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Na^+ K^+ Pump and Passive K^+ Transport in Large and Small Red Cell Populations of Anemic High and Low K^+ Sheep

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Reticulocytes, isolated by centrifugal elutriation from massively bled sheep and identified by cytometric techniques, were analyzed with respect to their cation transport properties. In sheep with genetically high K^+ (HK) or low K^+ (LK) red cells, two reticulocyte types were distinguished by conventional or fluorescence-staining techniques 5-6 days after hemorrhage: Large reticulocytes as part of a newly formed macrocytic (M) erythrocyte population, and small reticulocytes present among the adult red cell population (volume population III of normal sheep blood, Valet et al., 1978). Although cellular reticulin disappeared within a few days, the M-cell population persisted throughout weeks in the peripheral circulation permitting a transport study of in vivo maturation. At all times, M cells of LK sheep had lower K^+ and higher Na^+ contents than M cells of HK sheep. Regardless of the sheep genotypes, M cells apparently reduced their volume during their first days in circulation; however, throughout the observation period, they did not attain that characteristic for adult red cells. Both ouabain-sensitive K^+ pump and ouabain-insensitive K^+ leak fluxes were elevated in M cells of both HK and LK sheep. The increased K^+ pump flux was mainly due to higher K^+ pump turnover rather than to the modestly increased number of pumps as measured by [^{3}H]ouabain binding. In contrast, small reticulocytes enriched from separated volume population III cells by a Percoll-density gradient exhibited transport parameters close to their prospective mature HK or LK red cells. The data support the concept that the M cells derived from emergency reticulocytes while the small reticulocytes represented precursors of normal red cell maturation. The Na^+ and K^+ composition found in M cells of HK and LK sheep, respectively, suggest development of the LK steady state at or prior to the reticulocyte state, a finding consistent with that of Lee and Kirk (1982) on low K^+ dog red cells.

The high potassium (HK)/low potassium (LK) dimorphism of mature anucleate sheep red cells is characterized by different Na^+K^+ pumps and ouabain-insensitive, passive cation "leaks" both genetically associated with the M and L surface antigens [for detailed references, see recent review by Lauf (1982)]. The process by which the mature LK red cell develops in the hematopoietic system, i.e., the HK/LK transition, is still unclear. To address this problem, two experimental approaches have been used: first, the age-dependent replacement of fetal HK with adult LK cells in lambs (Evans and Blunt, 1961; Drury and Tucker, 1963; Dunham and Hoffman, 1971; Lauf et al., 1978; Valet et al., 1978; Lauf and Valet, 1980; Tucker et al., 1982), and second the study of membrane transport changes during maturation of reticulocytes to the final LK steady state cells in adult sheep (Lee et al., 1966; Dunham and Blostein, 1976; Kim et al., 1980; Blostein et al., 1981; Tucker and Young, 1981). In the present study, we followed the membrane transport events accompanying the in vivo maturation of stress-induced reticulocytes into both anucleate HK and LK red cells.

Our basic strategy was first to isolate by centrifugal elutriation and second to characterize by flow cytophotometry (Valet et al., 1978; Lauf and Valet, 1980) the reticulocyte populations newly produced in response to massive bleeding of both adult LK and HK sheep. In the cells thus separated and identified, we measured active K^+ pump and passive K^+ leak fluxes, and [^{3}H]ouabain binding. During the postanemic recovery phase we detected the simultaneous appearance in the peripheral blood of two distinct reticulocyte populations: one within a large, macrocytic cell population (population M cells), and a second within the adult population of LK red cells [here referred to as volume population "III" cells (Valet et al., 1978)].

With respect to their transport properties, the small reticulocytes were indistinguishable from adult type III red cells, while reticulocytes of the macrocytic red cell population of both LK and HK sheep were very much

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different. In LK sheep, M cells possessed a high cellular Na^+ content characteristic for adult LK cells, while in HK sheep they had much lower Na^+ steady-state levels. During volume reduction the passive K^+ permeability was increased in both LK and HK macrocytic cells. In regard to cell volume, M cells did not attain the final volume of adult sheep red cells during the period of observation. Preliminary reports of this work have been given elsewhere (Lauf and Valet, 1981).

MATERIALS AND METHODS

Experiments were carried out simultaneously on three adult whiteface sheep purchased from a local farm near Munich, West Germany. Two of the animals (No. 7 and No. 10) had LK red cells, while the third (No. 8) possessed HK cells. By immunologic tests with ovine anti-L or anti-M reagents and complement, LK sheep 10 and 7 were found to be homozygous and heterozygous for the L antigens, respectively, and the HK 8 red cells were M positive. During a span of 3 days, a total of 2 liters of blood/animal was removed (about 3% of body weight). Intramuscular iron injections were given to boost hematopoiesis. For subsequent analysis, not more than 5–10 ml of blood was taken from all animals at each time interval indicated.

