

Different Red Cell Populations in Newborn, Genetically Low Potassium Sheep: Relation to Hematopoietic, Immunologic and Physiologic Differentiation

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ABSTRACT Three red cell populations have been distinguished in genotypically low potassium (LK) newborn sheep by an improved electrical sizing method and were best approximated by a logarithmic normal distribution. Labeling studies with ⁵¹Cr and ⁵⁹Fe exclude transformation of the three red cell populations into each other. Population I, consisting of large red cells (mean volume 36 μm^3), with a comparatively slow electrophoretic mobility is present at birth and disappears within three to four weeks from circulation. These cells possess a high potassium (HK) steady state concentration, a K⁺ pump influx activity at least 5-fold greater than observed in adult LK red cells, very low amounts of the L antigens generally associated with the LK property, and do not respond to the stimulatory action of the L antibody. The first population is gradually replaced by population II comprising small red cells (mean volume 28 μm^3) of intermediate electrophoretic mobility and with a peak production around day 20 after birth. The potassium concentration, [K⁺]_c, in these cells appears to be lower than in the cells of population I but the L antigen content is increased. Formation of population III (mean volume 30 μm^3 and comparatively fast electrophoretic mobility) follows closely that of population II and is preceded by a sharp increase in reticulocytosis. The red cells of population III exhibit parameters characteristic for adult LK cells: low [K⁺]_c and K⁺ pump activity, fully developed L antigen content, and an almost maximal response to the K⁺ pump stimulating effect of anti-L. In L and M antigen positive LK red cells of newborn sheep, the development of the M antigen parallels that of the L antigen. The data are consistent with the hypothesis that cellular replacement and not maturation is the major factor in controlling the HK-LK transition in newborn sheep.

The erythropoietic differentiation in mammals after birth is characterized by changes of various erythrocyte parameters. For example, red cells from newborn sheep have osmotic properties (Widdas, '51), cell volume (Riegel et al., '61; Ullrey et al., '65; Moore et al., '66), enzymes (Kornfeld et al., '67), protein (Fésüs and Rasmussen, '71) and hemoglobins (Moore et al., '66; Huisman et al., '69; Huisman, '74; Blunt and Huisman, '75) different from those found in red cells of adult animals. A basic question is whether these parameters change independently, each due to a different stimulus, or simultaneously at a given time induced by one stimulus. The concept of a simulta-

neous switch of several parameters implies the existence of several production programs or gene regulation patterns in the precursor cell which, according to the needs of the organism, are activated or repressed. The general interest in such a mechanism stems from hematopoietic disorders characterized by an acute arrest or a deviation of differentiation.

A simultaneous differentiation switch in the hematopoietic system during postnatal development is supported by several investigations on mammalian organism. In newborn rats (Valet et al., '72a,b; Valet et al., '74;

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Hanser et al., '74), guinea pigs (Valet et al., '76), mice and rabbits (Valet et al., '74; Dorsey and Lofberg, '66) several erythrocyte populations of different mean volume were distinguished by an improved electrical sizing technique (Kachel, '76). These red cell populations of the newborn sequentially appear and disappear and are finally replaced by an erythrocyte volume population characteristic of the adult animal.

The present study was undertaken to investigate if different volume populations could also be distinguished in the newborn sheep. Sheep are particularly suitable for such an approach since the adult animals possess red cells with high potassium (HK) or low potassium (LK) steady state levels while all lambs are born with red cells always of HK phenotype (Blechner, '61; Tosteson and Moulton, '59; Tosteson, '66; see also reviews by Tucker, '71 and Lauf, '75a). The gene responsible for the LK character is dominant (Evans and King, '55; Evans, '57; Evans and Phillipson, '57). The "HK-LK transition," also observed in cattle red cells (Israel et al., '72), has been interpreted in terms of cellular replacement or maturation (Tosteson, '63, '66). A detailed analysis of the hematopoietic events occurring in the sheep during the first three months of life must involve measurements of the K^+ steady state levels, and of the Na^+K^+ transport systems which are also different in the two cell types (Tosteson and Hoffman, '60; Tosteson, '63). Additional markers are the M/L red cell antigens, genetically associated with the HK and LK red cell type (Rasmusen and Hall, '66; Rasmusen, '69; Tucker, '68; Ellory and Tucker, '69; Tucker, '71), which can be analyzed by immune hemolysis using anti-M and anti-L antibodies and complement (Lauf and Tosteson, '69; Lauf and Dessent, '73), and by the effect of anti-L to stimulate K^+ pump flux in LK red cells (Ellory and Tucker, '69; Lauf et al., '69, '70; Lauf et al., '71). The results reported in this study are consistent with the concept that cellular replacement equivalent to a switch of various parameters rather than maturation determines the erythropoietic differentiation in the young sheep.

MATERIALS AND METHODS

Animals and blood types

The red cells of four pregnant Merino Landrace ewes, obtained from different breeding farms near Munich, were of LK type and

possessed only the L antigen indicating homozygous LK animals. Each ewe gave birth to one lamb designated A, B, C and D. By progeny analysis lamb A was homozygous LL (LK) and lambs B, C and D heterozygous LM (LK). The hematological, immunological and sizing studies were done on lambs A-D. The red cells of the ewes served as controls. Lambs A and B were also chosen for the studies involving the use of ^{59}Fe and ^{51}Cr .

In addition, a parallel study on LL (LK) red cells of one Dorset ewe and her LM (LK) lamb, lamb E [sired by a MM (HK) ram], was carried out at Duke University in order to correlate active K^+ transport in the presence and absence of anti-L with the hematological, immunological and sizing data generated in Munich on animals A-D. The cationic and antigenic composition of the red cells of both the ewe and the ram were well known enabling us to predict the LM (LK) phenotype of the lamb E at Duke.

Blood was collected from the jugular vein in 5-ml portions in EDTA (ethylenediamine tetraacetic acid) coated polycarbonate tubes. The erythrocyte concentration in the blood was determined with a Coulter Model A electric particle counter at a dilution of 1:200,000 in a 300 mOsm NaCl, 5 mM Tris/Cl buffered solution of pH 7.4 (TBS). From hematocrits and red cell concentration the mean corpuscular volume (MVC) was calculated. Reticulocytes were stained with a 1% Brilliant Cresyl blue solution (Merck, Lot 1280, Darmstadt, Germany) and the number of reticulocytes per 500 or 1,000 red cells was counted.

