrinkage in the course of cellular maturation, which spanned several days.

Table I summarizes certain hematological indices of cells released into the circulation after phlebotomy. In agreement with findings on reticulocytes in other species (Kim et al., 1976), sheep reticulocytes (fraction 1, days 7 and 9) had a low MCHC. On the other hand, the MCH was high, reflecting the

larger size of these cells. At days 17 and 23, the middle fractions exhibited higher MCH values, again supporting the notion that the large cells had migrated down the density gradient.

In agreement with well-established findings (Lee et al., 1966; Blostein et al.,

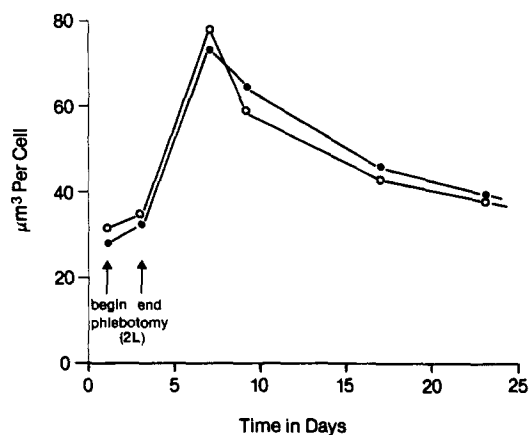


FIGURE 1. The volume of young cells released into circulation in response to the phlebotomy of LK sheep. The cells were separated according to their density by the procedure of Murphy (1973) after blood loss of 2 liters. The cell volume was measured on the uppermost 10% of cells. (●) Sheep 96. (○) Sheep 32.

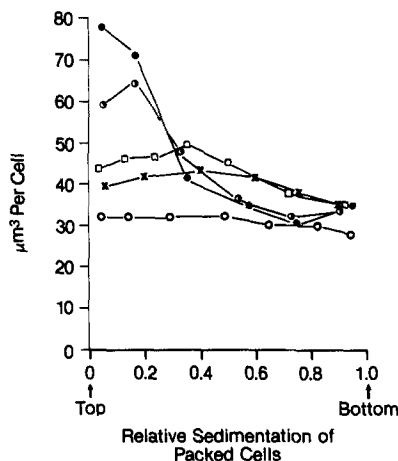


FIGURE 2. Effect of acute phlebotomy on the volume of LK sheep cells fractionated according to their density. (○) Control. (●) Day 7. (◐) Day 9. (□) Day 17. (×) Day 23.

1974; Dunham and Blostein, 1976), the reticulocytes of LK sheep were found to have a high cellular  $K^+$  concentration  $[K^+]_c$ , as shown in Fig. 3, in which  $K^+$  and  $Na^+$  compositions measured on the same aliquots of cells illustrated in Fig. 2 are shown. In an unbled control sheep, the  $[K^+]_c$  of the top 10% of the cells was 14 mmol per liter of cells and rose to 78 mmol per liter of cells at

the time of maximum reticulocytosis. In fact, the overall profile of the  $K^+$  levels in density-separated cells (cf. Fig. 3) remarkably resembled their volume changes (cf. Fig. 2), giving the impression that the large reticulocytes must have lost some of their  $K$  ions in the course of in vivo cell maturation.

The increases in  $[K^+]_c$  were counterbalanced to a large extent by concom-

TABLE I  
HEMATOLOGICAL INDICES OF LK SHEEP CELLS AFTER PHLEBOTOMY

Fractions	Day 0		Day 7		Day 9		Day 17		Day 23	
	MCH	MCHC	MCH	MCHC	MCH	MCHC	MCH	MCHC	MCH	MCHC
1	9.8	.310	17.4	.221	15.8	.269	12.8	.297	11.9	.310
2	10.2	.325	19.7	.276	18.0	.278	12.9	.283	13.1	.317
3	10.1	.322	12.5	.304	14.5	.306	13.3	.292	13.4	.312
4	10.4	.325	11.4	.328	11.6	.320	14.3	.299	13.4	.323
5	10.3	.343	10.3	.336	10.3	.323	14.1	.313	12.5	.331
6	10.3	.342	11.8	.340	11.0	.333	12.0	.332	11.6	.335
7	9.8	.346					11.7	.340		

Mean corpuscular hemoglobin,  $MCH = 10^{-12}$  g of hemoglobin per cell.

Mean corpuscular hemoglobin concentration,  $MCHC = \text{kg of hemoglobin per liter of cells}$ .

