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# Cation Transport in Different Volume Populations of Genetically Low $K^+$ Lamb Red Cells

P.K. LAUF AND G. VALET
*Department of Physiology, Duke University School of Medicine, Durham, North Carolina 27710 (P.K.L.), Max-Planck Institut für Biochemie, Abteilung für Experimentelle Medizin, D-8033, Martinsried, West Germany (G.V.)*

*ABSTRACT* During the first three months after birth lambs produce sequentially three erthryocyte populations of different mean volume as demonstrated by electric sizing methods (Valet, Franz, and Lauf, J. Cell. Physiol. *94* (1978) 215). We separated by centrifugal elutriation the small volume population (type II) red cells of a genotypically low $K^+$ (LK) lamb from the population containing the larger volume type I and III cells, an admixture of fetal (I) and adult (III) erythrocytes. The cells were separated at various time intervals after birth and analyzed with respect to their volumes, cation contents, and cation flux properties by means of $^{86}Rb$ uptake. The effect of anti-L on $K^+$ pump and leak fluxes was ascertained in unseparated and separated red cells. It was found that the small red cells of population II, transiently present for several weeks, were fully developed LK cells with $K^+$ pumps responding characteristically to the stimulatory action of anti-L. In constrast, the larger cells of population I and III were of high $K^+$ (HK) nature at early time points, the $K^+$ pump activities approximately ten times higher than adult LK cells. These cells constitute an admixture of type I fetal HK cells, and type III reticulocytes which are precursors for the final type III adult LK cells, since anti-L had a small stimulatory effect. At later times, however, only adult type III LK cells predominated. The data directly support our earlier finding that the HK-LK transition in genotypically LK lambs is primarily governed by cellular replacement.

The high potassium (HK) and low potassium (LK) steady-state levels in red cells of adult sheep (see recent reviews by Ellory, '77, and Lauf, '78) are maintained by kinetically and quantitatively different passive $K^+$ leak and $Na^+K^+$ pump fluxes (Tosteson and Hoffman, '60; Hoffman and Tosteson, '71; Joiner and Lauf, '78a,b). In LK cells $K^+$ pump fluxes may be modulated by the L antibody directed against the L antigen, which acts as an inhibitor of the LK pump (Lauf et al., '70; Glynn and Ellory, '72; Joiner and Lauf, '78a,b).

At birth, however, all lambs possess L antigen negative HK red blood cells, and only in those animals endowed with the dominant LK gene (Evans and King, '55; Evans, '57) do the red cells in circulation gradually assume LK character (Tosteson and Moulton, '59; Blechner, '61) and acquire L antigenicity (Tucker and Ellory, '70) during the first 3 months of life. This HK-LK transition has been interpreted in terms of cellular maturation or replacement (Tosteson, '66). Using electric sizing to measure cell volumes (Kachel, '76) we have previously shown that cellular replacement rather than maturation is the primary determinant of the HK-LK transition (Valet et al., '78). When the LK genotype lamb is born, it has almost exclusively a population of large fetal HK red cells which are gradually replaced by two consecutive cell populations, one of which is transient (28 $\mu m^3$, Population II), and one, the final adult LK red cell (30 $\mu m^3$, Population III). Fetal (Population I) and adult (Population III) red cells are easily distinguishable on basis of their different hemoglobins, volumes (Valet et al., '78), cellular cation levels, number of $Na^+$ $K^+$ pumps as measured by $^3H$-ouabain binding (Lauf et al., '78), and response to the $K^+$ stimulatory action of anti-L (Tucker and Ellory, '70; Valet et al., '78).

Dr. P.K. Lauf's mailing address is: Box 3709, Department of Physiology, Duke University Medical Center, Durham, NC 27710.

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We were able to separate and characterize the transient small type II red cells as compared to the large fetal and/or adult red cells on basis of their different volumes by counter current centrifugation (elutriation; Reckert, '74). This experimental approach has the advantage of separating sufficient numbers of cells for transport studies from small blood samples (5–8 ml) without disturbing the hematopoesis in the newborn animal. Analysis of the transport parameters distinguished the small red cell population II as an early LK cell type as opposed to the fetal HK population I and the adult LK population III. The transient fractional rise of these small LK cells contributed significantly to the HK-LK transition kinetics of the whole cell population. A preliminary report of this work has been given elsewhere (Lauf and Valet, '79).

## MATERIALS AND METHODS

### *Source of red cells and separation by centrifugal elutriation*

A mixed Suffolk/Merino lamb, homozygous for the low potassium gene ($Ke^{L}Ke^{L}$) and membrane L antigens (LL) of its red cells, was obtained from a farm near Munich about 15 days after birth. During the first several weeks not more than 5–8 ml of blood per day was removed into EDTA (ethylene diamine tetraacetic acid) coated polycarbonate tubes and kept ice-cold prior to further processing.

For separation by elutriation one milliliter aliquots of whole blood were diluted to a hematocrit of 5% (v/v) with isotonic 5 mM Tris/Cl buffered NaCl, pH 7.5, containing 5 mM glucose and 5 mM KCl ($K^+$ flux-medium), and injected into the separation chamber of the elutriator-rotor (Beckman) at a rotor speed of 5350 rpm at 4°C (Beckman Model J21B centrifuge). Following sample application the buffer flow rate was adjusted so that all red cells remained stationary in the chamber. About 50 ml $K^+$ flux-medium was pumped through the separation chamber at above flow rate to remove platelets and plasma proteins. The much larger white cells pelleted to the bottom of the chamber. Cell separation was effected by stepwise increasing the counterflow rate at constant rpm, causing the small cells to leave the separation chamber first, followed by cells of intermediate, and finally, of large volumes. About 250 ml of buffer were used per elution step. The cells were collected at the outlet of the centrifuge system into ice-cooled test tubes, and concentrated to a workable cell suspension density by centrifugation for 1 minute at 10,000 rpm prior to further analysis.

