The Transition from HK to LK Phenotype in the Red Cells of Newborn Genetically LK Lambs

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ABSTRACT Red cells from newborn lambs were separated into different age populations by centrifugation, and cells with fetal hemoglobin (Hb) were distinguished from those with adult Hb by an acid elution technique. Changes were followed during development in rates of K^+ transport (active and passive), numbers of Na^+/K^+ pump sites per cell, cell volumes, and numbers of L_p and L_l antigen sites per cell. These changes were correlated with the percentage of cells with adult hemoglobin. (The L_p and L_l antigens are associated with K^+ transport in that specific alloantibody against L_p, anti-L_p, stimulates active transport, and anti-L_l inhibits passive transport.) Active K^+ transport decreased during development because of a decline in number of Na^+/K^+ pumps (from measurements of ouabain binding) and because of an alteration in the affinity of the pumps for intracellular K^+ (from kinetic studies in which the intracellular K^+ concentration was varied). Cells with fetal Hb had fewer L_p sites and were larger than cells with adult Hb. As transport properties changed, the number of L_p sites increased and continued to increase after all the cells had adult Hb. Cells with fetal Hb had as many L_l sites as lamb cells with adult Hb, but the number of L_l sites was less than those found previously for adult sheep. A population of small cells with intermediate K^+ concentrations and intermediate numbers of L_p sites appeared soon after birth. The various points of evidence suggested that the developmental process leading to cells with adult transport properties was a gradual one and did not coincide precisely with the switch from fetal to adult Hb.

INTRODUCTION

It has been known for a number of years that the genetic polymorphism involving red cell potassium concentrations in sheep (HK and LK phenotypes) is not manifest in newborn lambs, but becomes detectable during the first 2 months of life (Evans and Blunt, 1961; Blechner, 1961; Drury and Tucker, 1963; Ellory and Tucker, 1969*a*). At birth the red cells of all sheep have high

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concentrations of potassium, and it is only when the blood group antigen L first makes its appearance on the cells that genetically LK lambs progressively assume the LK phenotype characterized not only by an alteration of Na^+ and K^+ concentrations, but also by a decline in active K^+ transport and the number of Na^+/K^+ pumps per cell (Dunham and Hoffman, 1971; Lauf et al., 1978). This observation provided the basis for the hypothesis that the L antigen acts by inhibiting active potassium transport in adult type LK red cells (Tucker, 1968). Further evidence lies in the fact that L is found only on the red cells of LK sheep and that binding of adult LK red cells with the alloimmune antibody anti-L can stimulate the Na^+/K^+ pump to a transport rate similar to that of HK cells (Ellory and Tucker, 1969*b*). HK red cells have an allelically associated antigen M which is also present in the red cells of heterozygous LK adult sheep, but no physiological role has yet been ascribed to M (Rasmusen and Hall, 1966; Dunham et al., 1980). The dominance of the LK allele is nearly complete; there is only a small difference in cellular K^+ concentrations between heterozygous and homozygous LK sheep (Evans et al., 1956). Therefore we have included in our study LK lambs of both genotypes (in each instance identifying them). None of the properties measured showed any difference between homozygotes and heterozygotes.

Recently, it has become clear that there are two specificities of anti-L that have different effects; one (anti-L_p) stimulates the pump, and the other (anti-L_l) inhibits the passive (ouabain-insensitive) K^+ transport (Ellory and Tucker, 1970; Lauf et al., 1971; Dunham, 1976*a*). Most hyperimmune anti-L sera have both antibodies. Preliminary studies suggested that it is L_p antigen and not L_l that is absent from the red cells of newborn lambs (Tucker et al., 1976). By making use of the fact that L_p but not L_l is destroyed by trypsinization of adult LK sheep cells (Lauf et al., 1971), it has been possible to prepare separate anti-L_p and anti-L_l reagents. These have been used in electron microscopy studies to measure the number of L_p and L_l sites on the red cells of adult LK sheep (Smalley et al., 1982).

In the present investigation, therefore, we thought it would be of interest to re-examine the changes in active K^+ transport that occur in growing lambs in relation to the number of L_p and L_l sites present on the red cells, and at the same time, by studying several different transport and hematological parameters, to try to determine whether the changes represent a switch to a discrete new population of adult type cells or whether there is a gradual replacement of the fetal cells by cells with increasingly adult characteristics. Previous studies ruled out a third possibility that fetal cells can change into cells with adult type K^+ transport (Tucker and Ellory, 1970).

Most of the data presented are correlated with cell hemoglobin type, using the presence of fetal hemoglobin (HbF) as the absolute criterion for fetal cells. Very little, if any, HbF is synthesized after birth in lambs, and new red cells coming into the circulation contain adult Hb (Drury and Tucker, 1963). The present work uses a simple centrifugation method that previous studies have shown enables cell populations containing mostly fetal Hb to be separated from cell populations containing mostly adult Hb (Drury and Tucker, 1963;

Tucker and Ellory, 1970). Use of this fractionation method has been made in the present investigation so that as much information as possible can be gained from each blood sample taken. Serological, electron microscopical, and cell sizing techniques were used as well as measurements of parameters related to active K^+ transport (ouabain binding and kinetics) in order to relate developmental changes in K^+ transport to cell size and numbers of L_p sites.

METHODS

General

Blood samples were taken from the jugular veins of lambs into heparinized evacuated tubes. Hemoglobin concentrations were determined as cyanmethemoglobin. Reticulocytes were stained supravitally with brilliant cresyl blue, a minimum of 500 cells being counted for each preparation. Cell volume measurements were made in a Coulter counter (model B; Coulter Electronics Inc., Hialeah, FL). Cells were counted at increasing thresholds using an orifice of 70 μm and a dilution of 1:10,000 in filtered saline. Serological tests were carried out as described by Tucker and Ellory (1970). Anti-L_p reagents were prepared by absorption of alloimmune anti-L sera with trypsinized homozygous *LL* type sheep red cells. Anti-L_l was obtained by acid elution at pH 3.0 from the trypsinized cells (Smalley et al., 1982). The separation of anti-L_p and anti-L_l was complete as judged by effects on passive and active K^+ transport (Smalley et al., 1982).

Acid Elution Test for Fetal and Adult Hemoglobin

Heparinized blood was diluted with an equal volume of saline and thin films were made on microscope slides. The films were treated with phosphate/citric acid buffer, pH 3.3, and stained with hematoxylin and eosin as described by Moore et al. (1966). Cells with HbF show up as deeply stained cells in contrast to cells with adult hemoglobin, which appear as faintly stained "ghosts." A minimum of 500 cells was always counted.

Electrophoresis

Hemoglobins were separated by starch-gel electrophoresis, at pH 6.5 in phosphate buffer for fetal hemoglobin (Drury and Tucker, 1963) and at pH 8.8 in a Tris-EDTA-borate buffer for adult hemoglobins (Gahne et al., 1960).

Separation of Fetal and Adult Cells by Centrifugation

Washed red cells were spun in polythene tubes at 2,500 *g* for 20 min at room temperature as described previously (Drury and Tucker, 1963). The packed cell column was sliced into four equal sections, and the buffy coat was discarded. The cells in each fraction were resuspended in buffered saline and prepared for the various tests. For Na^+ and K^+ estimations, the cells were washed three times in an ice-cold solution of 150 mM choline chloride, 10 mM Tris-HCl, pH 7.5. Sodium and potassium measurements were made in a flame photometer. It was shown that washing the cells in choline chloride did not result in measurable changes in intracellular concentrations of Na^+ and K^+.