The packed cells, which were segregated according to their density, were fractionated and consecutively numbered from top to bottom of the cell column.

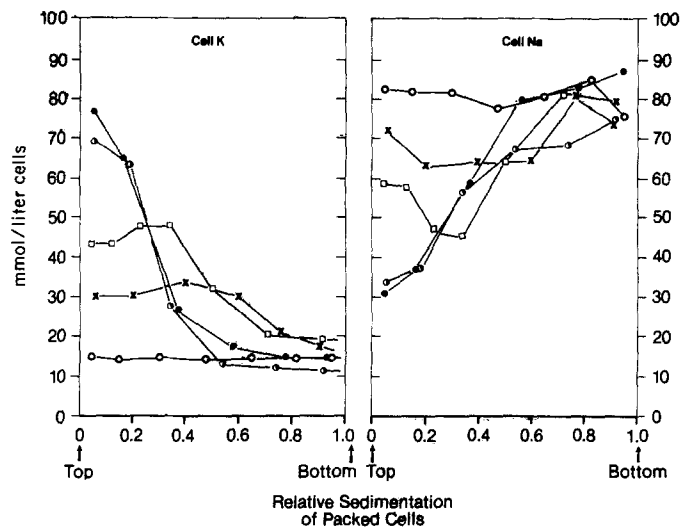


FIGURE 3. The cation compositions of density-separated cells after acute anemic stress. The same aliquots of cells depicted in Fig. 2 were used for the determination of  $K^+$  and  $Na^+$  concentrations. (O) Control. (●) Day 7. (◐) Day 9. (□) Day 17. (×) Day 23.

itant decreases in cellular  $Na^+$  concentrations ( $[Na^+]_c$ ), as shown in Fig. 3. The spectacular rise in  $[K^+]_c$  by 64 mmol per liter of cells from the normal young cells to the reticulocytes was almost entirely compensated for by the  $Na^+$  reduction of 51 mmol per liter. However, the reciprocal changes between  $K^+$  and  $Na^+$  were not identical in the two sheep studied. As is shown in Fig. 4,  $[Na^+]_c$  in the second sheep did not decrease below 70 mmol per liter of

cells, despite a large increase in  $[K^+]_c$  of 60 mmol per liter of cells. The data suggest that it is  $K^+$  which was always altered in the course of erythropoiesis, while the change in  $Na^+$  appeared to be much less predictable.

Fig. 5 presents  $K^+$  pump and leak fluxes for unseparated (A) and separated

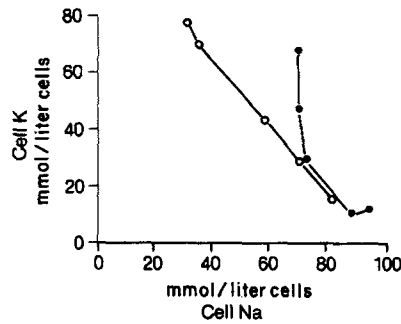


FIGURE 4. The relationship between the  $K^+$  and  $Na^+$  content of young cells in acutely bled LK sheep. The young cells refer to the top 10% of cells separated according to their density. (●) Sheep 96. (○) Sheep 32.

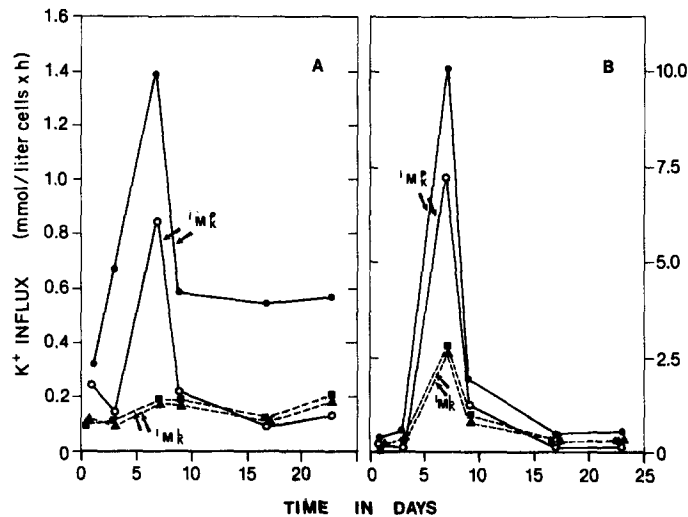


FIGURE 5. Effects of anti-L on  $K^+$  influx of the young and unseparated cells from the bled LK sheep. (A) Unseparated erythrocytes. (B) Top 10% fraction. (● and ▲) Plus anti-L. (○ and ■) Control.