### *Cellular parameters*

Erythrocytes were electrically sized at different times after birth by an improved Coulter method which uses hydrodynamic focusing of the particles (Kachel, '76). The erythrocytes were suspended in Tris/Cl buffered saline, pH 7.4 (TBS) with a specific electrical resistance of 62 Ωcm/25°C and sized with a cylindrical orifice of 60 $\mu$m diameter and 52 $\mu$m length of 0.35 mA electrical current, a suction of 0.1 kg/cm$^2$ and a flow rate of 1,000–1,500 particles/second at a temperature of 25°C with a METRICELL apparatus (Kachel, '76). The electrical volume pulses were amplified and classified in a 128-step multichannel analyzer (Telefunken, Ulm, Germany). The frequency histogram of the multichannel analyzer, referred to as volume distribution curve, was then approximated by a computer with one or several logarithmic normal distributions as described earlier (Valet et al., '78).

As described elsewhere (Joiner and Lauf, '78a), calculations of cation composition and ionic fluxes were based on mean corpuscular hemoglobin (Hgb) concentration, MCHC (Kg/L), and content/cell, MCH (g/cell), and on cell volume, $V_c$ ($\mu m^3$), and surface area, SA (cm$^2$/cell). The $V_c$ was determined with a coulter counter (model F Coulter counter) or by electric sizing as described above, and multiplied by the MCHC to yield the MCH. For estimation of SA, the hemolytic red cell volume, equivalent to that of a sphere ($V_h$), was determined by electric sizing of each separated and unseparated red cell under osmotic hemolysis conditions and calculated according to Eq. (1):

$$(1) \qquad SA = 4\pi\,(V_h \times \tfrac{3}{4\pi})^{2/3} = 4.84\,(V_h)^{2/3}$$

Cellular cations were measured as described elsewhere (Joiner and Lauf, '78a) using a Perkin-Elmer model 420 atomic absorption spectrophotometer, and computed in moles/kg Hgb or moles/cell, in particular when comparing data of small and large cells, or in concentration (moles/L. original cells) by dividing the cellular cation content by the $V_c$ value.

### *Isotopic flux measurements*

Unidirectional $K^+$ influx was measured using $^{86}Rb$ as tracer isotope after initially establishing that this isotope could satisfactorily replace $^{42}K$ (see Results). $^{86}$Rubidium, specific activity 2.2–2.4 Ci/g and $^{42}K$, specific activity, 0.27–0.56 Ci/g, were obtained from New England Nuclear Industries, Boston, Mass., and Amersham, Great Britain, respectively. De-

tails of the isotopic flux technique used have been described previously (Joiner and Lauf, '78a,b). In brief, separated as well as unseparated cells suspended at a hematocrit of 5–10% (v/v) in 5 mM $K^+$ flux-medium, in the presence or absence of $10^{-4}$ M ouabain, and with or without anti-L (5 mg IgG fraction of S42 anti-L/ml, Lauf et al., '77) were preincubated for 0.5 hour at 37°C prior to addition of 20–40 $\mu$Ci $^{86}Rb$ or $^{42}K$ to each cell sample. Tracer uptake was stopped 30 or 60 minutes after addition of the isotope (the time depended on cellular cation type under study) by separating the labeled cells from their medium using the dibutylphthalate technique (Joiner and Lauf, '78c). The total $K^+$ uptake/Kg Hgb was calculated from Eq. (2):

$$(2) \qquad K^+ \text{ uptake} = \frac{^{86}Rb_c}{X_o \cdot t}$$

where $^{86}Rb_c$ denotes counts per minute per Kg Hgb as calculated from the measured sample OD and extinction coefficient per Kg Hgb, $X_o$ the specific activity of $^{86}Rb$ in relation to $[K]_o$, the extracellular $K^+$ concentration, and t, the 30 or 60 minute interval over which $^{86}Rb$ (or $^{42}K$) uptake was measured. Total $K^+$ uptake was converted into total $K^+$ influx per liter original cells, $^iM_K^T$, by multiplying Eq. (2) with the MCHC.$K^+$ pump flux per liter cells, $^iM_K^p$, is the difference between $^iM_K^T$ and the ouabain insensitive leak flux, $^iM_K^l$. The latter was also expressed in terms of its rate coefficient, $^ik_K^l$, which is $^iM_K^l/[K]_o$, in order to make comparative analysis independent of small variations in $[K]_o$. Total $K^+$ influx per surface area, $J_K^T$, was computed according to Eq. (3):

$$(3) \qquad J_K^T = \frac{^{86}Rb_c}{X_o \cdot t} \times \frac{MCHC \times V_c}{SA}$$

Active or $K^+$ pump-mediated $^{86}Rb$ influx in terms of surface area then is given by the relation:

$$(4) \qquad J_K^p = J_K^T - J_K^{ou};$$

where $J_K^{ou}$ represents the $K^+$ influx ($K^+$ leak flux) in presence of $10^{-4}$ M ouabain. Most of the data are presented in terms of fluxes per surface area, permitting a volume independent, direct comparison of parameters between the separated cells.

