J. Membrane Biol. 76, 281–287 (1983)

The Journal of
Membrane Biology

The Correlation of Composition and Morphology during the High to Low Potassium Transition in Single Erythropoietic Cells

R. Gary Kirk, S. Brian Andrews and Ping Lee*

Department of Physiology, Yale University School of Medicine, New Haven, Conn. 06510 and
* Department of Physiology, West Virginia University Medical Center, Morgantown, West Virginia 26506

Summary. The change from high potassium dog erythroid cells to low potassium red blood cells during erythropoiesis was investigated by X-ray microanalysis of single cells. A correlation of morphology and composition, using freeze-dried cryosectioned preparations, showed that during normal erythropoiesis in dog bone marrow the switch from high potassium to low potassium occurs during the change from early to late nucleated erythroid cells, and in synchrony with the beginning of iron accumulation. In contrast, during rapid erythropoiesis in dogs with phenylhydrazine-induced anemia, the most prominent change in cation composition as well as the accumulation of iron occurs during the reticulocyte stage in the peripheral blood.

The determination of the absolute amounts of sodium and potassium per cell in stress reticulocytes of peripheral blood indicated that the changeover from high potassium to low potassium actually occurs by the loss of cellular potassium during volume reduction, with little change in the amount of cellular sodium. This suggests that maturation may involve a selective change in potassium permeability. Lastly, it was observed that not all cells followed the predominant pathway with respect to change in morphology, membrane permeability and hemoglobin synthesis. One particular subpopulation appeared to follow a sequence which expressed the complete HK to LK transition before the accumulation of any iron; this implies the possibility of completing protein synthesis in a low potassium intracellular milieu.

Key Words electron probe · X-ray microanalysis · bone marrow · red blood cells · transport · membrane · erythropoiesis · hemoglobin · blood

Introduction

Mature dog red blood cells contain low potassium (LK 10 to 15 mmol/liter of packed cells) and high sodium (80 to 100 mmol/liter) concentrations, whereas the erythropoietic stem cells contain high potassium (HK) and low sodium concentrations. Therefore, during erythropoiesis in this animal there must be a transformation of the membrane transport characteristic resulting in changes in composition. In previous studies on these membrane transport changes, we have shown that the reticulocytes isolated from the peripheral blood of dogs with phenylhydrazine-induced anemia show a dramatic drop in intracellular potassium and an increase in sodium as they accumulate iron (Lee & Kirk, 1982). From this study, it was concluded that the modification in membrane function must have been initiated before or during the formation of reticulocytes, at least in the phenylhydrazine-treated dog. In addition, this modification of cation transport appeared to occur after the initiation of hemoglobin synthesis but before the completion. These experiments were carried out using wavelength-dispersive (WDS) X-ray microanalysis on smeared cell preparations in which morphological identification of cell type was not possible. The degree of development of the erythroid cells was based on the accumulation of iron. Therefore it was not possible to correlate composition with the precise developmental stages of the erythroblasts.

To better define the relationship of composition to development, it is necessary to examine the composition of marrow cells of known cell type and developmental state. Energy dispersive X-ray microanalysis of cryosections is ideally suited for this task, since this technology permits quantitative elemental analysis of morphologically defined cellular and subcellular structures in heterogeneous cell populations. This paper reports the relationship between changes in the cytosolic sodium and potassium concentrations, the onset of hemoglobin synthesis, and the morphological development in erythroid cells. This study also describes the nature of the differences in developmental sequence that accompany accelerated erythropoiesis in anemic animals.