K^+ Influx Measurements

K^+ influx was measured using $^{86}Rb^+$ and microcentrifuge methods as described previously (Dunham and Ellory, 1980). Active K^+ influx is defined as the difference

in influx in the presence and absence of 0.1 mM ouabain. Samples were always run in triplicate and the incubation times varied from 10–60 min as appropriate for the magnitude of the flux. For binding with anti-L, cells (0.02–0.04 ml) were incubated at 37°C for 15 min with 1 ml anti-L (L_p + L_l) serum, washed once by centrifugation, and resuspended in flux medium.

Ouabain Binding

Numbers of Na^+/K^+ pumps per cell were obtained by measuring binding of ^3H-ouabain using the techniques of Dunham and Ellory (1980), relating percent pump inhibition to the amount of bound ouabain. Nonspecific binding was determined by preincubation with an excess of nonradioactive ouabain (0.1 mM).

Nystatin Treatment

Intracellular cation composition was varied using the nystatin method of Dunham and Blostein (1976). The loading medium contained 5–30 mM KCl, and 130–105 mM NaCl ([Na] + [K] = 135 mM), 65 mM sucrose, 10 mM Tris-HCl, pH 7.5, and 50 μg/ml nystatin.

Electron Microscopy

The L_p and L_l antigen sites on the cells were labeled with hemocyanin for electron microscopy by the method of Ostrand-Rosenberg (1975). Briefly, cells were first incubated with anti-L_p or anti-L_l. The bound antibody was then made visible microscopically by treatment with anti-ruminant IgG conjugated to hemocyanin (Smalley et al., 1982). Photographs were taken at a magnification of 10,000 ×. The total number of sites on the "face" of the cell seen in the photographs was counted. A background value of about five sites per face was observed in control preparations where LK-type red cells were treated with anti-M sera. This was subtracted from the total number of sites seen on cells pretreated with anti-L_p or L_l.

RESULTS

Detection of Cells with Adult and Fetal Hemoglobin

Preliminary experiments were carried out to determine whether the acid elution staining technique would be reliable for distinguishing those cells that contain HbF from those containing adult Hb. Sequential blood samples taken from three lambs over a period of 50 d after birth were tested by acid elution (Fig. 1). The number of cells containing adult Hb increased progressively to 100% at 50 d. These results correlated well with those obtained by electrophoretic separation of the hemoglobins in the same samples and could be more accurately obtained and quantitated. Fig. 2 shows a blood film made from one of the lambs at a stage when the blood had ~50% adult and ~50% fetal Hb as judged by both methods. The strongly stained cells with HbF were clearly distinguishable from the unstained cells with adult Hb.

Results Related to the Na^+/K^+ Pump Activity

Blood samples were taken from heterozygous (*ML*) type lambs aged 5, 18, 19, 32, 53, and 59 d; sequential samples were also taken from one homozygous *LL* lamb (9×443) at 4, 11, 16, and 31 d after birth. The blood was fractionated by centrifugation, and cells from fractions 2, 3, and 4 were analyzed for Na^+

and K^+ concentrations, active and passive K^+ uptake with and without pretreatment with anti-L, and for the number of ouabain binding sites per cell. Hematological measurements were also made. In these experiments fraction 1 was discarded because of the presence of reticulocytes in this sample; it is known that reticulocytes have very different K^+ transport characteristics from mature cells (Tucker and Ellory, 1971; Dunham and Blostein, 1976).

All of the data were analyzed by Genstat statistical routines (Alvay et al., 1977) for a correlation between the measured parameter and the percentage of cells with adult hemoglobin.

CELL NA^+ AND K^+ CONCENTRATIONS Initially, determinations of red cell

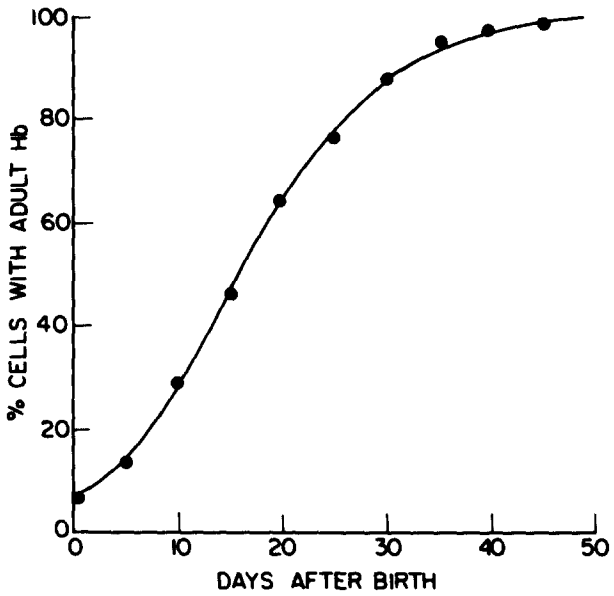

FIGURE 1. Percentage of red cells with adult hemoglobin in blood samples from lambs at intervals after birth. Mean values for three lambs. Cells with adult hemoglobin were distinguished from cells with fetal hemoglobin by the acid elution test.

Na^+ and K^+ levels were used as an index of ion transport, because the steady state internal ionic composition represents the balance between pump and leak fluxes. Fig. 3 shows the concentrations of intracellular Na^+ and K^+ plotted against the percentage of adult Hb-type cells. Essentially similar results were obtained whether K^+ concentrations were expressed in relation to the hemoglobin concentration or per unit cell volume. Simple regression analysis of the data gave a good correlation (significant at $P = 0.001$) for a linear *or* a quadratic relationship. Certainly inspection by eye would indicate a quadratic element. However, the departures from linearity could be a systematic effect of data from individual animals weighting a particular sector

of the curve, a fact that became apparent on more complex statistical modeling. An indication of complexity comes from Fig. 3C, where the sum of concentrations of cell Na^+ and K^+ is plotted against the percentage of adult Hb type cells. Here there is a significant decline from ~135 to 110 mM, over the range 0–100% adult Hb. This was not because of changes in cell volume because a similar correlation was obtained with ($Na^+ + K^+$) expressed per gram Hb. Also the MCHC (mean cell hemoglobin content) values showed no correlation with percentage adult Hb over this period.

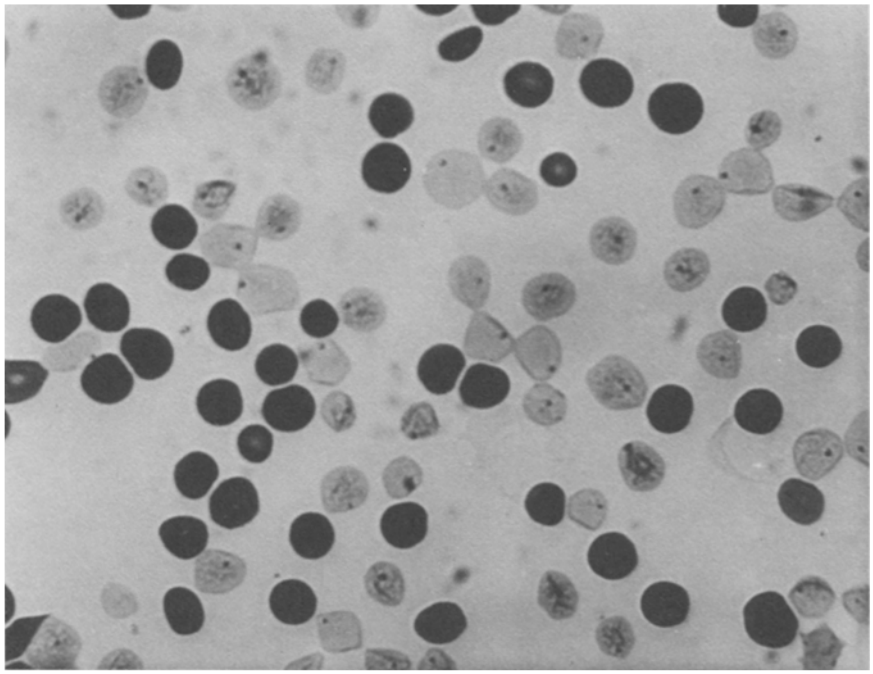

FIGURE 2. Blood film from a lamb aged 17 d stained to show red cells with fetal (dark cells) and adult (pale cells) hemoglobin. The acid elution test was used to distinguish the cells (see Materials and Methods). The micrographs are at a magnification of 2,000 ×.