## RESULTS

### *Comparison of $^{86}Rb$ and $^{42}K$ uptake*

In previous publications from this laboratory, (Lauf et al., '70, '77; Joiner and Lauf, '78a,b) $^{42}K$ was used as tracer to measure $K^+$ influx. However, because of the length of the experiments, $^{86}Rb$ was employed on the assumption that the active and passive $K^+$ transport systems translocate $^{86}Rb$ and $^{42}K$ indiscrimatorily. This assumption was substantiated by the data of Figure 1, comparing $^{86}Rb$ uptake (panel A) with $^{42}K$ uptake (panel B) into adult LK sheep red cells, in presence and absence of $10^{-4}$ M ouabain. Tracer uptake was nearly linear up to 1 hour incubation time, and ouabain exhibited the usual effect in blocking $K^+$ pump-mediated tracer exchange. Also shown is the dual effect of anti-L, i.e., the stimulation of total tracer uptake due to both activation of $K^+$ pump transport and slight inhibition of passive (leak) tracer exchange. Calculation of $K^+$ influxes (Eq. (2) × MCHC) for the two tracer isotopes used (Table 1) shows that $^iM_K^T$ was slightly higher with $^{42}K$, which was mainly due to a slightly larger passive leak flux. However, the pump-mediated $K^+$ influx values, $^iM_K^p$, were very close for the two tracers. Furthermore, Table 1 shows the characteristic four to five-fold stimulation of $^iM_K^p$ by anti-$L_p$ and the 20–40% reduction of $^ik_K^l$ by anti-$L_L$ in both parts of the experiment (Lauf et al., '77). Hence, all subsequent tracer experiments on lamb cells were carried out with $^{86}Rb$ replacing $^{42}K$ as isotope.

### *Volume distribution of unseparated lamb red cells*

Figure 2 shows the volume distribution curves of the lamb red cells constructed in a three-dimensional plot as function of time after birth. As shown previously (Valet et al., '78) for 15- to 20-day-old lambs, there was a bimodal erythrocyte volume distribution which has been attributed to the presence of large, progressively disappearing fetal red cells (Population I) and small cells (Population II) which were transiently produced until around day 90–100, when the final adult population of large erythrocytes (III) dominated in the peripheral circulation.

### *Separated small and large lamb red cells*

*Volume Distribution and Cellular Cations.* The volume distribution analysis, shown in Figure 2, revealed a sizable small red cell population (II) that has to be considered in terms of its contribution to the HK-LK transition in the newborn lamb. Thus far, definitive statements about the cation steady state were possible only with respect to the early type I and the final type III red cell population, which are fetal HK

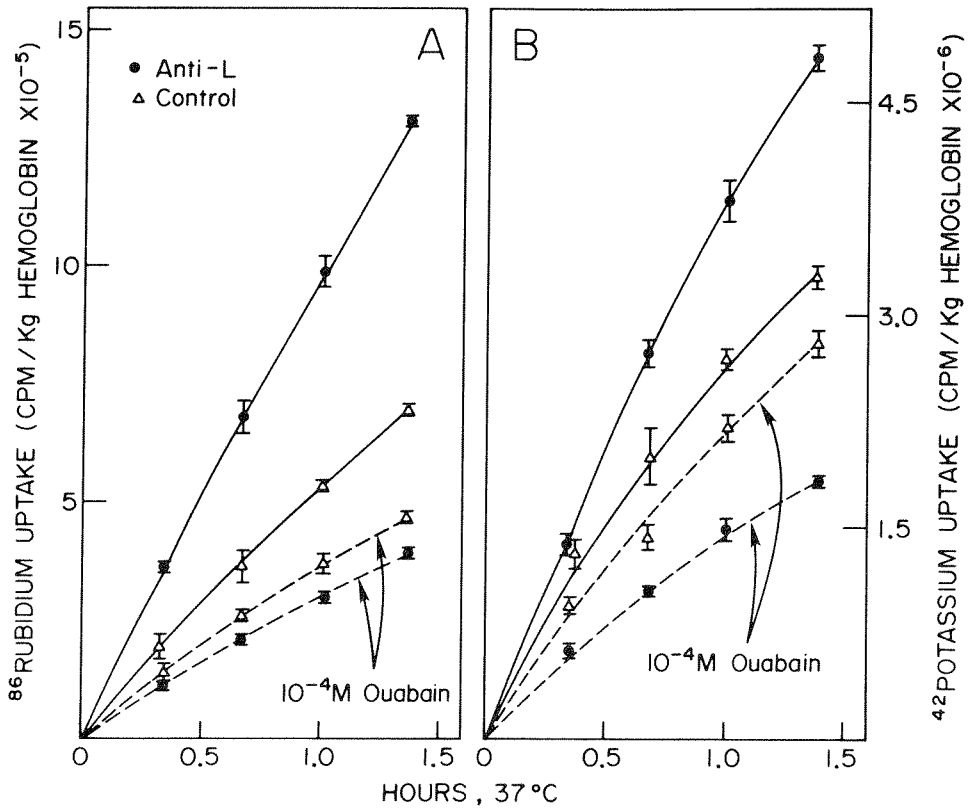


Fig. 1. Comparison of $^{86}$Rb (A) and $^{42}$K (B) uptake/kg hemoglobin as function of incubation time at 37°C in presence (filled circles) and absence (open triangles) of anti-L and without (solid lines) and with $10^{-4}$ M ouabain (broken lines).

*TABLE 1. Comparison of $K^+$ influxes in LK sheep red cells measured with $^{86}Rb$ or $^{42}K$ as tracers*

| Isotope | Anti-L | $^iM_K^T$ | $^iM_K^P$ | Pump Stimulation | $^iM_K^L$ | $^ik_K^L$ | Leak Reduction |
|---|---|---|---|---|---|---|---|
| $^{86}$Rb | – | 0.30 | 0.08 | — | 0.22 | 0.044 | — |
| | + | 0.52 | 0.36 | 4.5 | 0.16 | 0.033 | 0.75 |
| $^{42}$K | – | 0.43 | 0.08 | — | 0.35 | 0.063 | — |
| | + | 0.64 | 0.41 | 5.1 | 0.23 | 0.040 | 0.64 |

$^iM_K^T$, $^iM_K^P$, $^iM_K^L$ = Total, pump, and leak fluxes in mmoles/L. cells × hour.
$^ik_K^L$ = rate coefficient in hour$^{-1}$.

type and adult LK type cells, respectively, as previously reported. The transient small cell population always coexisted together with either type I or type III cells (Valet et al., '78), and hence their characterization requires separation from type I and type III cells.