Materials and Methods

The erythroid cells were obtained from the femur of dog by flushing the marrow cavity with ice-cold NaCl (0.155 M) containing heparin (1 unit/ml) and 10% plasma. Increased produc-

tion of reticulocytes was induced by injecting adult dogs daily with phenylhydrazine (10 mg/kg body weight/day) for four consecutive days. A reticulocyte-enriched fraction was obtained from the cell layer beneath the buffy coat (white blood cells) of centrifuged blood (5,000 × *g*, 30 min, 25° C). Cells were then packed by centrifugation (500 × *g*, 5 min), resuspended in plasma and packed again. The procedures for freezing, cryosectioning and analyzing thin samples are basically similar to the method of Somlyo, Shuman and Somlyo (1977), and have been verified for our laboratory use (Andrews, Kirk & Mazurkiewicz, 1983). Samples were frozen in Freon 22 precooled by liquid nitrogen and cryosectioned using an LKB cryokit (LKB, Bromma, Sweden). Sections were cut with glass knives at approximately −100° C. Sections were transferred onto carbon foil-coated copper grids and freeze-dried and carbon-coated in a vacuum evaporator (Denton Vacuum, Cherry Hill, N.J.). Energy-dispersive X-ray spectra were obtained using a JEOL 100-CX electron microscope equipped with a 30 mm^2 Kevex Si(Li) detector and a Kevex 7000 series X-ray spectrometer (Kevex Corp., Foster City, Calif.) interfaced to a PDP 11V03-L computer (Digital Equipment Corp., Maynard, Mass.). X-ray spectra were acquired from specimens at ambient temperature, accelerating voltage of 80 kV, and a beam current of 1 nA for 100 sec. The quantitation approach for the EDS data was the multiple least-squares (ML) method (Schamber, 1977) as implemented by Shuman, Somlyo and Somlyo (1976).

Concentrations (mmol/kg wet wt) were obtained directly by using a variation of the internal standard ratios method (Dörge et al., 1978). The isolated marrow cells contained numerous mature erythrocytes whose elemental concentrations and water content were known from chemical analysis. These erythrocytes were analyzed *in situ* in conjunction with the marrow cells, and subsequently employed as internal standards. Since characteristic X-ray intensities are directly proportional to elemental amounts in a defined microvolume, concentrations were calculated by comparing the X-ray intensities of the marrow cells and mature erythrocytes. This approach assumes constant section thickness and no differential shrinkage.

For determining the total amounts of K and Na in single blood cells, a smearing procedure which has been described by Kirk et al. (1979) was used. In this procedure, cells were washed four times by centrifugation (500 × *g*, 5 min) with sucrose solution (0.285 M) with 10% glycylglycine-$MgCO_3$ buffer (3.4 g magnesium carbonate, 5.1 g glycylglycine in 100 ml H_2O, pH 7.4) to remove external sodium and potassium. Cells were finally suspended in the same sucrose solution to obtain a cell/medium volume ratio of about 1 to 9. This suspension was smeared onto a preheated (70° C) pyrolytic graphite block (Fullam, Schenectady, N.Y.). Each cell was analyzed individually with a raster large enough to cover the entire cell. In order to visualize the cells the samples were exposed to an electron beam at low magnification and at 200 nA beam current for 30 sec to remove the sucrose layer coating the cells. The K, Na and Fe contents were measured using two wavelength spectrometers; these were required because their high peak to background ratio facilitates analysis of samples on solid supports. The wavelength spectrometers were calibrated by comparing the mean internal sodium and potassium concentrations from flame photometry with the X-ray intensities from X-ray microanalysis (Kirk et al., 1979).

Results

The bone marrow contains a number of different cell types and requires good visualization of the morphology for accurate discrimination of erythroblasts from myelocytes and for precise identification of developmental stages. Cryosectioned preparations were chosen to study this tissue, since only these provide the necessary morphological detail along with the preservation of ion distribution required for quantitative analysis of the cation concentrations by X-ray microanalysis. These sections were sufficiently thin (about 130 nm after freeze-drying) that the morphological characteristics of different cells are easily recognizable. As shown in Fig. 1 the distinction between the erythroid series and the myeloid series was evident. The upper panel of this Figure shows two representative cells of the myeloid series, one of the eosinophil line and the other of the polymorphonuclear leukocyte (PMN) line. These cells are characterized by the presence of the cytoplasmic granules and, in more mature cells, by a lobed nucleus. The lower panel of the Figure shows two cells of the erythroblastic series, which are characterized by the absence of cytoplasmic granules. The distinction between the earlier erythroid cells (early basophilic erythroblast, Fig. 1 *c*) and the more mature cell (a presumptive polychromatophilic erythroblast, Fig. 1 *d*) is less evident but equally certain, relying on the differences in cell size, degree and pattern of nuclear condensation, and the change in volume ratio of the nucleus to the cytoplasm. The late basophilic erythroblast was also categorized as a third, intermediate stage of development; this cell type (*not shown*), which was found less frequently than the other erythropoietic cells, was recognized by a sparse, very thin rim of cytoplasm.