Sizing of the erythrocytes

Sheep 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 TBS with a specific electrical resistance of $62 \Omega \text{ cm} / 25^\circ \text{C}$ and sized with a cylindrical orifice of $50 \mu\text{m}$ diameter and $40 \mu\text{m}$ length at 0.35 mA electrical current, a suction of 0.1 kg/cm^2 and a flow rate of 1,000-1,500 particles/sec at a temperature of 25°C with a Metricell (Kachel, '76) connected with a Telefunken sizing system (Coulter Company). The electrical volume pulses were amplified and classified in a 128-step multichannel analyzer. 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., '76).

Radiolabeled erythrocytes

⁵¹Cr studies

Ten millimeters of blood, collected from lamb B on day 5 after birth, were centrifuged at 3,000g for ten minutes. The supernatant plasma was removed and the erythrocyte sediment was washed twice with 50 ml TBS at 0°C. The erythrocyte sediment was then resuspended in 2 ml of the same buffer containing 270 μ Ci of Na₂Cr⁵¹O₄ (200-500 Ci/g Chromium, NEN, Co., Cambridge, Massachusetts). The suspension was incubated for one hour at 37°C, washed three times to remove the radioactivity not bound to cells. Finally the cells were resuspended in their plasma and reinjected intravenously into the animal. At various times after the injection blood was taken from the animal, the erythrocytes were washed twice in TBS, and resuspended at a concentration of 1×10^8 /ml. Of this suspension 0.2 ml were layered on top of a 10-ml syringe filled with TBS containing 5% bovine serum albumin. After two to three hours sedimentation at 4°C and one $\times g$, the content of the syringe was fractionated. Samples of all fractions were sized and the fractions containing mainly small erythrocytes were pooled. Between 80-90% pure small population II erythrocytes were obtained, counted for radioactivity and cell concentration. The radioactivity per 10^7 cells of the small volume population II (x) and of the large volume population I and III (y) erythrocytes, respectively, was calculated from the following equations:

$$(1) \quad a_1x + b_1y = s,$$

rearranged to

$$(2) \quad x = \frac{s - b_1y}{a_1}$$

$$(3) \quad a_2x + b_2y = n$$

Equation 3, was combined with equation (1) and resolved to

$$(4) \quad y = \frac{na_1 - sa_2}{a_1b_2 - a_2b_1};$$

where a_1 and b_1 represent small and large erythrocytes in the sedimented samples, a_2 and b_2 small and large red cells in the unsedimented erythrocyte suspensions (both determined by volume distribution analysis), and s and n are the cpm per 10^7 sedimented and unsedimented erythrocytes, respectively.

The cpm per population II and population I and III erythrocytes per animal (see table 1 under RESULTS) were calculated from the x

and y values, the total erythrocyte number per animal and the percent contribution of each erythrocyte population to the volume distribution curve.

⁵⁹Fe studies

Fifty μ Ci ⁵⁹FeCl₃ (2-40 Ci/g Fe, NEN Co.) in 5 ml TBS were injected intravenously into lamb A on day 27 after birth. Blood was taken at various time intervals. Preparation of the red cells for counting and calculation of data was done according to the procedure outlined for the ⁵¹Cr studies (see also below).

Radioactivity counting

Whole blood (50 μ l) was precipitated with perchloric acid and decolorized with hydrogen peroxide in polyethylene liquid scintillation vials at 60°C for one hour. Ethylene glycol and toluene with 0.6% PPO (2,5 Diphenyloxazol, Merck No. 2946) were added as described (Dorsey and Lofberg, '66). The radioactivity of the samples was determined in a liquid scintillation counter (SL30, Intertechnique, France). The counts were punched on paper tape. Background subtraction, corrections for decay and the external standardization were calculated with a digital computer from the paper tapes.

Electrophoretic mobility of erythrocytes

The mean electrophoretic mobility of the erythrocytes was determined with a Zeiss analytical cell electrophoresis system (Carl Zeiss, Oberkochen, Germany) using a cylindrical chamber of 2.4 mm internal diameter. The erythrocytes were suspended in a solution of 155 mM NaCl, 0.3 mM NaHCO₃ pH 7.4 with 66 Ω cm specified electrical resistance at 25°C. An electrical field of 3.5 V/cm was applied and the migration time of 25 or 50 erythrocytes over a distance of 20 μ m was measured and punched on paper tape. The electrophoretic mobility of each cell, the mean and standard deviation, and the distribution of the electrophoretic mobilities of all cells was calculated by a digital computer from the paper tapes.

Titration of L and M antigens

The cellular activity of the L and M antigens was measured by hemolytic titration using anti-L and anti-M antibodies and complement as previously described (Lauf and Tosteson, '69; Lauf and Dessent, '73). The two L antisera used were S 39* and S 32*, and the

M antiserum was S 31B. All three antisera were L and M monospecific and obtained from Doctor B. A. Rasmussen, University of Illinois, Urbana, Illinois. Red cells (0.2 ml, 5×10^7 cells/ml), 0.2 ml anti-L or anti-M serum of different dilutions (1/5-1/150), and 0.2 ml 1/10 diluted guinea pig serum as the complement source were incubated for two hours at 37°C. After centrifugation the degree of lysis was spectrophotometrically determined at 412 nm in the supernatants. Appropriate controls of red cells alone, guinea pig serum and red cells plus guinea pig serum were included in all experiments. Guinea pig serum was absorbed twice with 1/10 volume of LL (LK) sheep red cells in order to reduce L/M nonspecific hemolysis (anti-T mediated lysis: Lauf, '75b). Lysis was expressed in percent hemolysis of an equal number of cells hemolyzed in distilled water. The antigen titration of lamb A was performed with red cells which had been stored by freezing in the presence of 7.5% dimethyl sulfoxide (DMSO). After thawing the erythrocytes were washed once in a 500 mM sucrose solution followed by a second wash in a 300 mM sucrose solution and a final wash in TBS containing 5 mM KCl, 0.15 mM Ca^{++} and 0.54 mM Mg^{++} . This procedure was necessary to remove intracellular DMSO without substantial loss of red cells due to osmotic hemolysis. In pilot studies it was shown that such a treatment did not alter the immunological reaction significantly.

Cellular cations and K^+ influx measurements

In the experiments on lambs A-D, 1 ml EDTA blood was washed twice with 10 ml 300 mOsm MgCl_2 , adjusted to pH 7.4 with MgCO_3 . The final supernatant was discarded and the erythrocyte sediment lysed by the addition of 2 ml 1 mM CsCl solution. The lysates were frozen and stored at -20°C until measurement of cation concentrations by flame photometry. The computation of $[\text{Na}^+]_c$ and $[\text{K}^+]_c$, expressed in millimoles per liter of packed cells, is based on the microhematocrit of the original blood and the absorbance at 541 nm of hemoglobin in whole blood and in the diluted cell suspension, both hemolyzed in 1 mM CsCl, as previously reported (Lauf et al., '70).