Figure 3 illustrates the successful separation of the small type II cells from the large type I and III cells (henceforth called I + III cells) carried out with blood from the 20-day-old lamb. About 43% of all cells were type II and 57% were a mixture of type I + III cells (Fig. 3A). Due to a mean volume differential of about 1.6, the distribution curves of type II and type I + III cells (Fig. 3B), recorded after separation, practically did not overlap and hence permitted for the first time analysis of pure type II cells. Panels C and D of Figure 3 contain the volume distribution curves of samples A and B for cells that had been osmotically hemolyzed in 200 mOsm TBS as evident from the right shift of the volume distribution curves. Since these cells were ghosts with the hemolytic cell volume, ($V_h$), their shape was close to a spheroid. The surface area of the ghost was calculated as indicated under Methods.

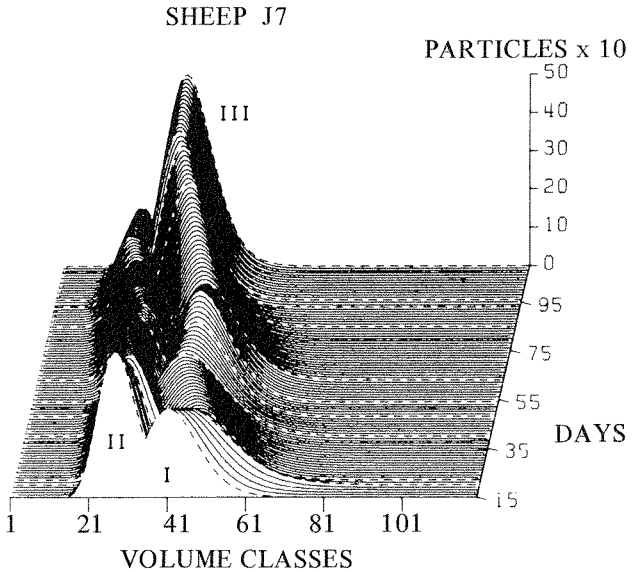


Fig. 2. Three-dimensional presentation of the temporal appearance of the erythrocyte volume populations in the LK genotype lamb of this study. The animal was obtained on day 15 after birth, at which mainly populations I and II were present. At later times population I diminished, followed by population II, until population III provided the dominant adult LK cell type. Solid lines are calculated by linear interpolation to generate the three-dimensional impression of the graph (see also Valet et al., '78).

In Figure 4, cellular volumes, surface areas, and cation contents are shown for separated type II and large type I + III cells as compared with unseparated cells at four intervals after birth. Around day 20, type I + III cells had 2.3 times greater volume and 1.5 times larger surface area than type II cells, while all populations together (I, II, III) assumed an intermediate volume and surface area (Fig. 4A,B). On day 95 after birth, these volume and surface area differentials fell to 1.7 and 1.3 respectively, indicating that the large cell population now contained cells with slightly smaller volumes. A striking difference existed in the cation content between large (I + III), small (II), and unseparated cells. Compared to type II cells, the $K^+$ content, $K_c$, of type I + III was tenfold greater on day 17 after birth and dramatically fell to about 1.8 times greater on day 95 (Fig. 4C). Simultaneously, the Na content, $Na_c$, was indistinguishably close in large and small cells and rose to 1.8 times greater by day 95 in large as compared to small cells.

It is interesting to compare the data of Figure 4CD with values from adult HK and LK sheep red cells. Table 2 shows that M antigen-positive HK cells from an adult HK sheep have a lower $K_c$ than type I + III cells of the 20-day-old lamb, in which at day 95, $K_c$ had been largely exchanged for $Na_c$. In contrast, population II cells were LK cells at all times, with $Na_c$ somewhat lower than in adult LK cells. Due to different volumes, the total cation contents of type I + III cells and type II cells were larger and smaller, respectively, than those of adult HK and LK cells. However, in terms of cellular $K^+$ concentration, $[K]_c$, population I + III cells (on day 17) were similar to HK cells, and cells of population II (days 17, 95) and population I + III (day 95) similar to LK cells.

The data of Figure 4 clearly distinguished the early type I + III cells as large HK cells with equivalent $[K]_c$ values of 79, 113, and 64 mmoles/L. packed cells on days 17, 21, and 28, respectively, from the LK cells of population II. The population II cells had $[K]_c$ values of 18, 25, and 22 mmoles/L. packed cells on the same days. As the animal matured (day 95) there was in the large cell population a substantial drop of cell volume and surface area together with a final $[K]_c$ of 17 and $[Na]_c$ of 75 mmoles/L. cells, suggesting that the large cell population had also assumed LK character.