Adult volume population III cells were separated by centrifugal elutriation from the macrocytic cells newly produced after bleeding. The fluid system of the elutriator rotor (Beckman, Fullerton, CA) contained a 300 mOsm NaCl solution buffered with 5 mM Tris-HCl to pH 7.35 (TBS). The elutriator centrifuge (Model J21B, Beckman Instruments, Fullerton, CA) was operated at 4°C and at a constant speed of 5000 rpm throughout the separation. The rotor chamber was filled with 2–3 ml of blood ($1–3 \times 10^{10}$ cells), and elutriation was started with a slow buffer flow rate to remove platelets, plasma, and cell debris. About 250 ml of buffer was used during this and each following elutriation step to assure complete separation of all cells to be collected at the following buffer flow rates: population III erythrocytes at 2 ml/min; an intermediate mixture of population III and population M cells that was discarded at 4 ml/min, and the large population M cells only, collected at 8 ml/min buffer flow rate. Usually six elutriator runs/sheep yielded sufficient numbers of cells for the subsequent flux experiments.

Cell volume distribution analysis

The cell volume distribution curves were determined by electrical sizing in a Metricell flow cytometer (Kachel, 1976) with a hydrodynamically focused particle inflow. Cells were suspended in TBS (16.5 mSiemens/25°C) and measured in a cylindrical orifice of 60 μm diameter and 52 μm length at a suction of 0.2 kg/cm^2 and an orifice current of 0.40 mA. Particle flow rates were between 500 and 1500 cells/sec. Volume distribution curves were recorded in a multichannel analyzer, transferred onto magnetic tape, and later fitted by logarithmic Gaussian normal distributions (Valet et al., 1976) in order to determine the mean volumes (in histogram channels) of the unseparated and separated cell fractions, the variation coefficients of the distributions, and the fractional contribution of normally sized populations III and population M erythrocytes.

Determination of absolute cell volumes

The mean cell volume in cubic micrometers was calculated from the mean channel number of the volume distribution curve, the conductivity of the suspending medium, the orifice radius and length, the form factor of the cells in the orifice, and the position of electric calibration pulses using a formula described earlier (Kachel, 1976).

Determination of cell surface

The Metricell flow cytometer (Cytomic) was filled with a hypotonic buffer obtained by diluting TBS 1+1 (v/v) with distilled water (150 mOsm, pH 7.35). The cells were suspended in this hypotonic buffer, and the volume distribution curve was determined after the erythrocytes reached the critical hemolytic (spheroid) volume and osmotic hemolysis ensued. The mean cell volume of the resealed ghosts measured in the flow cytometer was used to calculate the cell surface area (SA) as reported earlier (Lauf and Valet, 1980).

Reticulocytes staining

Two methods were used simultaneously. Sheep blood was mixed 1:1 (v/v) with a 1% brilliant kresyl blue solution in TBS and stained for 30 min at room temperature. A smear was then prepared on a glass slide, counterstained with Giemsa stain, and the number of reticulocytes/500 cells was counted microscopically. Alternatively 250 μl of 0.1% (v/v) cell suspension in TBS were stained with 5 μl of a 0.1% acridine orange solution in TBS for 15 min at room temperature. The cells were measured in a Fluvo-Metricell flow cytometer (Kachel et al., 1977), and the electric cell volume and the fluorescence of each cell were analyzed simultaneously. Reticuloctyes emitted fluorescence from their acridine orange-stained RNA while unstained mature erythrocytes did not. The data of the two parameter measurements were stored as a two parameter matrix on tape and analyzed by a computer program (Valet et al., 1976), which calculated the number of particles within partial areas of the histogram representing the fluorescent and nonfluorescent erythrocytes.

The fluorescence technique was 20% more sensitive than staining with brilliant kresyl blue. The advantage of the acridine orange dye lies in conjunction with the volume measurement allowing distinction of reticulocytes of population III and M (Valet and Lauf, 1980) and hence their quantitative separation (see below).

Cell separation by Percoll gradient centrifugation

To fractionate population III erythrocytes obtained by the first elutriation step further, a portion of these cells was centrifuged in a Percoll gradient. Percoll (Pharmacia Uppsala, Sweden) was suspended in TBS at the densities indicated. Population III cells were resuspended in Percoll/TBS with a density of 1.08 gm/ml and layered on top of a 25 ml continuous Percoll/TBS gradient (1.08–1.10 gm/ml) with a 1 ml cushion (1.12 gm/ml). After centrifugation at 1000g for 20 min at 4°C, the cells in the upper third of the gradient were pooled, washed twice in 25 ml TBS, and resuspended in the experimental media.