In the experiments on lamb E, 5-8 ml of blood was drawn by venipuncture into heparin containing tubes and on the day indicated under RESULTS. From the MCV, obtained from hematocrit and cell count (Coulter Counter,

Hialeah, Florida), and hemoglobin concentration, determined spectrophotometrically at 527 nm (isobestic point), the mean corpuscular hemoglobin concentration (MCHC) was computed. For measuring cellular cations, the fresh cells were separated from their plasma by centrifugation through dibutyl-phthalate ester as described earlier (Joiner and Lauf, '75). After pelleting, the cells were hemolyzed and $[\text{Na}]_c$ and $[\text{K}]_c$ determined by atomic absorption spectrophotometry (Perkin Elmer, Model 460). For computation of intracellular $[\text{Na}^+]_c$ and $[\text{K}^+]_c$ (in mmoles/L original cells) the amount of each cation in millimoles per Kg hemoglobin was multiplied by the MCHC.

The ^{42}K influx measurements were done as described in other reports (Lauf et al., '70; Lauf et al., '71; Lauf and Joiner, '76). Cells (3×10^9 /ml) were preincubated for 30 minutes in a medium containing in mM: 140 Na^+ , 5 K^+ , 5 glucose, 10 Tris/Cl, pH 7.4 (at 37°C), ± 0.1 ouabain, ± 0.02 anti-L IgG₁ (Snyder et al., '71; Lauf and Sun, '76). Following the addition of 20-40 μCi ^{42}K /ml (high specific activity) samples were taken at two time points (30 and 90 minutes), the cells separated from their medium by the phthalate method (Kepner and Tosteson, '73; Lauf and Joiner, '76; Joiner and Lauf, '75) and analyzed for ^{42}K uptake and hemoglobin. $^{42}\text{Potassium}$ influx (mmoles K^+ /L cells \times hr) was calculated from the radioactivity taken up by these cells between the two time points and from the specific activity of ^{42}K in the supernatant as described earlier (Lauf and Joiner, '76). By definition, K^+ pump influx (IM_K^p) is the ouabain sensitive component of the total K^+ influx measured in the presence or absence of anti-L. The anti-L concentration used here has been found to stimulate K^+ pump influx in adult LL (LK) red cells 4- to 6-fold (Lauf et al., '77).

RESULTS

Erythrocyte volume populations

The volume distribution of erythrocytes from an adult sheep (mother of sheep B) is best approximated by a logarithmic normal distribution of volume with a modal volume of $29.5 \mu\text{m}^3$, a median volume of $30 \mu\text{m}^3$, a mean volume of $30.5 \mu\text{m}^3$ and a linear coefficient of variation of 17.2% (fig. 1b).

In contrast, red cells of lamb B at 14 days of age showed a bimodal volume distribution (fig. 1a) indicating that a mixture of red cells of normal and small sizes was present in the