It is readily apparent that there were several events associated with the HK-LK transition in unseparated cells (types I, II, III, Fig. 4C, D). First there occurred a dramatic change in the cation steady-state composition within volume populations I + III while the small type II population of LK character was already present throughout the observation period. Second, there is a change in the fractional contribution of type II cells to the whole cell population as apparent in the three-dimensional volume distribution pattern of Figure 2. The fraction of each cell population, as well as its cellular parameters, are known at each point of analysis. A comparison of the sum of the parameters with the parameters actually measured in the unseparated cells revealed recovery ratios approximating unity as the population ratios of type II/(I + III) cells increased and decreased over the observation period (data not shown). Third, it is well known that about 3–4 weeks after birth whole blood analysis of lambs reveals a variable reticulocytosis which precedes the adult hematocrit value and red cell density (Valet et al., '78). Figure 5 shows these parameters for the lamb studied here. As our studies began with day 17, there existed the typical postnatal anemia with less than $10^{10}$ red cells/ml and a hematocrit of just above 20% (v/v). A significant reticulocyte peak occurred between days 20–40, followed by a sharp rise of the number of red blood cells to about $1.4 \times 10^{10}$/ml and of the hematocrit to about 37% (v/v) around day 60, at which time the reticulocyte count had dropped to insignificant levels.

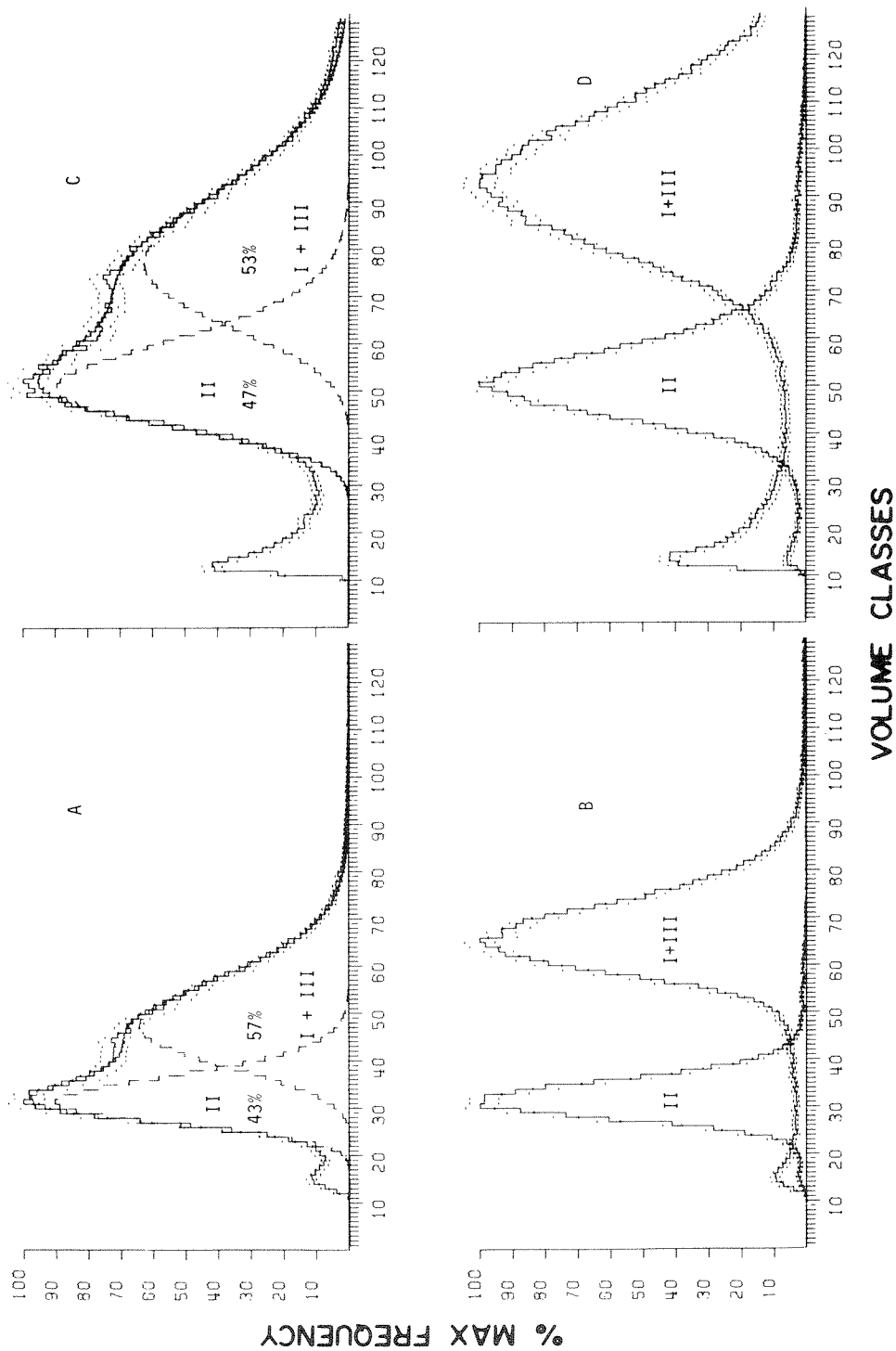


Fig. 3. Volume distribution curves (% of maximum frequency as function of volume classes) of erythrocyte populations from the 21-day-old LK lamb generated under isotonic (A, B) and hypotonic (C, D) conditions before (A, C) and after centrifugal elutriation of population II and population I + III cells (B, D). Conditions C and D provided the spherical volumes to calculate the surface area for each cell type. The distribution curves are fitted by logarithmic (A, C) or linear (B, D) Gaussian distributions to obtain the mean volume.