cells (B) in the presence and absence of anti-L. In unseparated cells,  $K^+$  pump fluxes on days 1, 3, 17, and 23 were between 0.1 and 0.24 mmol per liter of cells  $\times$  h, representing the characteristic values of adult LK red cells. In the presence of anti-L,  $K^+$  pump influxes were around 0.6 on days 3, 17, and 23, amounting to a threefold to sixfold activation by the antibody. The much lower stimulation of  $K^+$  pump flux by anti-L observed on day 1 was probably

due to some loss of anti-L activity in the L antibody preparation dialyzed for this day.

The most dramatic change in  $K^+$  pump flux occurred on day 7 when, in unseparated cells,  $K^+$  pump flux rose fourfold to eightfold as compared with the controls. Addition of anti-L further stimulated the  $Na^+$ ,  $K^+$  pump by 1.7-fold. It should be pointed out that this change was accompanied by a small upward shift in the ouabain-insensitive  $K^+$  leak flux. The data suggest that at day 7 a new cell population with much higher  $K^+$  pump and leak fluxes must have entered the peripheral circulation. Indeed, the immature cells, again referring to the top 10%, had their  $K^+$  pump activity increased by a factor of nearly 30 at day 7. Even this high  $K^+$  pump activity was further augmented 1.4-fold by anti-L.

In addition to the dramatic changes in  $K^+$  pump flux, we also found that on day 7, the  $K^+$  leak flux increased by a factor of 10 (Fig. 5 *B*). This becomes evident when the data is plotted in terms of rate coefficients (Fig. 6 *A* and *B*),

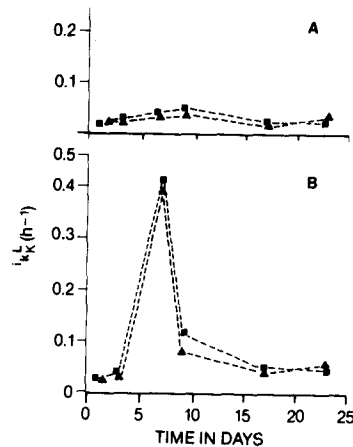


FIGURE 6. The rate coefficients of  $K^+$  influx of the young and unseparated cells after the phlebotomy. (A) Unseparated cells. (B) Top 10% fraction.

which also reveals only a small reduction in the  $K^+$  leak rate coefficients by anti-L. Hence, most of the passive permeability changes observed were not modulated by anti- $L_L$ , the antibody against the L antigen associated with the  $K^+$  leak pathway (Dunham, 1976; Lauf et al., 1977).

These findings unequivocally demonstrated that newly formed, large HK-type red cells, with extraordinarily high  $K^+$  pump and leak fluxes, had entered the peripheral circulation in response to bleeding. Shortly thereafter, cells having typical LK characteristics reappeared.

To monitor the  $K^+$  pump and leak fluxes accompanying cellular maturation, the top 10% cells harvested at day 9 were allowed to mature under *in vitro* tissue culture conditions. Fig. 7 shows  $[K^+]_c$ ,  $K^+$  pump, and leak fluxes in the top 10% cells obtained at day 9 from two LK animals and kept *in vitro* for 22 h at 37°C. These cells contained about 75% reticulocytes. From Fig. 5 it can be seen that their  $K^+$  pump flux and leak rate coefficient at day 9 had already been reduced to 15 and 30%, respectively, of the peak values at day



7. Once the flux data and the reticulocyte counts of day 7 and 9 were collected, we realized that the peak had just been passed with respect to both parameters, demonstrating the logistic difficulties of such experiments. Nevertheless, the data of Fig 7 are interesting. Whereas  $[K^+]_c$  stayed practically unchanged during the incubation period, the base  $K^+$  pump flux as well as the leak rate coefficient fell within the first 6 h rather significantly in both animals, approaching the typical ranges of LK red cells. Simultaneously, the  $K^+$  pump stimulation by anti-L decreased but maintained more than twofold augmen-