Cell cations, isotopic fluxes, and [^{3}H]ouabain binding

Cellular cations were measured using a Perkin-Elmer model 420 atomic absorption spectrophotometer (Joiner and Lauf, 1978a). Calculations of cation composition and ionic fluxes were based on mean corpuscular hemoglobin concentration, MCHC (kg/liter), and content/cell, MCH (gm/cell), and on cell volume, V_c (μm^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.

Unidirectional K^+ influx was measured using ^{86}Rb (specific activity 2.2–2.4 Ci/gm, New England Nuclear Industries, Boston, MA) by techniques described previously (Joiner and Lauf, 1978a,b; Lauf and Valet, 1980). Tracer uptake was stopped 30 min after addition of the isotope by separating the loaded cells from their medium using dibutylphthalate. Total K^+ uptake/kg hemoglobin (Hgb) was calculated from Eq. (2) of Lauf and Valet (1980) and converted into total K^+ influx per liter original cells, ${}^iM_K^T$, by multiplying with the MCHC. Active K^+ pump flux per liter cells, ${}^iM_K^P$, is the difference between ${}^iM_K^T$ and the ouabain-insensitive passive K^+ influx, ${}^iM_K^L$. Passive K^+ influx per surface area was computed from the relationship

$$ {}^iM_K^L = [({}^{86}Rb_c)/X_o \times t] / [(MCHC \times V_c)/SA], \quad (1) $$

from which the apparent K^+ permeability, P_K^{app}, was extracted by dividing ${}^iM_K^L$ by $[K^+]_o$. X_o is the specific activity of the tracer in the medium.

Binding of [^{3}H]ouabain to unseparated and separated red cells of all three sheep was studied by a method published earlier (Joiner and Lauf, 1978a,b). It was found that a time period of 30 min and a final ouabain concentration of 10^{-6} M were sufficient to yield maximal saturation of all Na^+K^+ pump sites. K^+ pump turnover was calculated from the relationship:

$$ PTO = ({}^iM_K^P \times N_A) / (N_c \times N_p \times 3600 \text{ sec}), \quad (2) $$

where N_A = Avogadro's number, N_c = number of cells/liter suspension, and N_P = number of [^{3}H]ouabain molecules/cell.

RESULTS

Red cell volume populations before and after phlebotomy

Figure 1 shows the cell volume distribution curves as a three-dimensional pulse height diagram as functions of time after massive hemorrhage. At day 0, the volume distributions of HK and LK red cells were of type III as previously classified for sheep (Valet et al., 1978) and other animals (Valet et al., 1976). It may be noted that the unbled LK 10 sheep possessed red cells with a mean corpuscular volume of about 37 μm^3 and hence were larger than red cells from LK 7 (31 μm^3) and HK 8 (33 μm^3). This fact was also supported by the basic hematological parameters of unseparated red cells listed in Table 1. The deviation seen in LK 10 sheep is not entirely clear and may be explained by assuming that prior to the experiment this sheep has been anemic (cf., hematocrit in Table 1).

Around day 6 after bleeding, bimodal cell volume distributions became evident due to the appearance of a large macrocytic red cell population (population M). Hence, there was an increase of the mean corpuscular volume, hemoglobin, and surface area at or shortly after the hematocrit had reached the lowest values (cf., Table 1). The newly produced M cells constituted about 20–25% of all red cells with maximal mean corpuscular volumes about 1.4- 2- and 1.7-fold larger than that of the population III cells in LK sheep 10, 7, and HK sheep 8, respectively (Table 2). The M cells disappeared only slowly from circulation, since 4 weeks after bleeding they constituted still about 10% of all cells.

The size of the cells of the M population permitted a nonoverlapping elutriation from the small population III cells. With a volume differential of 1.4–2.0, M cells were readily separated as shown in Figure 2B.

Evidence for two types of reticulocytes

Figure 3A,C shows that on day 6 about 15% of all peripheral red cells were reticulocytes in all three animals. Around days 6–8 those reticulocytes were preferentially among M cells but also in population III cells of all three animals (Fig. 3B,D). At later times, all reticulin-positive cells disappeared and those of the M-cell population did so with a time constant by far smaller than their volume reduction as monitored by the cytophotometer. On day 28 after bleeding, only a few percentages of reticulocytes were produced, irrespective of the sheep genotype.

Further evidence for two distinct reticulocyte populations was obtained by cytometric detection of acridine orange fluorescence. This finding was furter substantiated by hemoglobin analysis. The two LK sheep were of hemoglobin A type and the HK animal AB type. All three animals were tested by isoelectric focussing for stress-induced hemoglobin C formation. About 8–10 days after massive bleeding, hemoglobin C was present only in population M cells but absent in population III cells (Valet and Lauf, 1980). Hence, reticulocytes of population III were also biochemically distinct from those of population M.