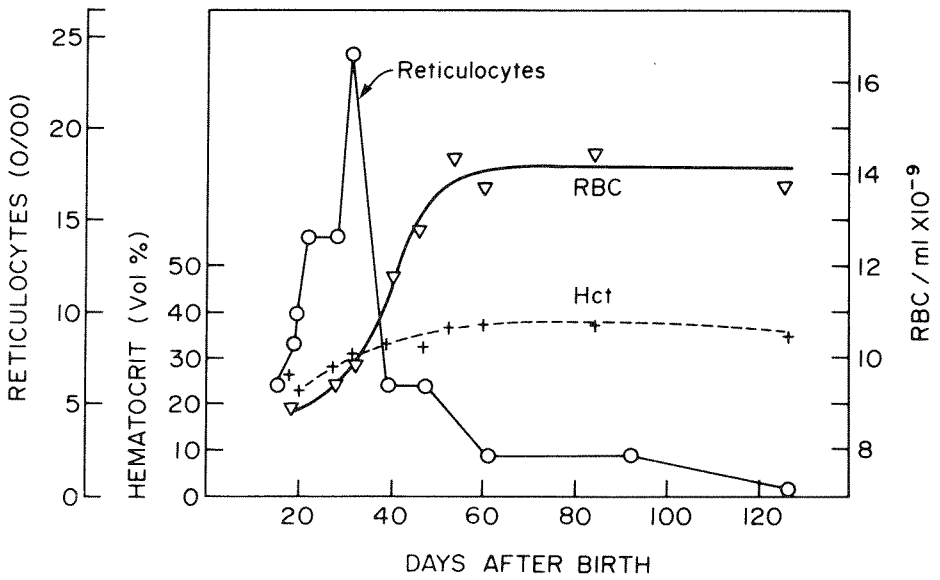


Fig. 5. Hematological parameters of LK lamb blood between days 15 and 126 after birth. (see Results for details).

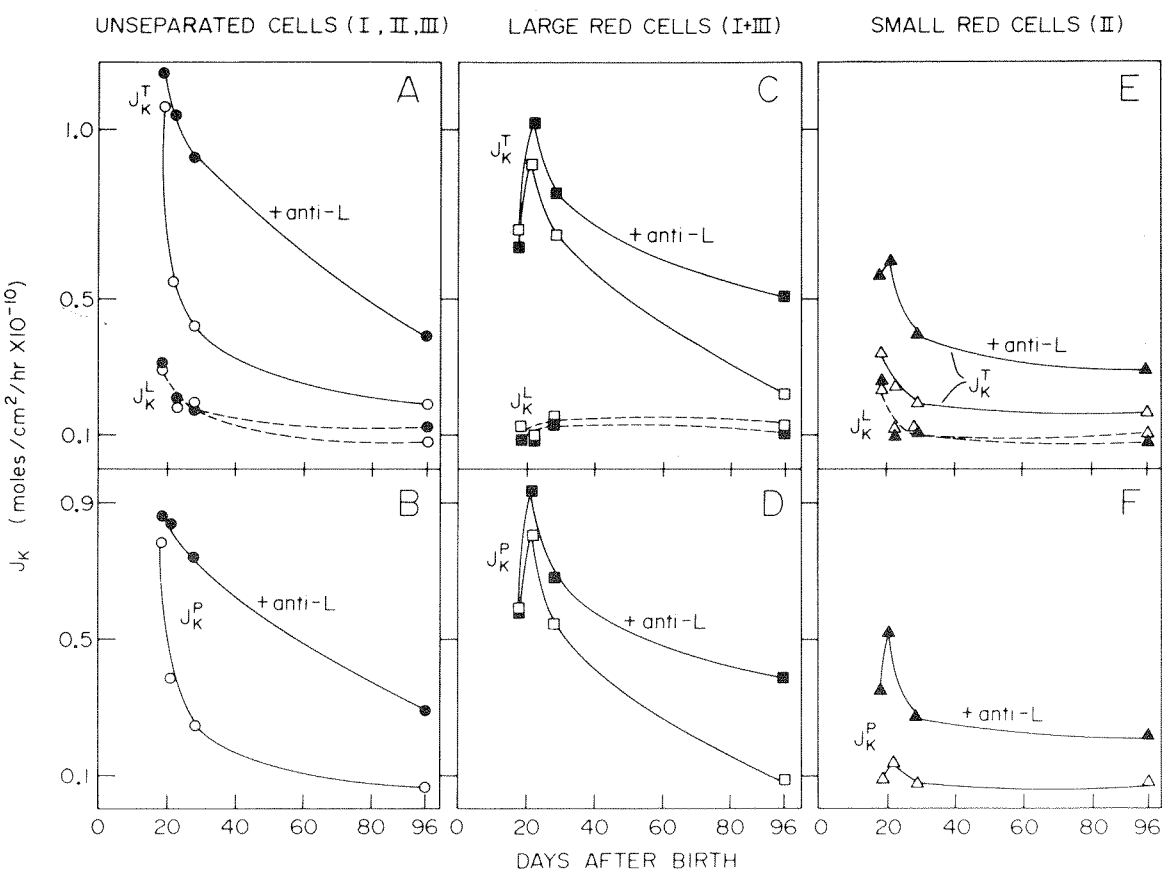


Fig. 6. $K^+$ influxes/surface area ($J_K$) in unseparated (A, B) and separated type I + III (C, D) and III (E, F) volume populations at days 17, 21, 28, and 96 after birth. Panels A, C, and E contain total influxes ($J_K^T$, solid lines) and leak fluxes ($J_K^L$, broken lines) in presence (filled symbols) and absence (open symbols) of anti-L. In panels B, D, and F: $K^+$ pump fluxes ($J_K^P$) in presence and absence of anti-L.