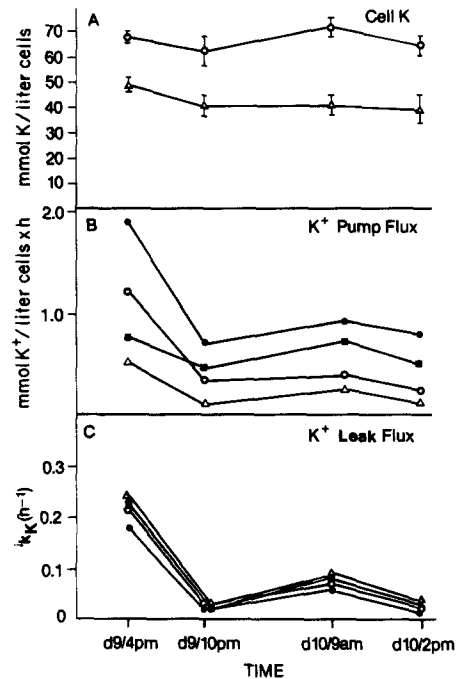


FIGURE 7. In vitro maturation of reticulocytes derived from acutely bled LK sheep. Effect of anti-L on  $K^+$  influx and the  $K^+$  levels were measured on the same aliquot of cells which were depicted at day 9 in Fig. 1. The reticulocytes were allowed to mature in an Ecollagen tissue culture chamber (New Brunswick Scientific Co., Inc.) under sterile conditions. (A)  $\circ$ , Sheep 32;  $\Delta$ , Sheep 96. (B and C)  $\circ$  and  $\Delta$ , Sheep 32 and 96 control;  $\bullet$  and  $\blacksquare$ , Sheep 32 and 96 anti-L.

tation, whereas the effect of anti-L on the leak rate coefficient again was small. These data suggest that newly produced cells kept in vitro maintained their cellular  $K^+$  concentration, while  $K^+$  pump and leak fluxes were approaching levels typical for LK cells. It is unlikely that this reduction was the result of depletion in the energy levels of the cells because anti-L did exert its effect throughout the observation period.

#### DISCUSSION

The results presented herein provide some new insight into the LK-HK-LK transition that occurs in the red cells of massively bled LK sheep.

In response to an acute anemic stress, the following physiological events defined the emergency recovery phase. (a) Large immature cells, 2.5 times the normal mature red cells, were released into the circulation. Some of these cells appeared to sustain their large size while gaining greater density in the course of cellular maturation spanning a few days. (b) The new cells had a high  $K^+$  concentration of nearly 80 mmol per liter of cells, with 30 and 10 times higher  $K^+$  pump and leak fluxes, respectively. In these cells,  $K^+$  pump flux was stimulated by anti-L. (c) The large cells seemed to maintain their high  $K^+$  content, while, in the courses of cellular maturation, the  $K^+$  pump and leak fluxes were rapidly approaching the levels typical for LK red cells.

The finding that the immature cells have high  $K^+$  content and  $K^+$  pump activity was first described by Lee et al. (1966). However, we determined that the  $K^+$  pump activity in the immature cells is 6–7 times greater than that reported by them. Also, in contrast to their finding that the  $K^+$  leak is only slightly increased, we report rather dramatically increased  $K^+$  leak fluxes of the immature cells. Moreover, that the immature cells were huge is, *prima facie*, at variance with this same study which reports “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.” In fact, these authors did find that in the lightest cells, cell water was increased about 1.5-fold 9 d after bleeding. However, they correlated this increment with the density of the new cells only (cf. Fig. 6, Lee et al., 1966). Aside from the general notion that sheep reticulocytes are larger than mature erythrocytes (Ellory et al., 1970), our findings are supported by the electric sizing studies of Valet et al. (1978), which revealed that bleeding of sheep induced a macrocytic erythrocyte population between 5 and 12 d after hemorrhage.

The reason for these discordant results may be related to the composition of harvested immature cells. It should be noted that the day of harvesting the top 10% cells most active in  $K^+$  pump transport is very critical, and “peak performance” of these cells may well be missed if one bleeds one day too early or too late. It is possible that the lightest fraction of cells in the earlier work (Lee, et al., 1966) did not contain as many reticulocytes as we observed (near 100%) and hence was more mature.

That anti-L stimulated  $K^+$  pump flux at all times in unseparated and top 10% cells confirms Dunham and Blostein's (1976) report, but not that of Tucker and Ellory (1971). The relative stimulation by anti-L was greater in unseparated cells, in particular after the new cell peak activity (day 7), than in the newly formed cells of the top 10% fraction. However, as was also pointed out by Dunham and Blostein (1976), the absolute stimulation by anti-L was much higher in the immature cells (2.5 mmol per liter of cells  $\times$  h) than in unseparated cells on day 7 (0.6 mmol per liter of cells  $\times$  h) or on day 17 (0.5 mmol per liter of cells  $\times$  h). Surprisingly, anti- $L_L$  had only little effect on the leak flux rate coefficient.