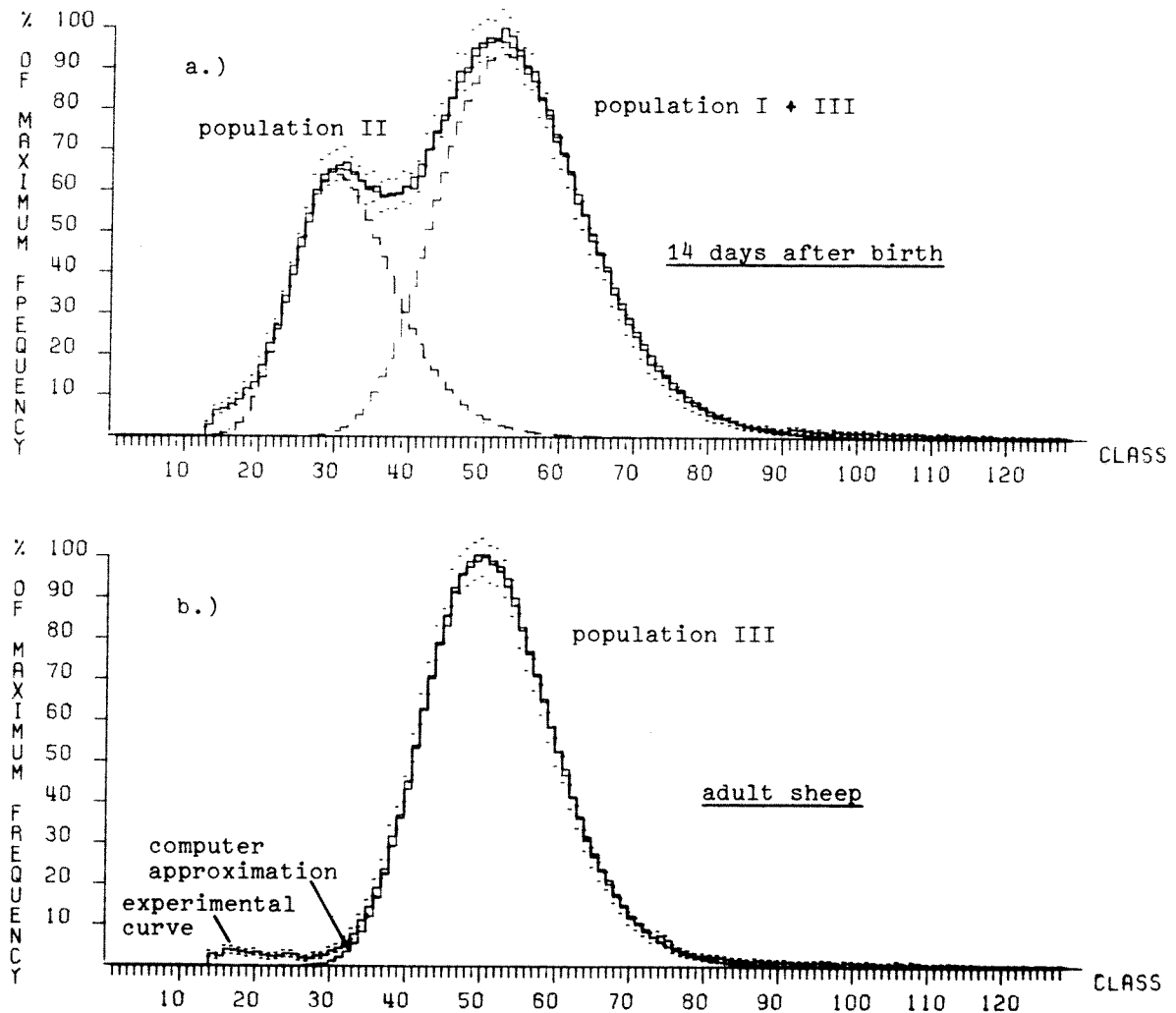


Fig. 1 Volume distribution curves of erythrocytes from 14-day-old sheep B (1a), and a normal adult sheep (1b). The experimental curves are approximated by one or two logarithmic Gaussian normal distributions. Drawn are the experimental curve, the logarithmic distributions and the sum of the single Gaussian distributions. The summative curve fits well the experimental curve in all examples and remains in the central parts of the curves well within the \pm three standard deviation limits indicated by the horizontal bars above and below the experimental curve. Standard deviations of each histogram class are calculated as the square root of the experimental class content. The young sheep (1a) has two erythrocyte populations of different mean volume, and the adult animal has one erythrocyte volume population (1b).

peripheral blood at this time and persisting for three months (fig. 2). The MCV of these populations diminished gradually as the lamb matured (fig. 3a). Similarly, the linear coefficient of variation of the larger and smaller red cells decreased with time (fig. 3B). A temporary increase of the coefficient of variation for the larger erythrocytes coincided with the appearance of the third and final red cell population, indicating less precision of the red cell volume during the phase of rapid production of population III. The MCV determined from the volume distribution curves, and those calculated from hematocrit and red cell counts (data not shown) agreed closely and showed that the formation of new volume populations

was not paralleled by dramatic changes of the MCV of the cell collective.

The curve analysis alone provided no information as to which population increased or decreased in the animal over a period of time. It also did not indicate whether the large or small red cell population in itself is heterogeneous. To obtain this information, the absolute number of large and small erythrocytes per animal was calculated from the product of the red cell concentration, the blood volume and the fraction of large and small erythrocytes as obtained from the volume distribution curves. The blood volume was assumed to be 7.6 body weight percent, a value interpolated from blood volume data re-

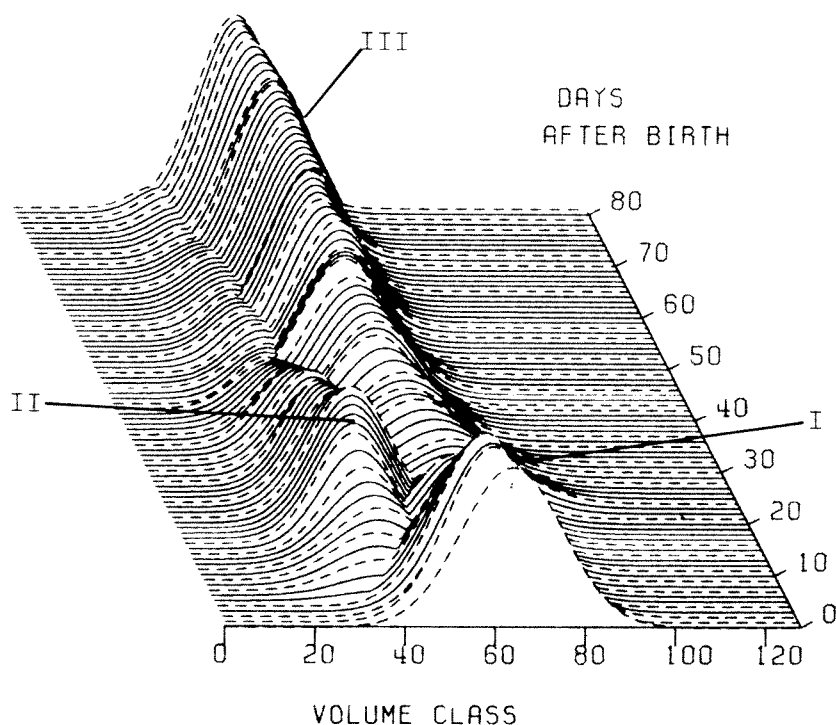


Fig. 2 Development of the erythrocyte volume populations in newborn sheep A. In this representation the measured volume distribution curves are plotted as dashed curves, the solid line curves are calculated by linear interpolation to increase the 3-dimensional impressions of the graph. In the newborn animal only large erythrocytes of population I are present. Beyond day 5 after birth the intermediate, smaller population II erythrocytes become apparent. Around day 20 they are gradually replaced by the final erythrocyte population III of the adult animal.

cently compiled (MacFarlane, '75). The total number of large red cells per newborn sheep (fig. 4A) decreased until day 22 after birth and then rose steeply accompanied by a transient reticulocyte peak corresponding to the highest daily production rates (fig. 4B). The initial decrease and subsequent increase of the large red cells suggested two different populations of large cells, one of which was present at birth and decreased continuously thereafter and a second which was produced from day 15 on and constituted the finite red cell volume population characteristic of adult sheep. In addition to the large red cell populations an intermediate population of small cells was formed beginning with day 5 after birth with a maximum of 31 days and a gradual decline thereafter. According to their time of appearance the large red cell populations were named I and III and the small population II.

The existence of several red cell populations in the blood of the young sheep raised the important question of the mechanism of production. Two possibilities were examined. First, red cell population II may have been derived from population I by volume reduction within peripheral circulation ("maturation"), and second, population II red cells were made

directly and independently in the hematopoietic organs and thus replaced the first population. To test the first possibility, i.e., transformation or maturation in the peripheral blood, red cells of 5-day-old lamb B were radiolabeled with ^{51}Cr and reinjected into the animal. At this time there were 93% large and 7% small erythrocytes. If transformation occurred the radiolabel should have gradually increased in the small population II red cells. However, as shown in table 1, the ^{51}Cr content decreased to 1/6 at day 26 after birth. Although selective elution of ^{51}Cr label from population I cells cannot be ruled out, several lines of evidence favor the assumption that most red cells of population II, appearing during this time (fig. 4A), could not have been derived from population I by volume reduction but rather were directly produced by the hematopoietic tissue. There was a transient increase of the reticulocyte counts (fig. 4A) which coincided with the appearance of population II and III red cells. The immediate ^{59}Fe incorporation into population II and III red cells of lamb A (table 1) indicated a direct production of these cells by the hematopoietic tissue. In addition, the total number of erythrocytes per animal (fig. 4A) increased continuously in the maturing