K^+ INFLUXES Since internal K^+ and Na^+ levels should reflect the balance between pump and leak fluxes, there should be a reciprocal relationship between ouabain-inhibitable K^+ influx and percentage adult Hb type cells, to be consistent with the cell Na^+ and K^+ determinations. Such a relationship is shown in Fig. 4, where active K^+ influx showed an inverse correlation, roughly linear, with percentage adult Hb-type cells. An alternative way of expressing these results is as the direct relationship between cell Na^+ and pump activity (ouabain-sensitive K^+ influx) (Fig. 5), where there is a dramatic rise in intracellular Na^+ as pump activity declines.

In this series of experiments, data were also obtained for passive K^+ influx. This mode of K^+ transport is not simply the electrodiffusional potassium leak,

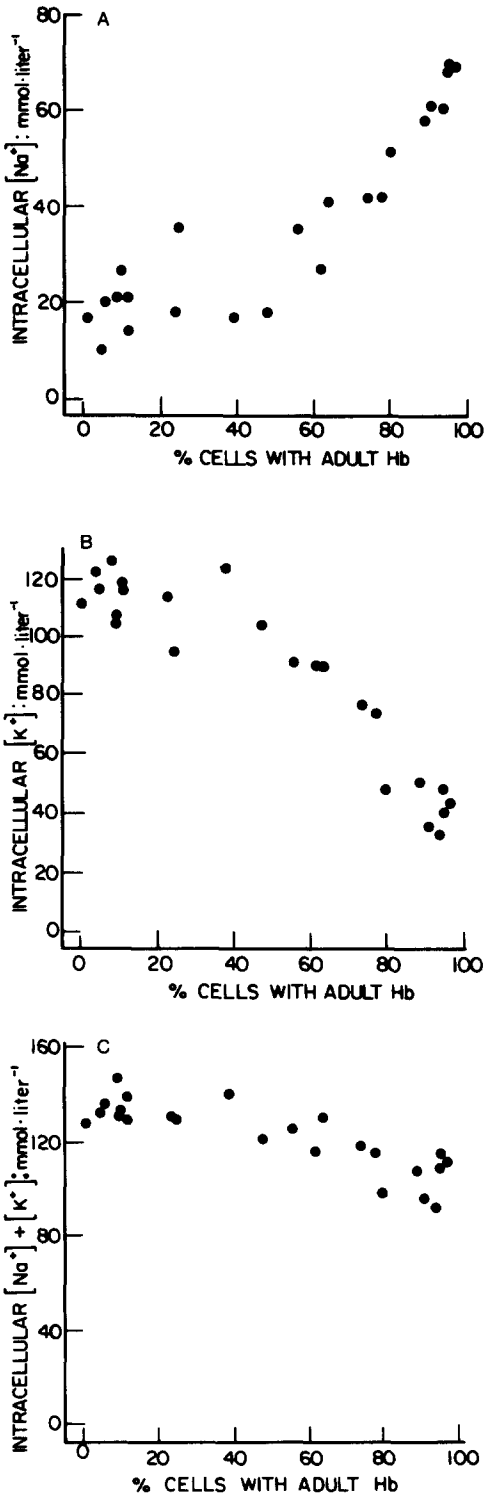

FIGURE 3. Relationship between red cell K^+ and Na^+ concentrations and the percentage of cells containing adult hemoglobin in fractions 2, 3, and 4 (combined) from lambs of different ages. (A) Na^+; (B) K^+; (C) $Na^+ + K^+$. The values are expressed in millimoles per liter of fractionated cells.

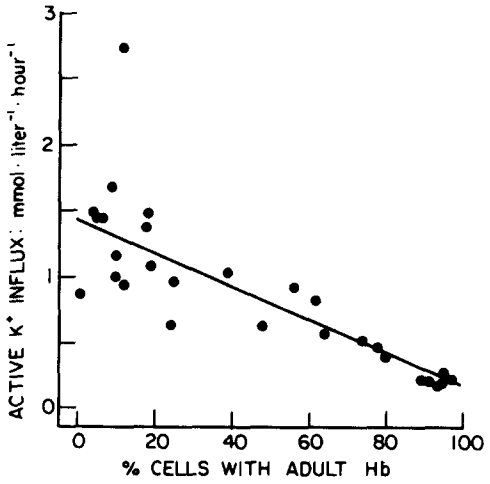

FIGURE 4. Relationship between active K^+ influx and the percentage of red cells containing adult hemoglobin in fractions 2, 3, and 4 (combined) from lambs of different ages. Active K^+ influx was defined as the ouabain-inhibitable component (ouabain concentration 0.1 mM; K^+ concentration 7.5 mM). The data were fitted to an equation of the form: $y = c - m \cdot x$, where y = active K^+ influx, x = percentage of cells with adult Hb, $c = y$ intercept, and m = slope. The computer fit yielded the following values: $c = 1.508 \pm 0.119$ (SE); $m = -0.0121 \pm 0.0021$, $P < 0.001$ (t statistic = -5.86, 42 df, degrees of freedom).

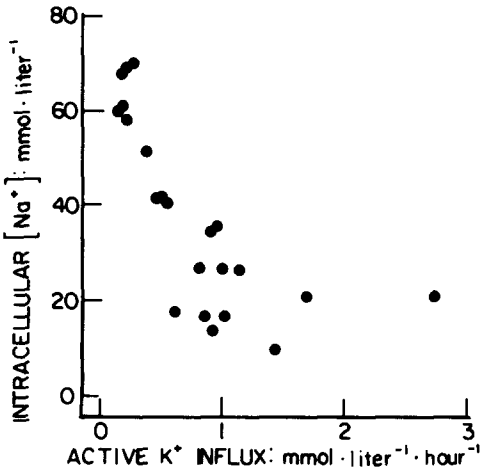

FIGURE 5. Relationship between intracellular Na^+ concentration and active K^+ influx in red cell fractions 2, 3, and 4 (combined) from lambs of different ages.

but includes a variable component that shows saturation kinetics in K^+ and is dependent on cell volume (Dunham, 1976*b*; Ellory and Dunham, 1980; Dunham and Ellory, 1981). When passive K^+ influx was plotted as a function of percentage adult Hb type cells there was no correlation, but the data

showed considerable scatter and the possibility of small volume changes from experimental manipulation affecting this parameter made it unprofitable to assess these data.