X-ray microanalysis indicated that while the myeloid cells are of high-potassium type, the erythroid cells exhibited a continuum of potassium concentrations, encompassing the entire range of anticipated potassium levels. This result was obtained from the analysis of either the nucleus or cytoplasm of marrow cells, because there were no significant differences in sodium and potassium concentrations between these compartments in any marrow cell type. There were, however, small but systematic differences in the distribution of chloride and phosphorus. Table 1 compares the nuclear/cytoplasmic elemental concentrations of two representative marrow cell types that were selected because of large differences in overall composition. The absence of cation compartmentalization is experimentally advantageous, since in certain cell types, e.g., mature myelocytes and late basophilic erythroblasts, it is often only possible to analyze the nucleus with the probe reliably located. Thus, nuclear concentrations are reported throughout this text as representative of cellular cation concen-

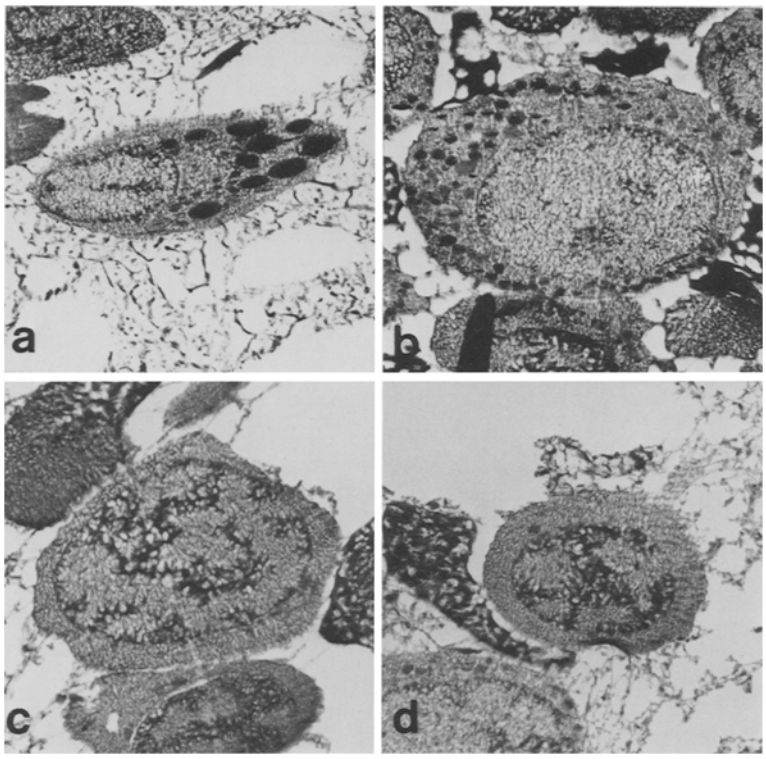

Fig. 1. Transmission electron micrographs of freeze-dried cryosections of erythroid cells from dog bone marrow. (*a*) eosinophil, (*b*) polymorphonuclear leukocyte, (*c*) early basophilic erythroblast and (*d*) polychromatophilic erythroblast

Table 1. Comparison of nuclear and cytoplasmic elemental concentrations in dog erythropoietic cells

	Na	P	Cl	K
	mmol/kg wet weight			
Anemic marrow HK erythroblast				
Nucleus ($n=10$)	20± 3.8	135± 6.4	25±1.6	108±5.0
Cytoplasm ($n=9$)	21± 4.6	119±10.8	33±2.6	105±6.2
Anemic marrow LK erythroblast				
Nucleus ($n=8$)	120±10.9	113±14.4	50±4.5	18±3.8
Cytoplasm ($n=7$)	128±10.9	108±14.4	49±7.3	18±2.1