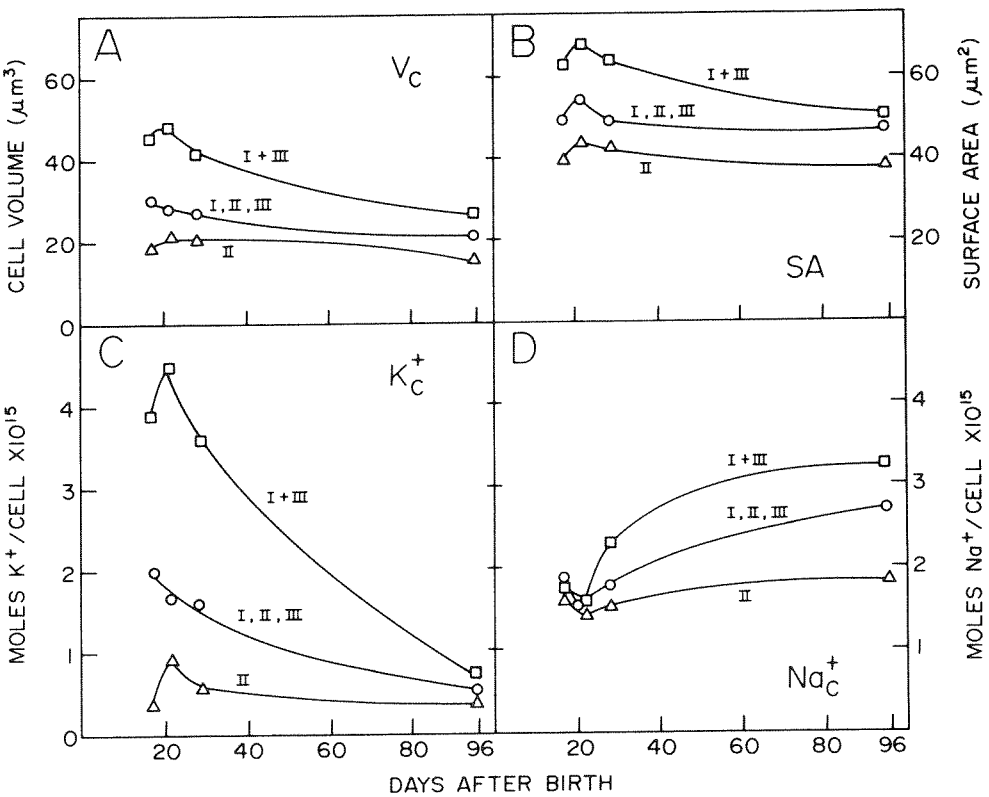


Fig. 4. Cellular volume (A), surface area (B), and cation content (C, D) of unseparated (I, II, III), and separated population II and I + III cells. Data in A obtained under isotonic conditions in part served for computation of data in C and D. Data of B from Figure 3D,E,F, using Eq. (1).

*TABLE 2. Comparison of cation levels in adult HK and LK sheep red cells, and in lamb red cell populations*

| Cation/antigen genotype (n = 4) | Age or population | Moles/cell × $10^{15}$ (± SE) $K^+_c$ | Moles/cell × $10^{15}$ (± SE) $Na^+_c$ | mM/L. Cells $[K^+]_c$ | mM/L. Cells $[Na^+]_c$ |
|---|---|---|---|---|---|
| HK (MM) | Adult | 2.33 ± 0.21 | 0.44 ± 0.05 | 77.6 | 14.7 |
| LK (LM) | Adult | 0.54 ± 0.02 | 2.90 ± 0.22 | 18.0 | 96.6 |
| LK (LL) | Adult | 0.31 ± 0.05 | 2.96 ± 0.33 | 10.3 | 98.6 |
| LK (LL) | 17-Day Lamb | | | | |
| | Pop. I + III | 3.87 | 1.76 | 78.5 | 35.7 |
| | Pop. II | 0.39 | 1.65 | 18.3 | 78.0 |
| | 96-Day Lamb | | | | |
| | Pop. I + III | 0.74 | 3.28 | 16.8 | 75.2 |
| | Pop. II | 0.39 | 1.85 | 20.9 | 97.9 |

*Active and Passive $K^+$ Influxes.* In unseparated red cells of LK lambs $K^+$ influx and particularly $K^+$ pump influx rapidly declined 20–40 days after birth (Tosteson, '66; Valet et al., '78; Lauf et al., '78), which for the unseparated cells of this study is illustrated in panels A and B of Figure 6. Plotting $K^+$ influx/surface area (Eq. (3)), $J^T_K$ first fell almost threefold (day 28) and finally reached a sixfold lower value (day 96). Although the passive leak flux, $J^L_K$, dropped gradually twofold (Fig. 6A, plus ouabain), most of the transport activity loss occurred in $J^P_K$, which at day 96 had fallen approximately tenfold to levels typical of adult LK cells. Concommitantly, $J^P_K$ became increasingly responsive to anti-L with a twofold enhancement on days 21 and 28, and a fivefold activation on day 96.

Striking differences between separated large, type I + III and small, type II cells were observed in $J^T_K$ and $J^P_K$ in presence and absence of anti-L as shown in panels C–F of Figure 6. In the large cell population, $J^T_K$ increased initially to $0.89 \times 10^{-10}$ moles/cm² × hour, and then fell

to $0.22 \times 10^{-10}$ moles/cm$^2$ $\times$ hour—a change entirely carried by the $K^+$ pump activity since $J_K^L$ remained rather constant (Fig. 6C). The stimulation of $J_K^P$ by anti-L became evident on day 21 and rose to fourfold on day 96 (Fig. 6D). In contrast, $J_K^P$ of the small cell population was around $0.1–0.2 \times 10^{-10}$ moles/cm$^2$ at all times (Fig. 6F), and hence of a magnitude characteristic of adult LK cells, while the elevation of $J_K^T$ at days 17 and 21 could be related to a twofold increase $J_K^L$ (Fig. 6E). Furthermore, like in adult LK cells, anti-L stimulated $J_K^P$ four to fivefold at all observation times (Fig. 6F). Again the recovery ratios computed for the individual $K^+$ influxes approached unity.