OUABAIN BINDING Tritiated ouabain binding studies have been used to determine the number of Na^+/K^+ pump sites on lamb and adult sheep red cells (Dunham and Hoffman, 1971; Lauf et al., 1978). In the present study we therefore measured ouabain binding to determine changes in the number of Na^+/K^+ pumps per cell during development; the data are presented in Fig. 6. Over the range of 0–100% adult Hb-type cells there was a two- to threefold decline in the number of pump sites. The slope of a straight line through the

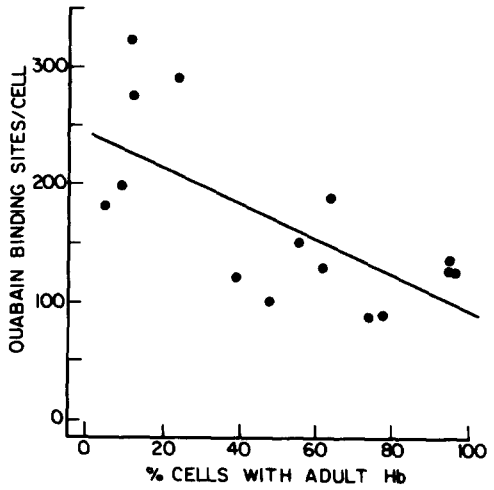

FIGURE 6. Relationship between number of ouabain binding sites per cell and the percentage of red cells containing adult hemoglobin in fractions 2, 3, and 4 (combined) from lambs of different ages. Ouabain binding is expressed as molecules bound per cell after correction for nonspecific binding (defined as ^{3}H-ouabain bound after preincubation with 0.1 mM nonradioactive ouabain). The data were fitted to an equation of the form: $y = c - m \cdot x$, where y = ouabain binding sites/cell, x = percentage cells with adult Hb, $c = y$ intercept, and m = slope. The computer fit yielded the values: $c = 24.88 \pm 2.72$, $m = -0.158 \pm 0.045$, $P < 0.01$ ($t = -3.52$, 13 df).

data was significantly different from zero ($P = 0.01$). The mean number of specific ouabain binding sites per cell for samples containing <12% adult Hb was 242 ± 32 ($n = 4$), whereas samples containing >70% adult Hb had 111 ± 10 ($n = 5$) sites per cell (mean ± SEM, number of determinations). The same samples were shown to have about a sevenfold reduction in ouabain-sensitive K^+ influx (from 1.45 to 0.22 mmol/liter/h), as will be shown in Table I. This indicates that a decrease in the number of pumps cannot account entirely for the reduced adult K^+ influx, and that changes in other kinetic properties of the pump are also taking place during development. An obvious possibility is that the affinity of the pump for intracellular K^+ is increasing,

thereby increasing the inhibition of the pump. This point will be dealt with below (Fig. 8; see also Sachs et al., 1974, and Cavieres and Ellory, 1977).

The number of sites per cell at 100% adult Hb in Fig. 6 was higher than values obtained in a recent study on adult sheep erythrocytes (Lauf et al., 1978). There is considerable variation in reported values for number of ouabain binding sites on LK sheep red cells (cf. Ellory, 1977, for a review); the differences may be related to, among other factors, the various breeds of sheep studied. However, our interest was focused on the changes in numbers of ouabain binding sites during development rather than on determinations of absolute numbers of sites per cell.

POTASSIUM TRANSPORT AFTER TREATMENT WITH ANTI-L Fig. 7 shows the

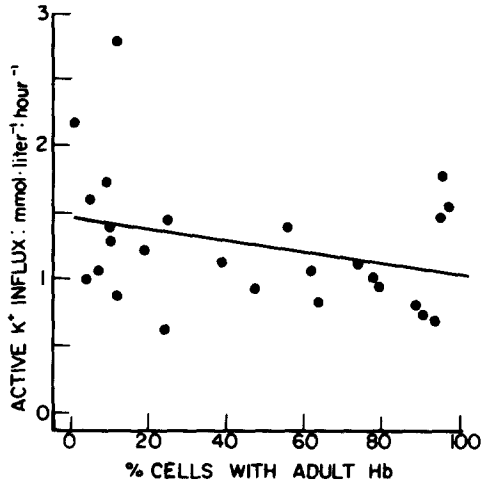

FIGURE 7. Relationship between active K^+ influx after anti-L sensitization and the percentage of red cells containing adult hemoglobin in fractions 2, 3, and 4 (combined) from lambs of different ages. Anti-L treatment was sufficient to give maximal stimulation. The data were fitted to an equation of the form: $y = c - m \cdot x$, where y = active K^+ influx, x = percentage cells with adult Hb, $c = y$ intercept, and m = slope. The computer fit yielded the values: $c = 1.763 \pm 0.145$; $m = -0.0061 \pm 0.0025$; $P < 0.02$ ($t = -2.44$, 42 df).

ouabain-inhibitable K^+ influx in anti-L-treated cells as a function of percent adult Hb type cells. There is considerable scatter, but the slope of the line for the anti-L-treated cells is much less than for the untreated cells (Fig. 4). To illustrate this point, we have calculated the mean values for ouabain-inhibitable K^+ influx in both control and anti-L-treated cells from the data presented in Figs. 4 and 7 from the extreme ends of the distribution of Hb types, i.e., the samples with <12% and >90% adult Hb. These data, given in Table I, indicate an eightfold stimulation of ouabain-inhibitable K influx by anti-L in the population containing adult cells, contrasting with no stimulation by anti-L in the fetal cell population.

Ouabain-insensitive K^+ influx varied greatly between individuals largely

because of the volume dependence of these fluxes mentioned above. For this reason it was not fruitful to analyze in detail the results on ouabain-insensitive fluxes. However, in our experiments, treatment with anti-L consistently had the effect of reducing ouabain-insensitive K^+ influx, as reported earlier (Dunham, 1976*b*). The degree of inhibition ranged from 20 to 70%, but was so variable that it could not be correlated with the fetal or adult hemoglobin type of the cells.

INTRACELLULAR K^+ AFFINITY OF THE NA^+/K^+ PUMP Because the ouabain binding experiments indicated that the changes in K^+ transport and intracellular cation content could not be explained fully in terms of a decrease in numbers of Na^+/K^+ pumps, a series of experiments was designed to investigate possible changes in affinity for K^+ (as an inhibitor) at the intracellular aspect of the pumps. Blood was taken weekly from two homozygous LK lambs, and their intracellular cation composition was altered by the nystatin method (Dunham and Blostein, 1976) to give cellular K^+ concentrations from 4 to 20 mmol/liter of cells and Na^+ concentrations from 80 to 95 mmol/liter (the sum

TABLE I

ACTIVE K^+ INFLUX WITH LK LAMB CELLS OF PREDOMINANTLY ADULT HEMOGLOBIN OR PREDOMINANTLY FETAL HEMOGLOBIN

	Ouabain-inhibitable K^+ influx	
	0–12% adult Hb	90–100% adult Hb
	mmol/liter/h	
Control	1.46±0.20 (9)	0.22±0.015 (6)
Anti-L-treated	1.53±0.31 (9)	1.17±0.20 (6)

The data were taken from Figs. 4 and 7. Values are means ± SEM (*n*). Shown are values for control samples of cells and samples that had been pretreated with anti-L.

of cellular Na^+ and K^+ concentrations was constant at ~100 mmol/liter cells). The data for one homozygous lamb (the other giving essentially the same results) are summarized in Fig. 8. It can be seen that at birth the ouabain-inhibitable K^+ influx was high and insensitive to inhibition by internal K^+. During development, the absolute pump activity declined dramatically (as shown also in Fig. 4) and sensitivity to inhibition of the pump by intracellular K^+ developed. Thus at 70 d the flux at 20 mM K^+ is only 30% of that at 4 mM, the familiar inhibition of the pump by internal K^+ in LK cells (Lauf et al., 1970; see Ellory, 1977, for a review). The time course of this change in K^+ affinity is consistent with the data presented in Fig. 7 for the development of sensitivity to anti-L.