Data are given as the mean ± SEM. The number of cells analyzed is indicated in parentheses.

trations. Iron was an important exception to this approach, since this element, when present, occurred only in the cytosol and never in the nucleus. Regarding the cation changes associated with erythropoiesis, the results are most clear-cut for the extreme members of the erythroblast population; Figure 2 illustrates the clear differences in X-ray spectra obtained from HK and LK erythroid cells. Cells in which the potassium concentration was >60 mmol/kg wet wt were pooled and averaged, and these results are presented in Table 2 as estimates of the limiting ion concentrations in HK cells; similarly, cells with $C_K < 10$ mmol/kg wet

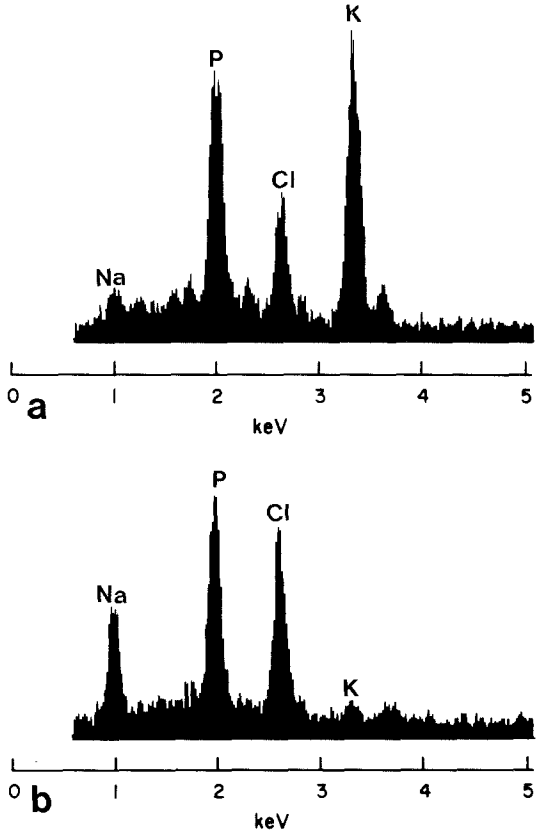

Fig. 2. X-ray spectra obtained from the nucleus of *a)* HK and *b)* LK erythroid cells

Table 2. Elemental concentrations in dog hematopoietic cells

	Na	P	Cl	K	Fe-positive fraction[a]
	mmol/kg wet weight				
Normal marrow cells					
Nuclei					
HK erythroblast ($n=24$)	28± 2.5	101± 4.0	31±1.7	90 ±2.6	0.22
LK erythroblast ($n=22$)	122± 4.3	144±10.4	48±3.0	5.9±0.8	0.76
Neutrophilic myelocyte ($n=21$)	19± 3.0	105± 7.9	32±2.1	96 ±4.4	–
Cytoplasm					
Red blood cell ($n=30$)	112± 3.1	36± 1.8	49±1.8	6.9±0.7	1.00
Anemic marrow cells					
Nuclei					
HK erythroblast ($n=19$)	22± 3.2	126± 6.7	38±3.1	108 ±4.3	0.00
LK erythroblast ($n=8$)	120±10.9	113±14.4	50±4.5	18 ±3.8	0.00
Neutrophilic myelocyte ($n=6$)	27± 4.2	135±11.2	34±4.0	114 ±9.2	0.00
Anemic reticulocyte fraction					
Nuclei					
HK erythroblast ($n=10$)	20± 3.8	135± 6.4	25±1.6	108 ±5.0	0.00
Neutrophils ($n=4$)	20± 3.7	122± 7.4	28± ±1.7	117 ±3.8	–
Cytoplasm					
HK reticulocyte ($n=19$)	24± 3.7	59± 4.5	37±1.4	92 ±2.8	0.56
LK reticulocyte ($n=43$)	108±3.0	27± 1.3	56±1.7	6.0±0.4	1.00

Data are given as the mean ± SEM. The number of cells analyzed is indicated in parentheses.