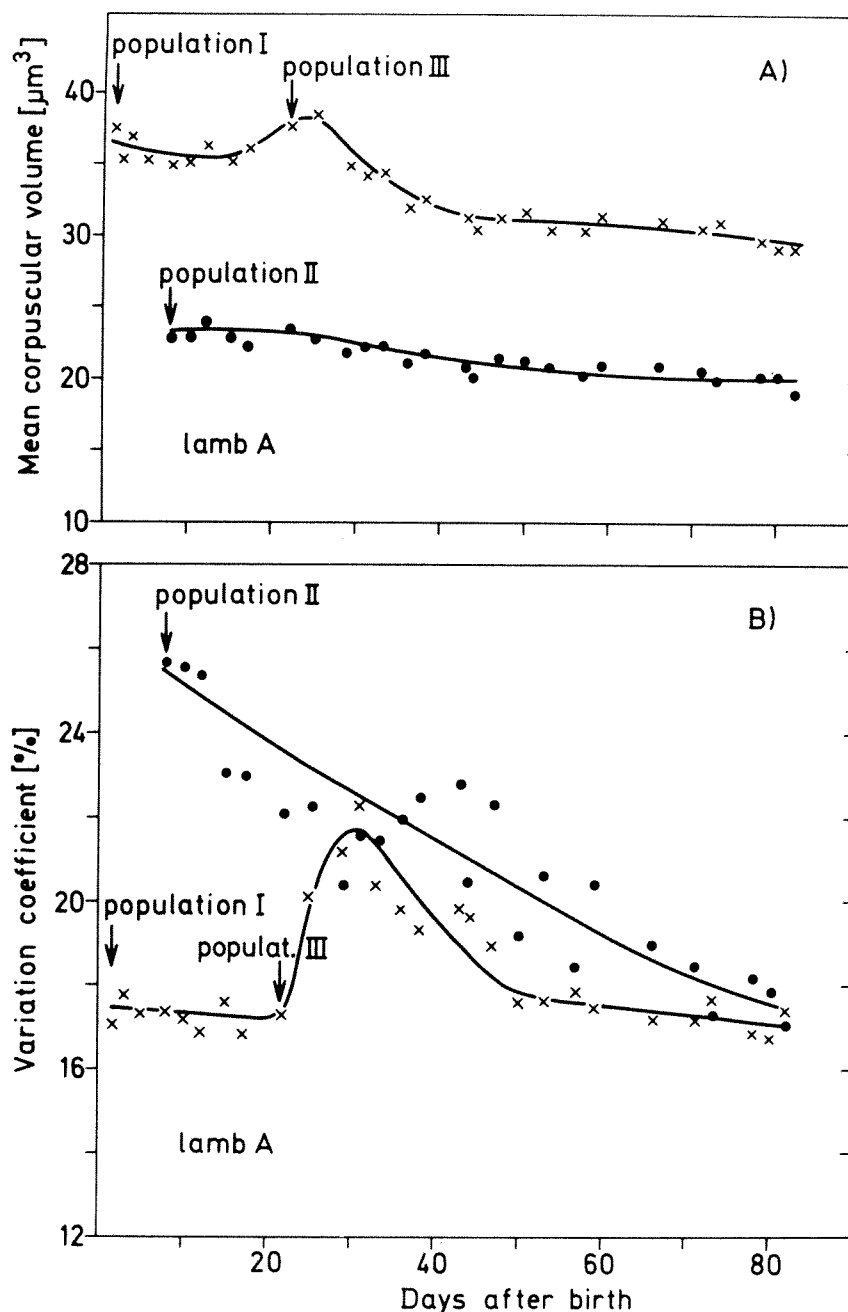


Fig. 3 Changes of the mean volume and the coefficients of variation of the erythrocyte volume populations in lamb A. The mean volume (3A) of the large and small erythrocyte population of the newborn animal decreases with time by 15-20% of the original values. The coefficient of variation (3B) decreases for the small erythrocyte population II of the newborn from 25.6 to 17.0%. For the large erythrocytes the coefficient remains constant around 17.5% except for a temporary rise to a maximum of 22.2% during the appearance of large population III cells around day 30 after birth. The arrows (3A,B) indicate the time of the first appearance in the blood of population II and III erythrocytes.

sheep, a fact compatible with the concept of new production of red cell populations II and III.

Electrophoretic mobility

It was of interest to determine whether other parameters such as the electrophoretic mobility would simultaneously indicate that the process of differentiation from the precursor cells is different for each red cell population. Figure 5 shows that for the first ten days

the mean electrophoretic mobility of the red cells was significantly lower than that after 22 days when values characteristic for the adult sheep red cell were approached. The increment in electrophoretic mobility coincided with the appearance of the final erythrocyte population III in the circulation suggesting that red cells of population I and II had a lower electrophoretic mobility than those of population III.