Figure 4 reveals that the mean cell volume of the isolated type III and M cells from all three sheep were distinct from each other at all times, the M cells never attaining the volume of the smaller type III cells. However, at the early sampling points, the M cells were of largest volume declining to a more steady value 8 days after bleeding (Fig. 4A,D). Comparatively, no volume changes were seen in the cells of population III of either LK or HK sheep. The volume changes of the large cells were accompanied by changes primarily in cell K^+ content (Fig. 4B,E) with much less fluctuation in the cellular Na^+ content. An important finding was that the Na^+ content of large M cells in the two LK sheep exceeded significantly that seen in the M cells of the HK animal (Fig. 4C,F). On the other hand, cellular K^+ levels of population III cells were rather constant at all times, measuring about one-fifth or less in LK cells (Fig. 4B) as compared to HK cells (Fig. 4E). A relationship for cellular Na^+ levels (Fig. 6C,F) existed inversely proportional between LK and HK type III red cells, respectively.

TABLE 1. Basic hematological parameters of phlebotomized sheep: Unseparated cells

Sheep (antigens)	Days after bleeding	Hematocrit (v/v × 100)	Mean corpuscular volume (10^{-15} liter/cell)	Mean corpuscular hemoglobin (10^{-12} gm/cell)	Mean surface area (10^{-8} cm^2)
LK 10 (LL)	0	0.28	37.2	12.1	55.1
	1	0.22	35.4	12.3	55.6
	2	0.19	34.7	12.7	56.0
	5	0.18	37.3	12.9	57.3
	6	0.19	39.5	12.9	58.6
	8	0.20	38.2	12.8	60.0
	14	0.21	36.6	12.3	56.0
	28	0.23	37.5	12.6	54.7
LK 7 (LM)	0	0.36	30.7	10.7	54.6
	1	0.33	32.1	10.7	54.8
	2	0.24	33.0	11.3	55.2
	5	0.23	32.8	11.5	56.5
	6	0.23	37.7	13.3	58.2
	8	0.24	33.6	11.2	56.5
	14	0.24	34.5	11.6	56.5
	28	0.35	34.6	11.9	59.9
HK 8 (MM)	0	0.36	32.9	10.7	53.6
	1	0.29	29.9	10.1	52.8
	2	0.24	31.6	11.9	54.4
	5	0.22	34.0	12.4	55.2
	6	0.23	36.1	12.1	56.6
	8	0.25	37.4	13.3	57.5
	14	0.28	31.0	11.3	53.9
	28	0.32	32.7	11.2	57.8

TABLE 2. Basic hematological parameters of phlebotomized sheep: Separated cells

Sheep (antigens)	Days after bleeding	Fractional population distribution		Mean corpuscular volume (10^{-15} liter/cell)	
		Pop. III	Pop. M	Pop. III	Pop. M
LK 10 (LL)	0	100	—	37.2	—
	1	100	—	35.4	—
	2	100	—	34.7	—
	5	85.6	14.4	35.0	51.1
	6	84.5	15.5	37.0	53.3
	8	74.4	25.3	33.0	52.0
	14	88.1	11.9	35.3	46.6
	28	90.0	10.0	36.5	46.8
LK 7 (LM)	0	100	—	30.7	—
	1	100	—	32.1	—
	2	100	—	33.0	—
	5	95.2	4.8	32.8	66.0
	6	90.0	10.0	34.6	65.6
	8	88.2	11.8	31.1	52.1
	14	77.7	22.3	31.4	45.5
	28	92.2	7.8	33.4	49.3
HK 8 (MM)	0	100	—	32.9	—
	1	100	—	29.9	—
	2	100	—	31.6	—
	5	90.0	9.1	31.9	55.8
	6	89.6	10.4	31.8	53.0
	8	74.3	18.3	32.8	51.8
	14	88.3	11.7	31.0	46.6
	28	90.3	9.7	31.0	45.0

Active and passive K^+ fluxes and [^{3}H]ouabain binding

Figure 5 shows about 6 days after massive bleeding, the appearance of erythrocytes with higher K^+ pump activities (Fig. 5A,B) and increased apparent K^+ permeabilities (P_K^{app}) (Fig. 5C). The changes in the passive K^+ permeability seemed to trail those in the active K^+ transport, which returned to lower values as P_K^{app} still was rising. Anti-L_p stimulated LK pump fluxes throughout the observation period confirming our earlier findings that reticulocytes from LK animals possess the Lp antigen (Kim et al., 1980). There was practically no effect of anti-L_L on P_K^{app}, which may be due to the possibility that the cells may have been slightly shrunken. Cell shrinkage is known to reduce anti-L_L-sensitive, passive K^+ fluxes in LK cells (Dunham and Ellory, 1981). More experiments are required to assess the failure of anti-L_L to reduce ouabain-insensitive K^+ influx.