The *in vitro* maturation experiments were initiated in the hope of finding a time differential between the reduction of  $K^+$  pump and leak fluxes. However, both processes declined simultaneously within 6 h of culture time, which may account for the fact that the  $K^+$  content in these cells remained

rather constant while  $K^+$  pumps and leaks reached activities typical of mature LK cells (Fig. 7). Inasmuch as anti-L stimulated  $K^+$  pump transport in these cells, it is unlikely that we observed an artifactual decline of these transport processes resulting from our incubation conditions.

The density change, which caused the newly formed cells to sediment at a faster rate, may be determined by several factors. The total cation content in these cells declined from about  $7.7 \text{ mol per cell} \times 10^{15}$  on day 7 (fraction 1) to about  $4.25 \text{ mol per cell} \times 10^{15}$  on day 23 (fraction 3) due to a three-fold drop in cellular  $K^+$  content, a finding compatible with the possibility of cell shrinkage. However, it is difficult to assess to what extent salt loss effected volume reduction, because these newly formed cells coseparated with the general red cell population as they, themselves, became denser. We also have independent evidence that the newly formed cells did not assume the volume typical of normally produced mature LK cells (Valet et al., 1978; Lauf and Valet, unpublished data). The density change may also be considered in terms of continued hemoglobin synthesis as recently shown for red cells of anemic dogs (Kirk et al., 1978). In addition, it is known that elevated levels of impermeable organophosphate anions cause a chloride and water shift out of the cell, which results in cell volume reduction (Parker, 1971). Hence, elevated levels of adenosine triphosphate, glutamate, and glutathione may also contribute to volume and density changes. Smith (1977) has shown that their levels increased significantly in red cells of sheep 5–7 d after phlebotomy, roughly in correlation with the time course of reticulocytosis.

Lack of precise volume data, in particular for the large cells as they assumed higher densities, precluded a meaningful computation of  $K^+$  fluxes in terms of surface area.  $K^+$  pump and leak fluxes per liter of large cells were more than an order of magnitude greater than those of control cells, which contrasts with an estimated twofold to threefold enlargement of membrane surface area. This difference was also apparent in conversion of our data to fluxes per cell. Although an increase in the volume:surface area ratio certainly would have lowered the flux:area differential between large and mature red cells of normal size, the immature red cells must have had a  $K^+$  pump and leak site density much in excess of that found in adult LK cells and even in adult HK cells (Joiner and Lauf, 1975). Although still to be verified by [ $^3\text{H}$ ]ouabain binding experiments, this hypothesis is supported by recent analyses of  $K^+$  pump activities in red cell populations of growing lambs (type I/III cells; see Lauf and Valet, 1979). It cannot be decided at present whether all or only a fraction of these pumps were stimulated by anti-L. However, the fact that anti-L did significantly stimulate active  $K^+$  transport in HK-type erythrocytes of LK sheep indicates that at least some of these pumps already may have been altered with respect to the apparent  $\text{Na}^+$  loading site affinity (Lauf et al., 1970; Joiner and Lauf, 1978 *a* and *b*).

Because the newly formed cells appeared to stay large for some time (Lauf and Valet, unpublished data; Valet et al., 1978), the reduction in  $K^+$  pump activity indicates a major membrane involution of  $\text{Na}^+$ ,  $K^+$  pumps by an as yet totally unknown mechanism rather than a splenic effect upon the red cell surface area (Crosby, 1959). A similar membrane modification affected the

K<sup>+</sup> leak pathway, which may explain the relatively short-term persistence of high K<sup>+</sup> contents of these cells *in vitro*.

The presence and rapid decline of the high K<sup>+</sup> leak fluxes in the newly formed cells may be rationalized in terms of a unified pump-leak concept (Tosteson and Hoffman, 1960; Lew and Beauge, 1978). However, the fact that the initially augmented leak was not affected by anti-L speaks against this concept, and we have shown earlier that the pump- and leak-associated L antigens may be immunologically and functionally separated (Lauf et al., 1977). Nevertheless, one could assume that those pumps and leaks which are turned off during circulation are very much different from those remaining in the adult LK cells. Some kinetic evidence seems to support this assertion (Dunham and Blostein, 1976).

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