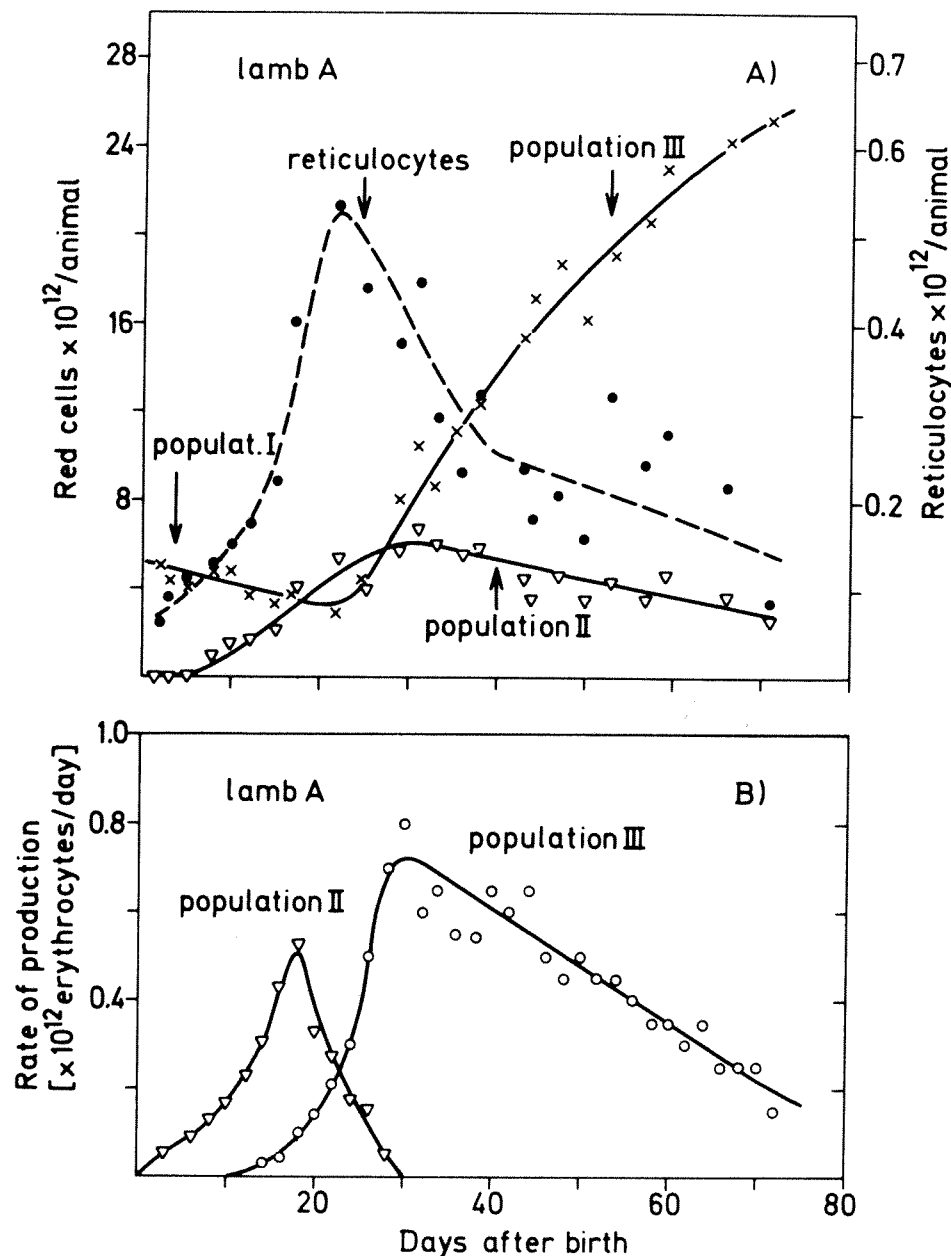


Fig. 4 Total number of small and large erythrocytes and reticulocytes per animal in the newborn lamb A (4A) and the rate of production (4B). The large erythrocytes in the newborn animal (4A) decrease continuously from birth until day 22 after birth and then rise again steeply, indicating the existence of two different kinds of large erythrocytes, comprised of population I and III cells. Population I contains the erythrocytes prevailing at birth and declines thereafter steadily with a half life of 28 days. Population III erythrocytes are produced from day 15 after birth on. An intermediate population II of small red cells appears on day 8, peaks on day 31 and disappears slowly with a half life of 42 days. During the time of maximal production of population II and III erythrocytes (4B) a transient reticulocyte peak is observed (4A).

Cellular cations and active K^+ transport

The typical fall of intracellular $[K^+]_c$ and rise of $[Na^+]_c$ at rather constant total mean cation concentration (fig. 6) correlated well with the change in the erythrocyte populations. It is likely that the slower fall of $[K^+]_c$ observed between day 5-15 was related to the appearance of volume population II followed by population III as indicated in figure 6. As population III became predominant the $[K^+]_c$

levels dropped to around 12 mM corresponding to a $[Na^+]_c$ level of around 110 mM/L cells. It should be pointed out that the time course by which $[K^+]_c$ fell and $[Na^+]_c$ rose, varied between the newborn sheep analyzed. This was evident from the $[K^+]_c$ and $[Na^+]_c$ analyzed on the red cells obtained from lamb E. At days 3 and 17 the $[K^+]_c$ levels were higher (table 2) than in the red cells of lamb B (fig. 6). Differences between animals existed also for the

TABLE 1
Kinetics of ⁵¹Cr loss and ⁵⁹Fe uptake in lamb red cells of populations I, II and III

Parameter analyzed	Days after birth	Days after injection	Total radioactivity (cpm × 10 ⁷ ± 1 SD ¹) bound to each red cell population per lamb	
⁵¹ Cr loss (Lamb B)	5	0	Population I	Population II
	16	11	1.30 ± 0.1	0.098 ± 0.003
	21	16	0.94 ± 0.02	0.056 ± 0.002
	26	21	0.85 ± 0.02	0.026 ± 0.001
⁵⁹ Fe uptake (Lamb A)			Population II	Population III
	29	4	1.94 ± 0.05	6.28 ± 0.18
	31	6	2.14 ± 0.06	7.18 ± 0.21
	33	8	3.68 ± 0.12	9.40 ± 0.17
	36	11	2.30 ± 0.08	11.6 ± 0.27
	38	13	2.36 ± 0.07	11.0 ± 0.22
	47	21	1.61 ± 0.03	11.8 ± 0.26
	53	28	1.36 ± 0.04	10.4 ± 0.23

¹ One standard deviation. The standard deviation is calculated as square root of the total counts of the radioactivity measurement. 10³ or more counts were counted per measurement.

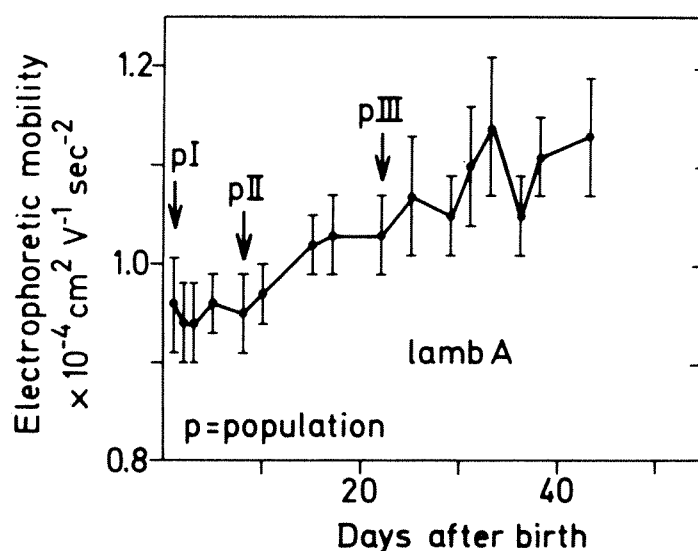


Fig. 5 Mean electrophoretic mobility of sheep erythrocytes in lamb A. The electrophoretic mobility of the erythrocytes of the newborn sheep is lower at birth than in the adult animal. It increases when population II erythrocytes come into circulation at day 8 (see arrow) and later rises again when population III erythrocytes appear. Each mean ± standard deviation was calculated from the electrophoretic measurements of 50 red cells.

production of populations II and III, which was slower in lamb A (fig. 4A) than in lamb B (fig. 6). These findings suggest biological variation between the randomly selected sheep in the temporal appearance of the different red cell populations.