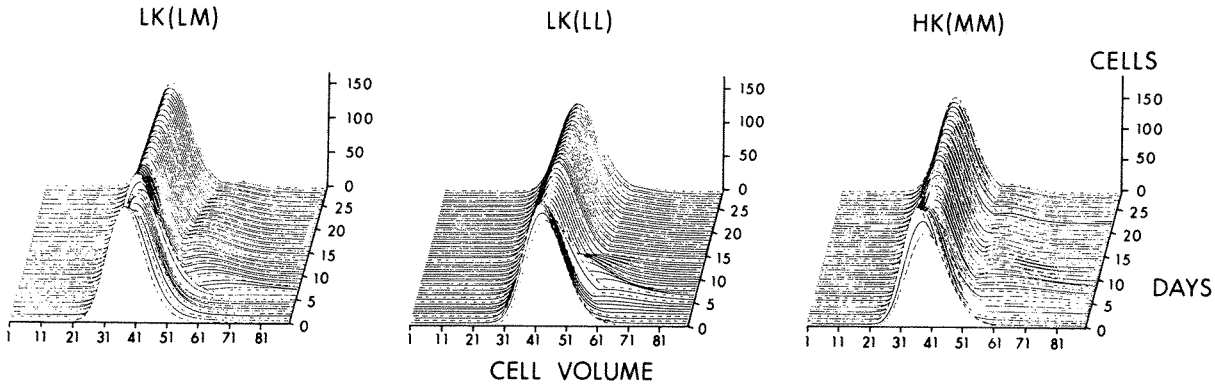

Fig. 1. Cell volumes μm^3 (x-axis) of unseparated red cells in sheep LK 7 (LM), LK 10 (LL), and HK 8 (MM) as function of time after massive hemorrhage (day 0, Z-axis). The y-axis represents the number of cells measured in each run. Note the presence of the dominant type III volume populations of adult red cells with mean peak volumes of about 33 μm^2 in the LM and MM and about 39 μm^2 in the LL animal. To the right of the main peak, there appears about 6 days after bleeding the shallow volume population of macrocytic (M) cells with volumes almost twice that seen in the adult type III cells. These M cells were barely detectable until the end of the experiment (day 28).

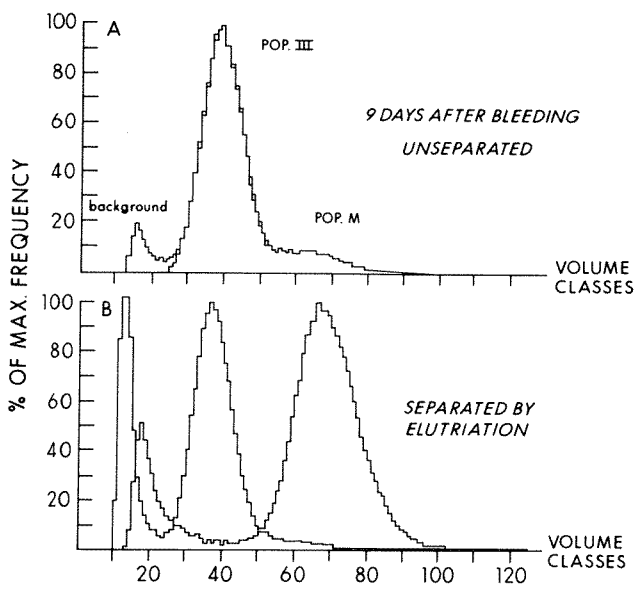

Fig. 2. Cell volume profiles of normal type III and macrocytic (M) erythrocytes of sheep 10 (LL) before (A) and after (B) separation by centrifugal elutriation. Relative cell volume classes (recording channels, x-axis) red cell numbers normalized to 100% (y-axis). Panel B shows a complete separation of the M cells from type III cells, which was feasible due to a ratio of mean volumes greater than 1.4 between the two cell types.

Panels D and E of Figure 5 show that in HK 8 red cells active and passive K^+ fluxes underwent changes qualitatively similar to those in the LK animals. Initially, HK red cells had a higher K^+ pump flux than LK cells. Around days 5–8 K^+ pump influx increased significantly followed by elevated P_K^{app} values, which gradually fell to base levels around day 28.

By comparison, there were rather large discrepancies between the P_K^{app} values measured in LK 10, LK 7, and HK 8. As expected, P_K was severalfold larger in normal LK cells than in HK cells. The passive basal K^+ fluxes were about fivefold different between LK 7 (Fig. 5C) and HK 8 cells (Fig. 5E). However, the P_K^{app} values measured in LK 10 (Fig. 5C) by far exceeded those of LK 7.