[a] The presence of iron was based on cytoplasmic analysis. A dash indicates neutrophilic cells which were, and were expected to be, iron free.

wt were considered representative of terminal LK concentrations. In addition, the population averages for red blood cells and polymorphonuclear leukocytes are included in Table 2. PMN's were always found to be of high potassium type whereas the red blood cells were of low potassium type. These cells were analyzed together with erythroid cells in order to serve as controls.

An analysis of the nucleated erythroid cells in the normal dog bone marrow shows that a significant number of nucleated erythroid cells have already switched from HK to LK type (Fig. 3). As illustrated in Fig. 4, most of the LK nucleated erythroid cells contain low levels of iron whereas the majority of the HK nucleated erythroid cells contain no detectable amount of iron. The data in Fig. 3 were obtained from all nucleated erythroid cells without regard to their maturational stage. In order to focus on the difference between early and late erythroid cells we have categorized the erythroid population into three different developmental stages on the basis of morphological criteria previously described. Table 3 shows the results concerning the relative abundance of cells of different cation composition in the various categories, which are defined as follows: HK cells have a potassium concentration (C_K) > 60 mmol/kg wet

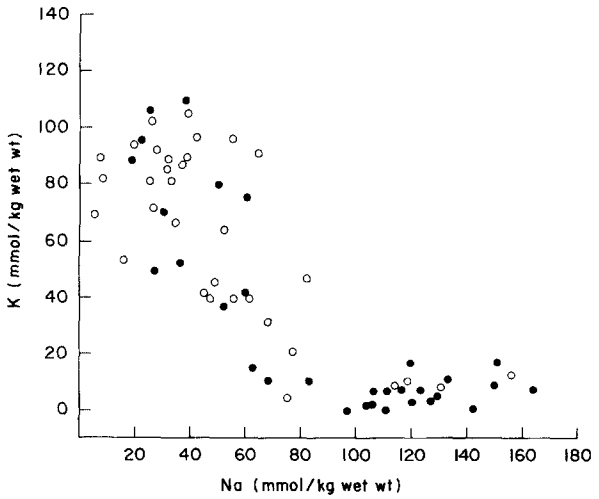

Fig. 3. Relationship between the potassium and sodium concentration in nucleated erythroid cells from marrow of normal dog bone marrow. Solid circles locate single cells which contain iron. Open circles locate single cells without iron

wt; intermediate (IK) cells have C_K between 10 and 60 mmol/kg; and LK cells contain $C_K <$ 10 mmol/kg. The percentages of cells with detectable amounts of iron are also shown in this Table.

As seen in Table 3, early erythroid cells are of HK type and most show no trace of iron. In con-

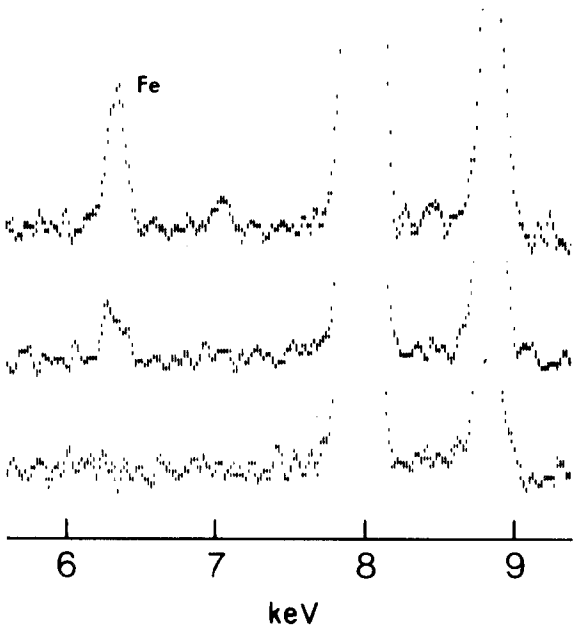

Fig. 4. X-ray spectra showing varying levels of iron with highest level from a denucleated red cell (upper trace), intermedite level from a LK basophilic erythroblast and lowest level from a HK basophilic erythroblast (lower trace)