If indeed the HK-LK transition shown in figure 6 is based on the appearance and exchange of three distinct populations of red cells, one may find: (a) the K⁺ pump (iM_K^P) flux in a red cell population predominantly of type I cells to resemble that of HK cells, the iM_K^P of population III to be similar to that found in adult LK cells, and the iM_K^P of mixed cell populations obtained between days 10-50 to be of

intermediate value; (b) anti-L, the antibody binding to and stimulating iM_K^P in adult cells (Ellory and Tucker, '69; Lauf et al., '69, '70; Lauf, '75a), may not activate iM_K^P in the first but increasingly in the subsequent populations. In spite of the different time courses in the HK-LK transition between lambs B and E (fig. 6, table 2) our data clearly show that the HK-like cells of the 3-day-old lamb E possessed an iM_K^P which was about five times higher than that of red cells taken on day 121 and thus closely resembled HK type iM_K^P values. Anti-L did not affect iM_K^P in the early cells but did stimulate iM_K^P about five times in the final red cell population. It can be seen

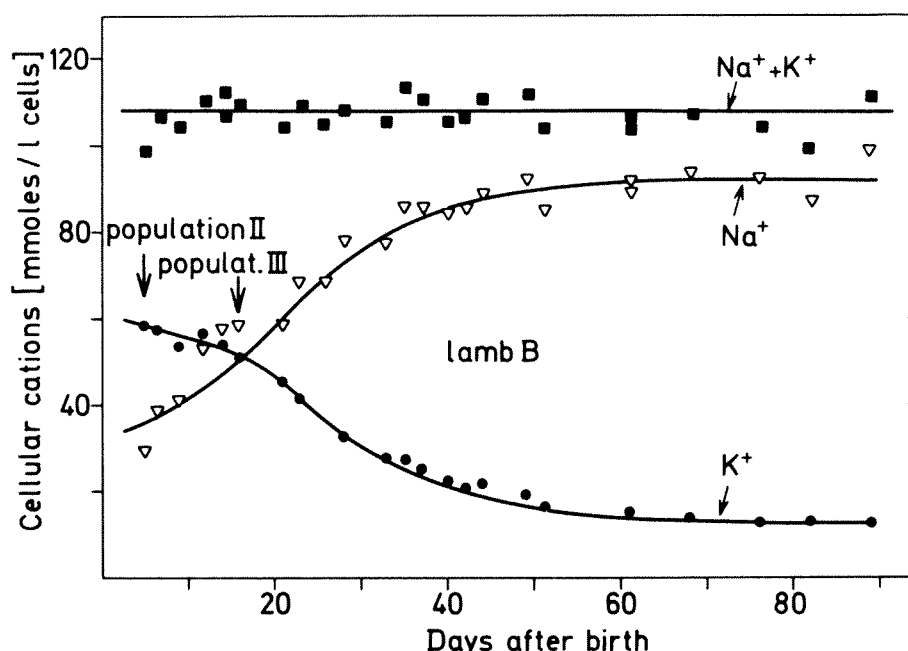
Na⁺ and K⁺ concentration in sheep erythrocytes after birth

Fig. 6 Sodium and potassium concentration in the erythrocytes of the newborn sheep B. Arrows indicate entrance of population II and III erythrocytes into blood circulation, respectively. For further explanations, see text.

TABLE 2

Cellular cations and K⁺ pump influx in genotypically LM (LK) red cells of Lamb E

Day	Sheep	[K] _c (mM/Lcells)	[Na] _c	iM_K^P (mmoles/L·cells × hr)	
				Control	Anti-L
0	Mother (LL 115)	17.5	109.0	0.17	1.31
3	Lamb (LM)	121.0	7.5	0.81	0.81
17	(LM)	115.9	24.1	0.90	0.98
36	(LM)	43.9	61.2	0.30	0.77
121	(LM)	16.4	—	0.14	0.83

that the anti-L effect on the K⁺ transport system becomes apparent after day 17 (table 2).

Occurrence of L and M antigens

The titration curves with the early red cells of predominantly large volume (Type I) exhibited a very weak response to anti-L and complement in L (fig. 7A) and M (fig. 7B) sheep while the anti-L dose response as well as the maximum hemolysis was higher in red cells appearing later and composed of type II and particularly of type III volume populations (figs. 7A,B). Figure 7C indicates that also the M antigen was weakly expressed on the early erythrocytes but strongly on the final red cells of lamb C. The temporal changes

of the L and M antigen expression correlated with the appearance of population II and III, in particular with population III cells which appeared in lamb A (fig. 4A) and lamb C around days 25 and 16, respectively. Coincident with the fast increment in population III cells, the L and M antigen activities rose to their final levels (fig. 7D).

DISCUSSION

The reports of erythrocyte volume differentials in newborn rat (Valet et al., '72a,b), guinea pig (Valet et al., '76), mouse (Valet et al., '74), rabbit and goat (Valet, unpublished), humans (Valet, unpublished), and antigenic differences in chicken (Blanchet, '76) suggest