## DISCUSSION

The HK-LK transition in peripheral blood cells of the maturing LK genotype lamb is marked by a series of complex events among which cellular replacement as well as fundamental changes in the cation transport properties of individual cells assume central importance (Tosteson, '66). Together with our previous work (Valet et al., '78; Lauf et al., '78), our data reported herein permit the following macroscopic scheme of events during the HK-LK transition: The large fetal erythrocyte volume population I present at birth is HK-like, possessing high $K_c$ values and $K^+$ pump fluxes but lacking the L-antigen. This population is gradually replaced by the two consecutive LK cell populations II and III, the latter being the final, adult LK cell population.

An unexpected finding of this study was that the transient population II consisted of small LK-type red cells present already on day 17 after birth. Since the fraction of these cells still increased to 71% of the total population (day 28), their LK cation steady-state concentration and $K^+$ influxes significantly contributed to the overall HK-LK transition in the peripheral blood until beyond day 100, when the larger adult LK cell is the dominant peripheral cell type (Fig. 2). These type II cells then must have had sufficient time to develop the LK character and L antigenicity prior to entering peripheral circulation.

The rapid changes of cation steady-state composition, $K^+$ influxes, and their response to anti-L, observed in separated type I + III cells, require consideration in light of the accompanying hematological parameters. Between days 20 and 40 a burst of reticulocyte release preceded the rise of hematocrit and cell density to adult levels (Fig. 5). The temporal appearance of reticulocytes closely followed the peak values of $K_c$ and $K^+$ pump influx in large type I + III cells, and hence makes it likely that these immature cells constitute the actual precursors of the final type III cell population. This assumption is supported by the finding of others (Blostein et al., '74; Benderoff et al., '78) that reticulocytes of LK sheep are HK-like cells which, however, respond to the $K^+$ pump stimulatory action of anti-L (Dunham and Blostein, '76). While the HK-like large cell population I + III did not react with anti-L on day 17, it did so on day 21 and 28 (Fig. 6, CD). This finding could mean that from day 21 on, a sizable fraction of the type I + III cells were indeed reticulocytes of the incoming type III cells, which like the still-remaining fetal red cells, were initially of HK type. The quick fall of $K_c$ and of $K^+$ pump flux combined with the appearance of the full anti-L effect on the pump on day 96 suggests that all HK cells as well as the precursor reticulocytes for type III cells were phased out, and that the large cell volume population now consisted entirely of adult LK type red cells, an assumption borne out by the comparative data of Table 2.

The presentation of the data of Figure 6 in terms of $K^+$ pump flux/surface area is a measure of the relative pump site density. On day 17 and 21, type I + III cells possessed six and nine times more $K^+$ pump activity than on day 96. This dramatic fall in $K^+$ pump activity cannot simply be accounted for by assuming that there was a corresponding loss of surface area combined with a reduction in the number of pumps since the surface area of type I + III cells (Fig. 4B) fell only by 25% on day 96. Rather, the large pump site differentials between type I + III cells, analyzed around days 20 and on day 96, indicate that on the latter day typical adult LK cells dominated in the periphery. The high $K^+$ pump density on days 17 and 21 then is compatible with the presence of fetal red cells (day 17) known to have about fourfold more ouabain binding sites/cells (Lauf et al., '78) and the admixture of reticulocytes (days 21 and 28) reported to have up to tenfold, and higher, $K^+$ pump fluxes than normal adult LK cells (Dunham and Blostein, '76; Kim et al., '79).

Earlier work on red cells from 10 to 60-day-old LK lambs had shown that the parameter $\beta$, the pump ($^iM_K^P$) to leak ($^iM_K^L$) ratio fell from about five to less than one, roughly paralleling the reduction of $[K]_c$ (see Fig. 9 in Tosteson, '66). It was concluded that fetal HK cells are gradually replaced by adult LK cells—however, with the caveat that this interpretation may not be the sole explanation of the behavior

*TABLE 3. Comparison of the pump/leak ratios (β parameters)*

| Day after birth | $^iM_K^p/^iM_K^l$ (β measured) | | | |
|---|---|---|---|---|
| | Unseparated cells I, II, III (this study) | Populations I + III | Populations II | Unseparated cells (Tosteson, '66) |
| 18 | 2.7 | 4.9 | 0.3 | 3.8 |
| 21 | 4.9 | 9.6 | 1.1 | 2.8 |
| 28 | 1.2 | 3.6 | 0.7 | 1.1 |
| 60 | — | — | — | 0.2 |
| 96 | 0.5 | 0.7 | 0.9 | — |

of the LK lamb red cells (Tosteson, '66). Table 3 compares the β parameters calculated from our studies (Eq. 2 × MCHC) with those extrapolated from Fig. 9 of Tosteson ('66). It can be seen that β of unseparated cells changes similarly to the published values. However, β of the large cells remains HK-like until day 28, while β of the small type II cells was below unity on days 17, 28, and 96, and hence characteristic for LK cells. There was a considerable surge in β on day 21 in both unseparated and the larger type I + III cells which may be related to the reticulocyte burst around this time (Fig. 5).

The behavior of the β parameter also appears to be compatible with the hypothesis advanced further in this study that the HK-LK transition kinetic is primarily determined by the cellular interchange of populations I, II, and III. A similar conclusion was reached for the maturation process in dog red cells (Kirk et al., '78) which exhibit a functional and cellular heterogeneity after maturation (Castranova and Hoffman, '79). Our results extend a finding of our earlier work (Valet et al., '78), that cellular replacement governs the HK-LK transition, suggesting that a variety of other membrane transport changes such as nucleoside and glucose transport (Mooney and Young, '78) and the calcium induced $K^+$ efflux (Brown et al., '78) may be also interpreted in terms of this mechanism.

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