Serological Studies

L_p SITES Because expression of the L_p antigen on LK cells is believed to be responsible for the change in kinetics of the pump shown above (Fig. 8), it is important to try to monitor during development the appearance of L_p sites

on the lamb cells. Therefore, blood samples were taken from two homozygous LK lambs at 2, 53, and 137 d after birth and the red cells were examined for the number of L_p sites by electron microscopy. At these three ages there were, respectively, 11, 99, and 100% adult Hb-type cells present in one lamb (9X457) and 4, 99, and 100% adult cells in the other (9X458). Fig. 9 shows the number of L_p sites plotted against cell surface area. In the samples at 2 d the number of L_p sites was very low, with mean values of 4.6 ± 0.8 and 8.0 ± 1.2 (n = 20, ± SEM) sites per face for each of the lambs. The mean areas of the cell faces were, respectively, 11.1 ± 0.5 μm^2 and 11.4 ± 0.5 μm^2. In the 53-d samples the mean number of L_p sites had increased to, respectively, 34.3 ± 2.5 (31) and 54.3 ± 2.6 (39) (± SEM [n]) but with a large scatter between individual values. Mean values for area of cell face were 10.8 ± 0.3 and 11.3 ± 0.3 μm^2, respectively. On day 137 the number of L_p sites was significantly

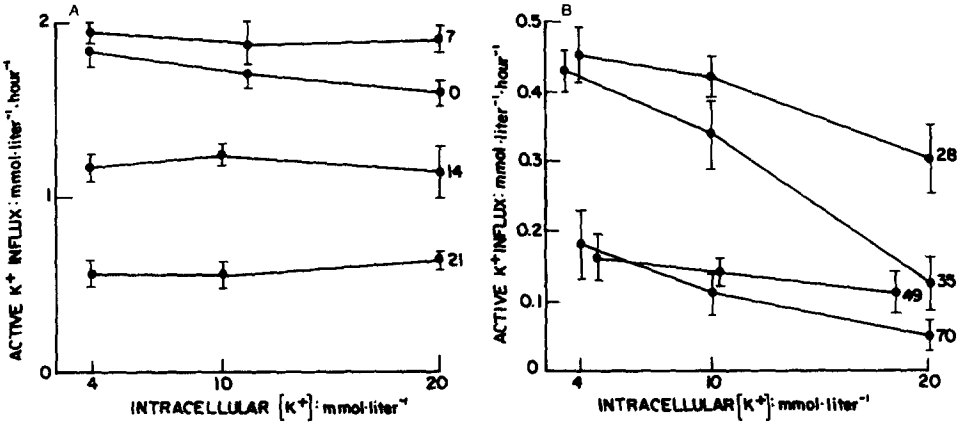

FIGURE 8. Active K^+ influx at different intracellular K^+ concentrations in red cells from one lamb (homozygous) at various ages: 0, 7, 14, and 21 d (A) and 28, 35, 49, and 70 d (B) after birth. The intracellular cation concentrations had been altered by treatment with nystatin.

higher than at day 53 in both lambs, with mean values of 73.9 ± 6.8 (12) and 76.4 ± 5.0 (12), respectively. The cells were also smaller (mean values for area per face of 9.35 ± 0.25 and 7.22 ± 0.34 μm^2). These results therefore indicated that the number of L_p sites on the red cells increased as the lambs aged and that this increase continued after the population had attained 100% adult Hb cells. The values at 137 d were close to the mean value of 78 obtained previously for cells from an adult *LL* sheep (Smalley et al., 1982).

To obtain a more direct comparison of numbers of L_p sites in different cell populations from the same blood sample, blood had been taken from these two lambs at day 12 and fractionated into fraction 1, "young cells," and fraction 4, "older cells." The results for L_p sites are given in Table II. In each case fraction 1 consisted mostly of cells with adult Hb and numbers of L_p sites were significantly higher (three- to fourfold) than those of fraction 4, which contained mostly cells with fetal Hb. It could be argued that the presence of

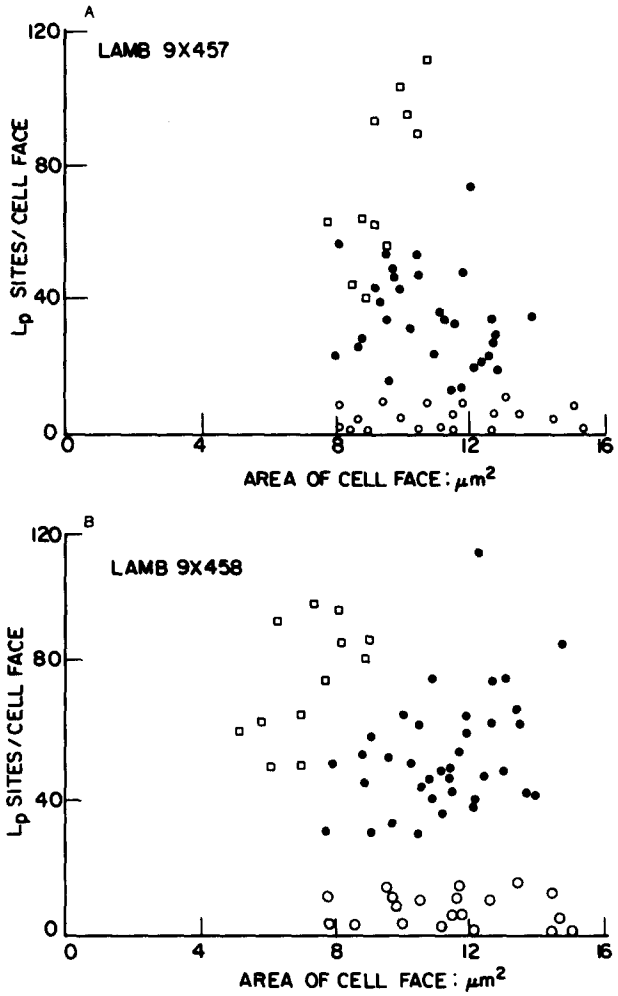

FIGURE 9. Numbers of L_p sites per cell face in relation to cell area on red cells from two lambs, (A) 9X457 and (B) 9X458, at 2 (○), 53 (●), and 137 (□) d after birth. Red cells were labeled with anti-L_p and examined by immunoelectron microscopy.

TABLE II

NUMBERS OF L_P SITES ON FRACTIONATED CELLS FROM TWO 12-DAY-OLD HOMOZYGOUS LK-TYPE LAMBS

Lamb	Fraction	Adult Hb cells	Reticulocytes	L_p sites (sites/face)
		%	%	*mean ± SEM (n)*
9X457	1	82	8	54.2±4.5 (20)
	4	11	0	14.1±3.2 (20)
9X458	1	94	2	40.4±2.8 (20)
	4	6	0	13.9±2.2 (20)

reticulocytes in fraction 1 influenced this result. However, because there were only 8 and 2% reticulocytes, respectively, and the cells were selected as far as was possible in random manner for electron microscopic examination, it is unlikely that this influence could have been great.

The above samples were tested for complement lysis by anti-L_p reagent. Red cells taken at 2 d, and fraction 4 of the 12-d samples, showed no lysis at all. Fraction 1 of the 12-d samples gave hemolysis scores of 12 and 15 for 9X457 and 9X458, respectively, with no further increase in lysis on days 53 and 137, in spite of the fact that L_p sites as judged by electron microscopy became more numerous.

L_l SITES Red cells from the above experiment were also tested with anti-L_l reagent. In contrast to the results with anti-L_p, no difference was seen between any of the samples, the 2-d samples giving similar hemolysis scores to those of the 137-d samples, both ~38. Thus L_l was shown serologically to be present at birth.