Figure 6 shows that large differences existed in K^+ pump and leak fluxes of separated M and type III cells, respectively. The K^+ pump activities of the type III red cells were of the magnitude known for adult LK and HK sheep red cells. In sharp contrast, however, K^+ pump fluxes in M cells of all three animals (days 5–8) were much higher than those of type III cells. In particular in LK 10 sheep, $^iM_K^P$ of M cells was more than an order of magnitude higher than the pump activity seen in type III cells. Anti-L stimulated K^+ pump flux at all time points in both type III and type M cells of LK sheep 7. The absolute magnitude of the anti-L effect was largest in the type M cells toward the end of the observation period and smallest at the time of their first appearance in the peripheral circulation. Hence, the ratio of $^iM_K^P$ in the presence to that in the absence of anti-L_p increased between days 5 and 28 in M cells, while it remained rather constant in type III cells. It is not quite clear why in LK 10 anti-L_p had only a marginal effect.

The ouabain-insensitive K^+ leak fluxes (P_K^{app}) were also strikingly different in the M type and small type III cells (Fig. 6B,D,F). The M cells of LK 10 showed the largest changes in P_K^{app} falling from 2×10^{-8} to 6×10^{-9} cm/sec. As compared to the P_K^{app} values of LK 7 type III cells P_K^{app} of the small type III cells of LK 10 was also considerably elevated. In M cells of the HK animal, passive K^+ influxes were clearly elevated, but declined to the low values of the mature type III cells around day 28 posthemorrhage (Fig. 6F).

Figure 7A,C,E shows the number of [^{3}H]ouabain molecules (normalized per surface area) bound to unseparated and separated type III and M cells in the two LK and one HK sheep studied. Consistent with our previous work (Joiner and Lauf, 1978a), unseparated LK and HK red cells obtained prior to bleeding bound about one and two molecules ouabain per square micrometer of surface area, respectively, corresponding to about 50 and 110 Na^+K^+ pumps per LK and HK cell, respectively. These

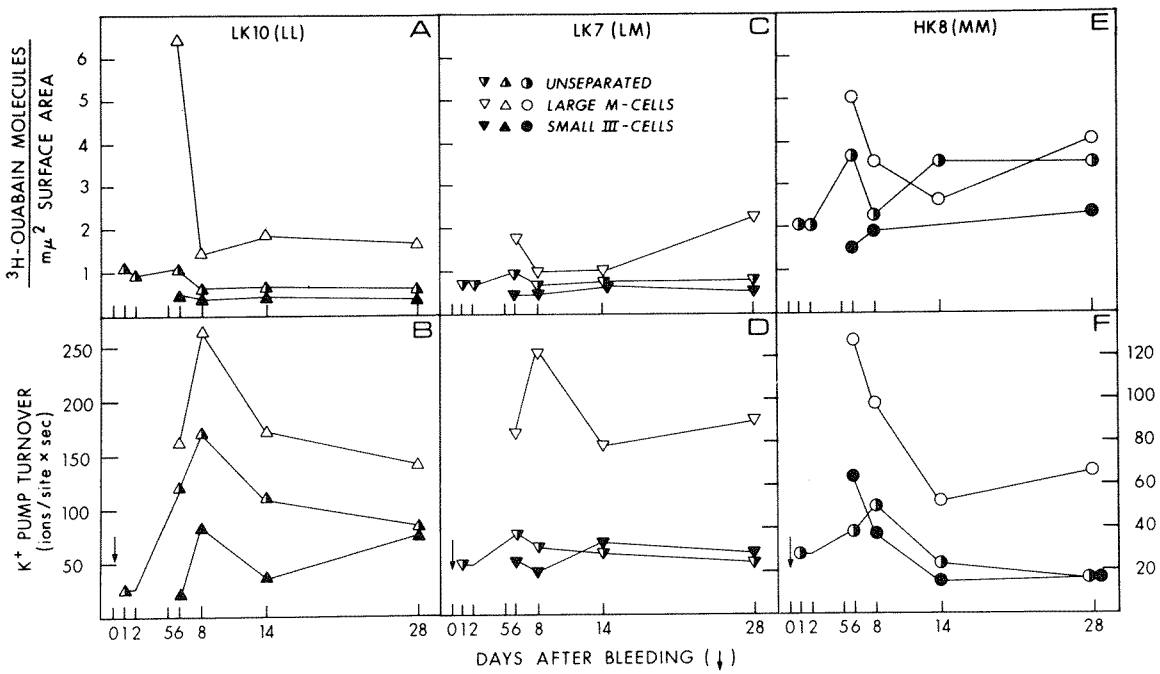

Fig. 7. The number of [^{3}H]ouabain molecules bound per unit surface areas and K^+ pump turnover in unseparated and separated M and type III red cells of anemic sheep. Open symbols stand for M cells, half-filled symbols for unseparated cells, and filled symbols for small type III cells. Note the expanded scale on the y-axis for (D) and (F). For details see text.

numbers changed little as the large M cells entered circulation around day 5, although there were some fluctuations in unseparated HK cells (Fig. 7C). In all three animals, the small type III cells had always the lowest numbers of ouabain-binding sites, while the large M cells of the two LK sheep had about twofold elevated [^{3}H]ouabain-binding sites during the entire observation period. An exception were the M cells of LK 10, which on day 6 had more than 300 sites/cell. In M cells of the HK sheep, [^{3}H]ouabain binding was initially elevated but then could not be distinguished further from unseparated cells.