Table 3. Distribution of erythropoietic cell types by morphology and composition in normal dog marrow

Erythroblasts by cation composition	Erythroblasts by morphology		
	Early	Intermediate	Late
	(percent)		
HK cells ($C_K > 60$ mmol/kg)	29	8	2
IK cells ($10 < C_K < 60$ mmol/kg)	6	13	4
LK cells ($C_K < 10$ mmol/kg)	6	6	25
Fe-positive cells	6	10	25

The distribution is expressed as percentages; that is, the sum of all values in the 3×3 matrix is 100%. 48 cells are represented in this Table. The sum of values in the last row is 41%, because 20 of 48 cells were Fe-positive.

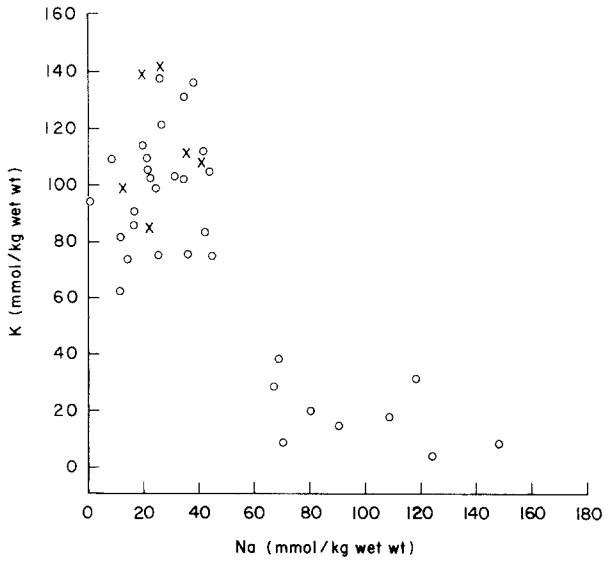

Fig. 5. Relationship between the potassium and sodium concentration in nucleated erythroid cells from marrow of anemic dogs. The open circles indicate that all cells were without a trace of iron. Single polymorphonuclear leukocytes are located by X's. These were always of HK type and served as controls

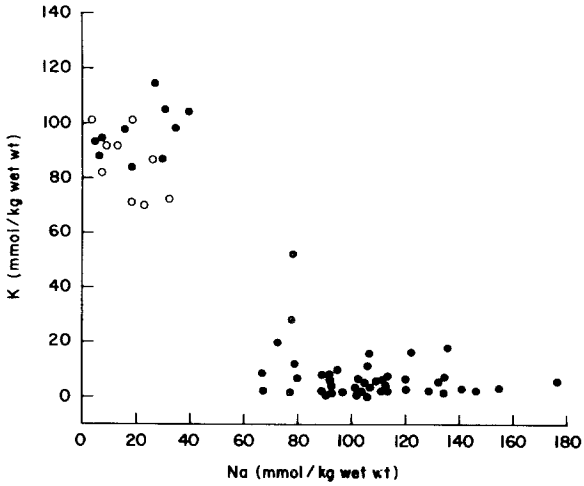

Fig. 6. Relationship between the potassium and sodium concentration in single reticulocytes from peripheral blood of anemic dog. Solid circles locate cells which contain iron

trast, the later erythroid cells are frequently of LK type; these low potassium cells show detectable but low levels of iron. The denucleated red cells are distinctly LK, and contain high levels of iron (Fig. 4). These results indicate that in normal adult dog the switch from HK to LK type occurs primarily in nucleated cells, relatively quickly, and in close synchrony with the onset of iron accumulation. However, most of the iron incorporation appears to occur after denucleation of the normoblast.