In another experiment aimed at comparing numbers of L_p and L_l sites and minimizing age differences as far as possible between samples containing fetal and adult cells, a blood sample taken from one lamb (L107) aged 17 d was fractionated. Fraction 3, containing 50% adult and 50% fetal Hb-type cells, was examined by electron microscopy for L_p and L_l sites. Under such circumstances, according to the simple replacement theory, one would expect to find half of the cells with virtually no L_p sites but with L_l sites and half of cells with the adult complement of sites. Fig. 10A shows the number of L_p sites plotted against cell size. In general, the smaller cells have more L_p sites than the larger cells. If cells with areas $<10\ \mu m^2$ are considered to be those with adult Hb, then it can be seen that there is a range of L_p sites from 1–100 sites per face with a mean of 51.8 ± 4.6 (30). Cells above 10 μm^2, presumed to be those with HbF, ranged from 0–88, with a mean of 18.9 ± 4.4 (27) sites per face. This result again showed that, although 50% of the cells had adult Hb, L_p site numbers had not reached full adult values.

The distribution of L_l site numbers was quite different from that of L_p sites per cell face (Fig. 10B). No correlation with cell size was apparent; the mean value for cells with areas $<10\ \mu m^2$ was 72.5 ± 6.4 (32), and $>10\ \mu m^2$, 78.6 ± 8.0 (51) sites per face. There was a wide range in L_l values, from 6 to 225 but the mean value for all the cells, 76.8 ± 5.6 (83), was significantly less than the number of L_l sites per face obtained for cells from adult sheep (132 ± 6 [20]) (Smalley et al., 1982).

Detailed Study of Developmental Changes in One Lamb

The developmental changes in the red cells that occurred in one *LL* type lamb (9X443) were studied in detail. This lamb was also homozygous Hb*AA* type. Blood samples taken at 4, 16, and 31 d after birth were fractionated, all four fractions were tested serologically for L_p and L_l sites, and measurements were made of cation concentrations and K^+ fluxes with and without anti-L. In addition, hemolysates were subjected to electrophoresis to determine the presence of HbC (Table III), and the distribution of cell volumes was measured on the Coulter counter (Fig. 11). In the samples at day 4 the percentage of

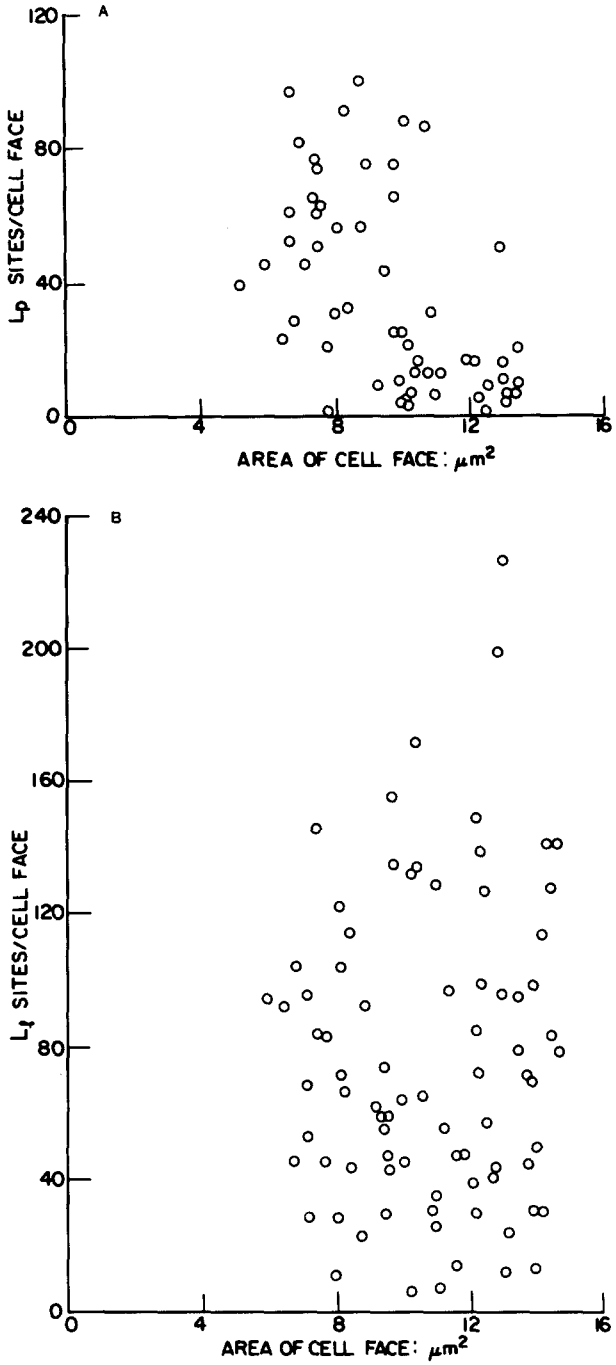

FIGURE 10. Numbers of L_p (A) and L_l (B) sites per cell face in relation to cell areas on red cells from a lamb (homozygous) aged 17 d, 50% of which had adult hemoglobin. Red cells were labeled with anti-L_p or anti-L_l and examined by immunoelectron microscopy.

adult Hb cells showed a gradation down fractions 1, 2, 3, and 4, being 56, 10, 6, and 1%, respectively. No HbC was present and L_p antigen was not detected serologically, although cells of all four fractions were lysed by anti-L_l. The number of L_p sites, determined by electron microscopy on unfractionated cells, was relatively low: 8.6 ± 0.9. Fraction 1 was complicated by the presence of 10% reticulocytes, which could account for the relatively high K^+ concentration, high flux rate, and weak stimulation of K^+ transport by anti-L in spite of the presence of 56% adult cells (see Table IV). Coulter counter sizing showed that cells in fractions 2, 3, and 4 were of similar size and were larger than the majority of cells in fraction 1.

At day 16, 96% adult cells were present in fraction 1 and only 10% in fraction 4. By this time, hemoglobin C was present in fractions 1 and 2. In fraction 1, intracellular potassium concentration and ouabain-inhibitable K^+ influx were still higher than in red cells from adult sheep in spite of the fact

TABLE III

HEMATOLOGICAL AND SEROLOGICAL STUDIES OF FRACTIONATED RED CELLS FROM ONE LAMB (9×443) AT 4, 16, AND 31 DAYS AFTER BIRTH

Age	Fraction	Adult Hb cells	Reticulocytes	HbC	Hemolysis scores		L_p sites (sites/face)	Cell face area	
					Anti-L_p	Anti-L_l			
d		%	%	%			*mean ± SEM (n)*	*(μm^2) mean ± SEM (n) [range]*	
4	1	56	10	0	0	38			
	2	10	<1	0	0	36	8.6±0.9 (11)	13.6±0.4 (11)	[11.8–15.7]
	3	6	0	0	0	36			
	4	1	0	0	0	35			
16	1	96	11	12	6	37	42±3.5 (2)	10.7±0.3 (20)	[8.4–13.4]
	2	89	1	10	0	40	—	—	
	3	25	0	0	0	40	—	—	
	4	10	0	0	0	40	10±1.5 (20)	12.3±0.4 (20)	[9.5–15.2]
31	1	97	5	15	11	40	33±3.2 (21)	8.7±0.4 (21)	[6.9–12.8]
	2	94	2	19	6	40	—	—	
	3	91	<1	23	6	40	—	—	
	4	80	<1	17	6	40	23±3.3 (23)	9.0±0.2 (23)	[6.9–11.6]

that most of the cells were of adult Hb type. A twofold stimulation with anti-L was obtained, however. According to previous studies (Tucker and Ellory, 1971), the presence of 11% reticulocytes in an adult *LL* sheep would raise the K^+ concentration to ~0.1 mmol/g Hb. The value found in this lamb was 0.22 mmol/g Hb, a concentration one would expect to find if 30% reticulocytes were present, which strongly suggests that the K^+ concentration of the cells in this fraction was not yet at normal adult values. Fraction 2 with 89% adult cells (<1% reticulocytes) had somewhat lower values of K^+ (0.19 mmol/g Hb) and K^+ uptake was stimulated 3.6-fold by anti-L. Fraction 1 cells lysed very weakly with anti-L_p and the L_l scores were high in all fractions. Electron microscopy studies on fractions 1 and 4 also showed a difference in size and a marked difference in the number of L_p sites between the fractions. The Coulter counter measurements showed a progressive increase in cell size down the fractions, because fraction 1 cells were smaller at this time than fraction 1