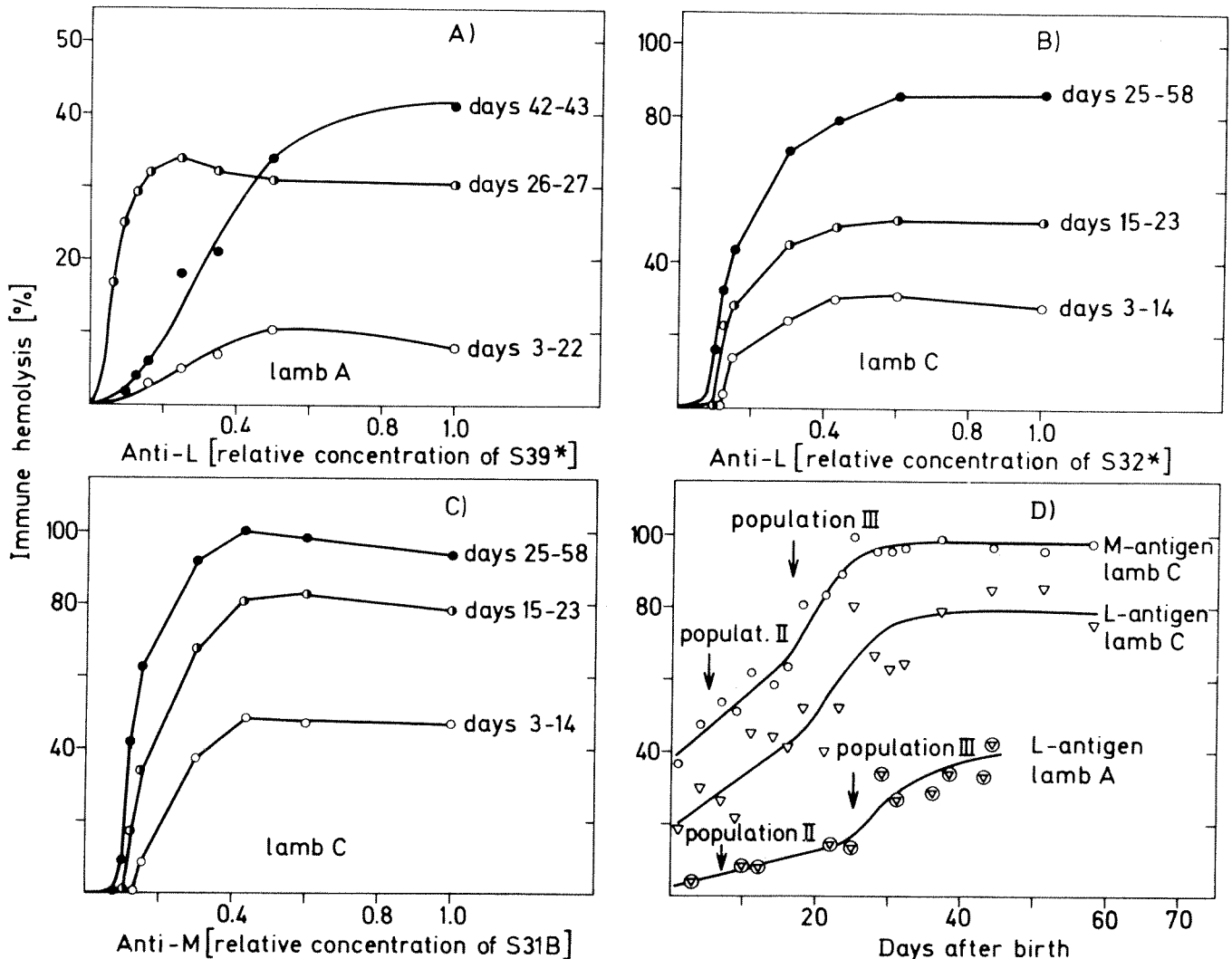


Fig. 7 Titration curves of the L and M antigen (7A,B,C) and changes of the maximum L and M antigen expression in the newborn sheep A (LL) and C (LM). For further explanations, see text.

that a ubiquitous heterogeneity of red cell precursors may exist in the early states of development in mammals and birds.

For the studies reported in this paper, the following findings warrant emphasis: 1. In the newborn, genotypically LK sheep, electrical sizing studies revealed three different red cell populations which were sequentially produced in the hematopoietic tissue and were not derived from one another in the peripheral blood. 2. The naturally occurring change from HK to LK type red cells thus was related to the appearance of red cell populations of different K^+ steady state levels and K^+ pump activities. 3. Moreover, each red cell population exhibited differences in the genetic markers typical for sheep red cells such as Na^+K^+ transport associated membrane antigens and hemoglobin pattern. 4. The electrophoretic mobility of the three cell populations was different.

The formation of several distinct erythrocyte populations, different in several parameters, suggests discrete steps in the postnatal hematopoietic differentiation. The transition from fetal (HbF) to adult (HbA, HbAB or HbB) hemoglobin of the transient appearance of hemoglobin C (HbC) is another important parameter studied in the newborn lamb, in particular in relation to the HK-LK transition (Blostein et al., '74). There is a striking resemblance of the time course of development and disappearance of HbC (Blunt and Huisman, '75) and of population II erythrocytes (fig. 4A) which suggests that HbC is located in the population II red cells.

Two explanations can be offered for the sudden change of parameter expression: the change of gene expression occurs in a *common* hematopoietic precursor cell or *separate* precursor clones. For anemic sheep the hemoglo-

bin A-C switch has been explained by nuclear events occurring early in the erythroid maturation (Nienhuis et al., '74) and involving different messenger ribonucleic acids (Baldy et al., '72; Nienhuis et al., '74) and pretranslational control (Elson et al., '74). Both hemoglobins A and C were demonstrated to coexist in the same cell (Garrick et al., '74; Nienhuis and Bunn, '74) supporting the concept of an intracellular mechanism of gene activation whereby the hemoglobin switch occurs in a single stem cell line rather than involving several stem cell clones (Kabat, '72). In contrast to the concept of "non-cellular" selection processes, other reports on erythropoiesis in newborns have stressed hemoglobin changes in terms of a change in cell populations (Baglioni, '66; Boyer, '70; Boyer et al., '75).

Our findings, which correlate cation composition, K^+ pump fluxes, antigenic patterns and electrophoretic mobility with three different erythrocyte volume populations in newborn lambs (fig. 4A) seem to be more compatible with the hypothesis that type I, II and III cells arose from different clones of erythroid stem cells. It is possible that in the newborn sheep such a switch occurs only in a particular erythroid cell line which, when stimulated by a single event, produces cells which are also different in various other hematological, immunological and biophysical parameters. Further evidence for this hypothesis would be the confinement of a particular hemoglobin type to a discrete erythrocyte population. Indeed preliminary work indicates that after separation from each other only red cells of population I contain hemoglobin F.

Of particular interest with respect to erythropoiesis as well as the molecular genesis of the LK steady state cell, is the signal which brings about such profound alteration in erythropoietic proliferation. Investigations in the rat show that the leucopoietic system is also influenced by the appearance of the new erythrocyte populations (Hanser et al., '74; Valet, unpublished). Hence specific extra- or intracellular signals may trigger distinct production programs in one or several different cell lines of hematopoietic precursors (stem cells) affecting both the erythro- and leukopoietic systems. Studies (in progress) on red cell populations in adult sheep exposed to severe anemic stress should decide whether one can also induce in adult animals the formation of several erythrocyte populations

similar to those reported here for the postnatal period.

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