Figure 7B,D shows that the initial K^+ pump turnover of the two unseparated LK cells was between 20 and 40, and 60 for the HK cells. These numbers are compatible with our earlier report (Joiner and Lauf, 1978a). As between days 6 and 8 reticulocytes entered circulation, the K^+ pump turnover in unseparated LK 10 cells reached a maximum and increased parallel in type III and M cells followed by a decline in all cells (Fig. 7B). The increase to 250 ions/site × second on day 8 is recognized to be somewhat high and may be due to an artifactual underestimation of the number of ouabain-binding sites. In comparison to LK 10, the cells of LK 7 revealed much less fluctuation of K^+ pump turnover numbers, which were similar in unseparated and type III cells. In striking contrast, the M cells of this sheep had high K^+ pump turnovers at all times (Fig. 7D). The changes in K^+ pump turnover seen in HK sheep 8 were similar to those of LK sheep 7 (Fig. 7E,F).

Cation transport in type III reticulocytes

Using a Percoll gradient centrifugation following the elutriation step, the small reticulocytes in the type III volume population of days 5 and 6 were enriched up to 28% as shown in Table 3. The cellular K^+ contents indicated the presence of rather mature LK 10 and LK 7 red cells, and typical HK red cells. Active K^+ pump fluxes, pump density, and turnover measured on day 6 were characteristic for LK cells stimulated by anti-L_p (fourfold in LK 7, and much less in LK 10 sheep). The elevated $^iM_K^P$ value of HK 8 type III reticulocytes apparently was due to a pump density higher than that found in adult HK sheep red cells.

DISCUSSION

Separation of red cells by volume permitted to follow the process of reticulocyte maturation in HK and LK sheep by an in vivo approach contrasting with in vitro studies by others on the same (Tucker and Young, 1980; Kim et al., 1980) and other mammalian red cells (Zeidler and Kim, 1982). The following observations warrant emphasis: (1) About 5–6 days after massive phlebotomy of the sheep, large erythrocytes belonging to a new macrocytic red cell population (population M) were detected in the peripheral circulation by flow cytophotometric analysis (Fig. 1). The mean corpuscular volume of M cells was at least twice that of population III cells (Figs. 2 and 4, Table 2). (2) At early times, the large M cells were primarily large reticulocytes (Fig. 3). Although M

TABLE 3. Transport parameters of reticulocyte enriched volume population III cells

Sheep	Days after bleeding	Reticulocytes (%)	$[K^+]_c$[1]	anti-L	$^iM_K^P$[2]	PTO[3]	P/SA[4]	P_K^{app}[5]
LK 10	6			−	0.19	25		6.5
(LL)		18.0	0.25	+	0.24	32	0.8	6.1
LK 7	6			−	0.14	35		1.2
(LM)		28.0	0.55	+	0.47	120	0.4	1.3
HK 8	6	27.0	2.48	−	1.53	48	3.6	1.4
(MM)								

[1] 10^{-15} mol/cell.
[2] mM/liter cell × hr.
[3] K^+ ions/pump × sec.
[4] pumps/μm^2 surface area.
[5] cm/sec × 10^9.

cells harvested at later times were of somewhat reduced volume, they did not attain that characteristic of that of adult erythrocytes in volume population III. (3) In contrast, and inversely related to M cells of HK sheep, M cells of LK sheep had lower K^+ and comparatively higher Na^+ contents, the latter already characteristic for adult LK red cells (Fig. 4C,F). Nevertheless, volume reduction observed in M cells of both HK and LK sheep appeared to be accompanied primarily by changes in cellular K^+ (Fig. 4B,E) and much less in Na^+ levels (Fig. 4C,F). (4) In red cells of LK and HK sheep, K^+ pump and ouabain-insensitive K^+ leak fluxes rose at about 5–6 days posthemorrhage and fell gradually thereafter (Fig. 5). The increased K^+ fluxes were primarily due to the appearance of M cells in the peripheral blood (Fig. 6). In general, the greater K^+ pump flux activity was attributed to K^+ pump turnover rather than to the modest increase in the number of K^+ pump sites as estimated by ouabain binding. (5) A second cohort of *small* reticulocytes was identified in volume population III with membrane transport properties and cation steady state levels close to those of adult LK or HK red cells (Fig. 3, Table 3).