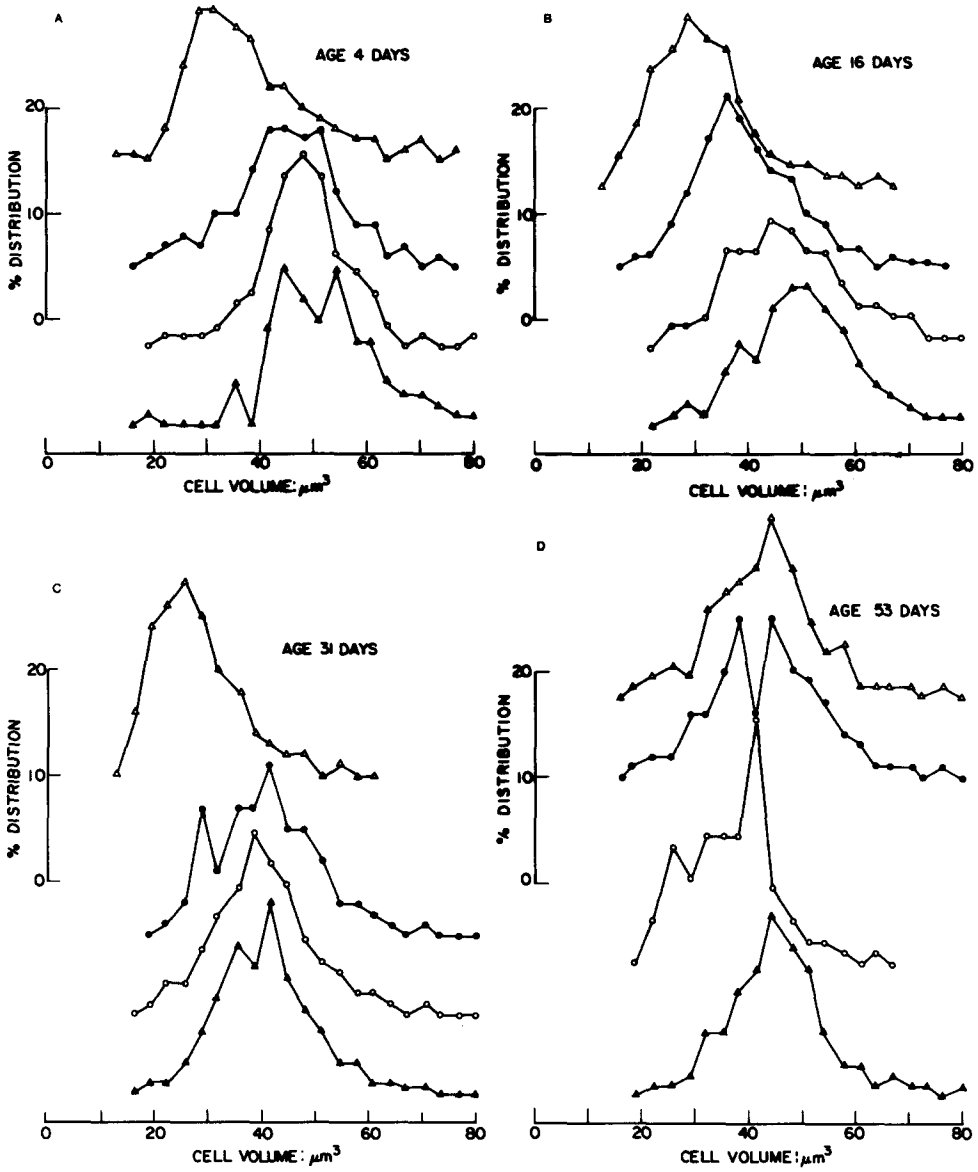

FIGURE 11. Cell volume distributions (from Coulter counter) in fractions of red cells from a lamb (9X443) at 4, 16, 31, and 53 d after birth. Fraction 1 (△) consisted of the "youngest" red cells, fraction 4 (▲) the "oldest," and fractions 2 (●) and 3 (○) contained cells of intermediate age.

cells at day 4. Fraction 2 cells were of similar volume to those in fraction 1 on day 4.

By day 31 all four fractions contained mostly cells with adult Hb and HbC had spread to all four fractions, but K^+ and Na^+ concentrations had not yet

reached normal adult values. K^+ transport in the cells in all fractions was stimulated by anti-L. Between days 16 and 31 there was no significant increase in the numbers of L_p sites determined by electron microscopy in cells from fraction 1, although the number of sites had doubled in cells from fraction 4. Most of the cells in fraction 1 of the day 31 sample were extremely small both by Coulter counter and electron microscopic measurements and these presumably corresponded to the small cells described by Valet et al. (1978). Because only 15% of the hemoglobin in this fraction was HbC, this would seem to eliminate HbC-containing cells as being specially connected with this small cell population. The cell volumes in fractions 2, 3, and 4 were all remarkably alike and close to those expected in an adult cell population.

A further sampling from 9 × 443 was made at day 53 for serological studies and determinations of cell volumes. By this time fraction 1 cells, containing 100% adult Hb, were similar in size to the cells in fractions 2, 3, and 4;

TABLE IV

Na^+ AND K^+ CONCENTRATIONS AND ACTIVE K^+ INFLUX IN FRACTIONATED RED CELLS FROM ONE LAMB (9×443) AT 4, 16, AND 31 DAYS AFTER BIRTH

Age	Fraction	K^+	Na^+	Active K^+ influx	Fractional pump stimulation with anti-L
d		*mmol/g Hb*	*mmol/g Hb*	*mmol/liter/h*	
4	1	0.314	0.121	0.900	1.64
	2	0.429	0.106	1.145	1.20
	3	0.374	0.066	—	—
	4	0.355	0.054	0.870	—
16	1	0.221	0.251	0.554	2.00
	2	0.193	0.225	0.226	3.58
	3	0.354	0.134	0.964	1.49
	4	0.412	0.108	1.080	1.29
31	1	0.110	0.233	0.223	3.65
	2	0.134	0.256	0.165	4.08
	3	0.137	0.243	0.196	3.76
	4	0.168	0.186	0.388	2.41

however, fraction 3 cells had a component of smaller cells, possibly the same cells that had been present in fraction 1 on day 31. The anti-L_p hemolysis scores of the cells of all four fractions had risen to 15, but the numbers of L_p sites determined electron microscopically on unfractionated cells did not indicate any further increase between days 31 and 53 (L_p sites being 34 ± 2.5 [31] per face). However, a blood sample taken at day 143 from this lamb showed that the number of mean L_p sites had increased to 66 ± 5.6 (12) per face.

DISCUSSION

Valet et al. (1979) and Lauf and Valet (1980) have also studied the changes in cation transport that take place in newborn LK lambs. They separated red cells of lambs on the basis of their differing volumes and examined the transport properties of the separated populations of large and small cells. The

results indicated the appearance at about day 17 of a transient population of small cells (28 μm^3) with adult K^+ characteristics, which, it was suggested, might be cells with HbC known to occur in Hb *AA* or Hb *AB* type lambs of about this age (Blunt and Huisman, 1975). Very large cells present in the blood (36 μm^3) were presumed to be fetal cells that, together with the small cells, were eventually replaced by another population of large cells comprised presumably of reticulocytes and mature adult LK type cells (30 μm^3). However, the separated cells were not identified either by their hemoglobin type or by the presence of reticulin (Lauf and Valet, 1980).

In the present investigation, we separated cells primarily on the basis of their age rather than volume by taking advantage of the differing densities of "young" and "old" red cells. Previous experiments with lambs using ^{59}Fe and electrophoretic separation of hemoglobin had shown that the method of differential centrifugation in saline achieved a satisfactory separation of cells with fetal Hb (older cells) from cells with adult Hb (younger cells) (Drury and Tucker, 1963). The acid elution technique used in our present study for identifying cells with adult and fetal Hb had the advantage of providing a direct visual quantitative measurement of the percentage of cells with each type of hemoglobin. A possible objection to the method is that a false estimate could be made if both types of hemoglobin are present in the same cell. However, the two cell types were so clearly distinguishable (see Fig. 2), with intermediate-staining cells being seen only occasionally (possibly reticulocytes), that we decided that the method was reliable. Certainly the results agreed well with those of electrophoresis.

Analysis of changes in the blood of lambs is complex because the newborn animal is in a dynamic growth phase, rapidly increasing its volume of circulating red cells so that the mean age of the nonfetal cell population decreases over the first weeks after birth (Drury and Tucker, 1963). The presence of circulating reticulocytes, a reflection of this phase of rapid erythropoiesis, also presents problems because such cells have very different properties from mature cells (Tucker and Ellory, 1971; Dunham and Blostein, 1976). We were therefore careful to monitor all samples for the presence of reticulocytes.

When cell K^+ content was plotted against the percentage of cells with adult Hb in samples from lambs of age 5–59 d, there was an inverse linear correlation, with cell Na^+ showing a reciprocal relationship. Such a linear correlation is consistent with a simple replacement of one population by another. At first sight the data seemed to show a more complex relationship, possibly giving a quadratic function. However, this was not amenable to further study because the scatter derived from pooling data on individual animals made it impossible to resolve the situation. The hint of curvilinearity does, however, leave open the possibility of a more complex conversion during development than simple replacement by one adult population.

The K^+ influx data paralleled the data on K^+ content in terms of an inverse correlation with adult Hb; similarly, the number of Na^+/K^+ pumps, obtained by measuring ouabain binding, showed a threefold decline as HbF disap-

peared. When intracellular Na^+ was plotted as a function of active K^+ influx, it was found, as expected, that high Na^+ cells had low K^+ pump activity. Although this low pump activity correlated with the low number of pump sites, there was an additional factor involved, namely changes in affinity of the pumps for K^+ at their intracellular aspect. In the separate series of experiments, where the kinetic characteristics of the internal cation loading site of the Na^+/K^+ pump were examined as a function of internal K^+ at high internal Na^+, the results clearly showed that the decrease in K^+ transport was caused not only by a reduced number of Na^+/K^+ pumps, but also by altered affinities of the pumps. It has previously been shown (Sachs et al., 1974; Cavieres and Ellory, 1977) that LK goat cells show marked inhibition of their pumps by intracellular K^+, which is consistent with a paradoxically higher internal affinity for K^+ as a dead-end inhibitor than for Na^+ as the substrate; the situation in LK sheep cells is apparently the same (Lauf et al., 1970; Dunham, 1976*b*). The present kinetic studies show that this marked inhibitory effect of internal K^+ was absent in fetal cells, which had more pumps per cell and kinetic characteristics of the pumps typical of HK cells, i.e., only moderate inhibition by cellular K^+. In contrast, the new cells of the postnatal lamb had increasingly LK-type kinetics, and fewer pumps per cell.

The hemocyanin-labeling technique, like the acid elution method for hemoglobin, had the advantage that individual red cells could be studied. It was not possible to know whether the individual cells examined in the electron micrographs contained HbF or adult Hb, but if enough cells were studied, and the percentage of cells with adult and fetal Hb was also ascertained for the sample, it was possible to relate numbers of L antigen sites to the other parameters measured. Moreover, estimation of the surface area of the individual red cell on an electron micrograph enabled the number of sites to be related to cell area and gave an independent measure of cell size, which could be compared with the determinations of cell volume made on cell populations by Coulter counting.

In general, cells from samples with predominantly fetal Hb had fewer L_p sites and were larger than cells from samples with predominantly adult Hb. During the period over which the transport properties changed, the numbers of L_p sites increased. Although serologically the fetal cells gave no observable hemolysis with anti-L_p, the hemocyanin method indicated the presence of some L_p sites on these cells. Significantly, L_p site numbers appeared to show a progressive increase, a finding not consistent with simple replacement by one adult cell type. Also, numbers of L_p sites per cell (but not hemolysis score) continued to increase after 100% adult Hb cells had been attained. In the hemocyanin-labeling experiment where L_l sites were compared with L_p sites in cell samples comprising 50% adult and 50% fetal cells as judged by acid elution, there was no obvious correlation of L_l site numbers with cell size. There was a wide scatter between individual cells but nothing to suggest that cells with fetal Hb were different from cells with adult Hb in respect of L_l. This agrees with the serological findings, and with the K^+ transport studies. However, the mean number of L_l sites in this 17-d-old lamb was significantly

less than that previously found for adult cells (Smalley et al., 1982), a point worthy of further investigation.

The results obtained both by electron microscopy and from the Coulter counter confirmed Lauf and Valet's (1980) observation that small cells appear in the blood of newborn lambs. Their results showed that these cells had adult K^+ transport characteristics. The small cells that we examined in fraction 1, however, appeared to have intermediate K^+ transport properties and intermediate numbers of L_p sites in spite of having adult Hb. Our small cells were in fraction 1, and hence by definition were "young cells," even though they were certainly not all reticulocytes. This could account for the intermediate values. However, taking all the results together, we consider it more likely that they are indicative of a gradual conversion to a completely adult-type cell, brought about by the release into the circulation of cells with progressively increasing adult K^+ properties. Furthermore, the results suggest that the switch to adult Hb synthesis does not coincide exactly with the switch to adult K^+ transport. It is perhaps not surprising that the cells with adult Hb varied in size. The small cells were produced during the rapid recovery phase from the postnatal anemia through which all lambs pass. They may simply be a reflection of cells produced under stress. The presence of HbC in some but not all of the small cells supports this idea, for this hemoglobin is only produced in adults after episodes of severe anemia (Blunt and Huisman, 1975). For further discussion of these various points, see Valet et al. (1978) and Lauf and Valet (1980).

The results described in this paper provide further information on the separate functional properties of antigens L_p and L_l. As mentioned in the introduction, the original hypothesis that antigen L acts by inhibiting active K^+ transport was largely based on the observation that it is only weakly developed on the red cells of newborn lambs (Tucker and Ellory, 1970). It so happened that the anti-L serum used for that study was a virtually pure anti-L_p; had we used a hyperimmune serum, it would certainly have contained anti-L_l and we would not at that time have been able to put forward the hypothesis. It is obviously very important to separate and identify these two specificities in any studies involving the use of anti-L sera.

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