The large cells (M cells) of the present study isolated by centrifugal elutriation exhibited volumes (about 60 μm^3) similar to those reticulocytes with low buoyant density studied earlier (Kim et al., 1980). However, in the older study, a further analysis of cellular maturation of these cells was difficult due to their density changes accompanying maturation. Moreover, no distinction was made between the two reticulocyte populations identified here by two different methods. Several findings support the concept that the reticulocytes of the M population were emergency cells quite different from the small reticulocytes of population III: M cells exclusively contained hemoglobin C while population III cells did not (Valet et al., manuscript in preparation), and M cells displayed different electrophoretic mobilities (Valet and Lauf, 1980) than population III reticulocytes. Population III reticulocytes, on the other hand, were the final products with respect to their physiological LK and HK cation transport properties and hence may be considered physiologically representative for normal erythrocyte maturation.

One of the most intriguing findings of this study was that, with approximately similar total cation contents, the early M cells (reticulocytes) of LK sheep already possessed a cellular Na^+ content that was typical for that of adult LK erythrocytes and severalfold higher than that in HK reticulocytes. This finding speaks for Na^+ accumulation by the prospective LK cells prior to or at the beginning of its reticulocyte stage by a mechanism not yet understood. Interestingly, Lee and Kirk (1982) and Kirk et al. (1983), analyzing the cell cation/iron ratios in reticulocytes of phenylhydrazine-injected dogs, concluded that modification of the membrane transport function leading to the LK cell status must have occurred before or during the reticulocyte formation. These common observations of these authors as well as of the present work are of interest with respect to the question of whether or not high cellular K^+ levels are required for protein synthesis (Cahn and Lubin, 1978; Moscatelli et al., 1979: Frantz et al., 1980).

The apparent passive K^+ permeabilities were found to reach values in the order of 10^{-8} cm/sec. In vitro maturing reticulocytes of newborn pigs also display volume reduction associated with K^+ loss (Zeidler and Kim, 1982). In addition, rat reticulocytes have severalfold higher passive K^+ fluxes than adult cells (Furukawa et al., 1981), and Panet and Atlan (1980) have attributed the presence of a high ouabain-insensitive K^+ flux in rabbit reticulocytes to a furosemide-sensitive specific K^+ carrier that is lost upon cellular maturation. Recent findings from this laboratory suggest that a substantial fraction of the ouabain-insensitive K^+ fluxes in LK and HK reticulocytes is Cl^--dependent and may be stimulated by the thiol group reagent N-ethylmaleimide (Lauf, 1983). The continuous presence of Cl^--dependent (Dunham and Ellory, 1981) and N-ethylmaleimide-stimulated (Lauf and Theg, 1980) K^+ transport in mature LK but not in HK red cells coupled with the higher Na^+ content in reticulocytes of LK sheep poses an interesting problem for the origin of the LK steady-state cell.

Compared with other biological cells, mature sheep red cells are known to have the lowest number of Na^+K^+ pumps/cell surface area (Joiner and Lauf, 1978a,b). We expected that the reticulocyte precursors of both HK and LK cells would display a much higher number of pumps than adult cells, particularly in light of reports of increased numbers of ouabain binding sites in immature human red cells of dyserythropoeitic anemias (Wiley and Shaller, 1977; Wiley, 1981). However, the total number of ouabain-binding sites of the separated M cells in both LK and HK sheep was only modestly increased

with exception of one measurement (LK 10, day 6). There are no data available on ouabain binding to bone marrow cells. From work on nucleated cells with high metabolic activities (Becker and Willis, 1979; Kirk et al., 1983), one may infer that cell stages prior to the reticulocytes have higher pump numbers, which then become considerably reduced during maturation to the anucleate cells as shown for the rat by Furukawa et al. (1981). Our data emphasize changes in K^+ pump turnover more than in K^+ pump sites.

If the maturation process observed in the M cells resembles to some degree that occurring in the small reticulocytes (i.e., the actual precursors of normal adult LK or HK cells) the following processes may be considered part of the HK–LK transition: An early acquisition in reticulocytes of a Na^+ content characteristic for the prospective adult LK or HK cells, and a partial volume reduction perhaps mediated by K^+ loss in both LK and HK precursor cells perhaps by a K^+/Cl^- carrier. Considerably high K^+ pump turnover compensates for an apparently high passive K^+ permeability. The correlation between K^+ pump turnover and passive K^+ permeability, mostly evident in LK 10 sheep, is heartening with regard to the proposed pump/leak concept (Tosteson and Hoffman, 1960) and deserves further attention.

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