The erythroid cells in dogs with anemia induced by phenylhydrazine injection show a different pattern in the switch from HK to LK. Figure 5 shows the K and Na concentrations of nucleated erythroid cells from the marrow of anemic dogs. Two major differences exist as compared with normal dogs (Fig. 3). First, none of the nucleated erythroid cells in Fig. 5 contain iron. Secondly, over 2/3 of the erythroid cells in anemic dogs are of HK type. Figure 6 shows the K and Na concentrations of the reticulocytes from the peripheral blood of the anemic dog. It is clear that this reticulocyte population consists of both HK and LK type cells and that the LK cells are all iron-containing but only

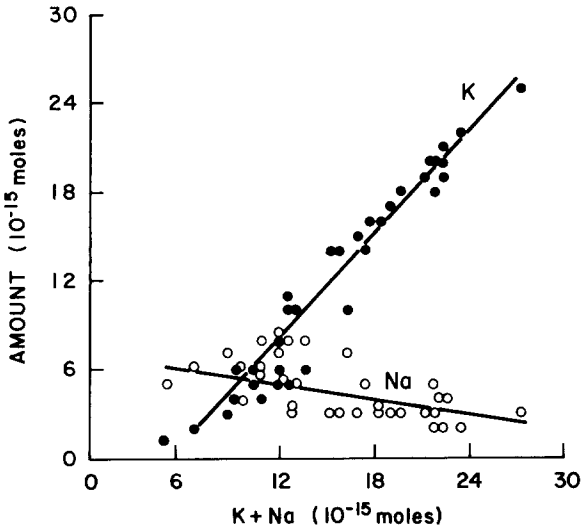

Fig. 7. Relationships between the amounts of potassium and sodium with the total amount of (K + Na) in single cells from peripheral blood of anemic dog

about half of the HK reticulocytes have detectable amounts of iron. Additionally, these spectra reveal that the LK reticulocytes contain much higher levels of iron than the Fe-positive HK reticulocytes. These results indicate that in anemic dogs the morphological maturation of erythroid cells occurs earlier than the switch of the membrane transport properties, resulting in the appearance of HK reticulocytes in peripheral circulation.

The enriched reticulocyte fraction from anemic dogs is also suitable for demonstrating the nature of ionic movements responsible for the change in composition. Figure 7 shows the results of wavelength-dispersive X-ray analysis of the contents of individual reticulocytes using the smear technique to measure the total amount of intracellular potassium and sodium. It is useful to determine the total potassium and sodium because it has been shown previously that the cell volume is proportional to the amount of cellular (K + Na), (Funder & Wieth, 1966; Kirk & Lee, 1981). Plots of total K and Na *vs.* K + Na may be regarded as indicating the change in intracellular K and Na contents as a function of cell volume. As shown in Fig. 7, the K content dramatically decreases as the total (K + Na) decreases, whereas the Na content increases only slightly with decrease in (K + Na). However, it should be noted that the Na radiation is energetically very weak (1.04 keV) and some of the Na increase could be obscured by self absorption as the dry mass increases due to hemoglobin synthesis. It is known that as the erythroid cells mature they decrease in size. Therefore, it appears that this drop in size is primarily associated with a loss of K without an equivalent gain in the Na content.

Discussion

Previous studies on normal dog marrow had indicated a correlation between the initial accumulation of iron and the change in cellular cation composition. The present investigation confirms this relationship; moreover, the morphological information available from cryosectioned preparations associates this compositional transformation with the developmental transition from basophilic ("early") erythroblast to polychromatophilic or orthochromatophilic ("late") erythroblast.

In the case of late erythroid cells, the X-ray spectra frequently showed a distinct iron peak, representing <20% (*ca.* 2 mmol/kg) of the iron concentration present in mature red blood cells. When the iron was present, it occurred only in the cytosol; this is in contrast to sodium and potassium, which were uniformly distributed between the nucleus and cytoplasm in all cell types. The situation is different in the marrow of dogs with an accelerated rate of erythropoiesis due to chemically induced anemia. In this case, most of the nucleated erythroblasts are HK, all of them are Fe-negative, and there is a distinct paucity of late erythroid cells. Instead, the HK to LK transition, along with the beginnings of iron accumulation, can be demonstrated in the reticulocyte fraction from the peripheral blood of the phenylhydrazine-treated animal; this result is qualitatively consistent with previous conclusions from the analysis of smeared cell preparations. Thus, erythropoiesis in the anemic dog appears to involve a large acceleration in the morphological aspects of red blood cell production with less of an effect on the development of mature biochemical and transport functionality. It is also possible that the alterations in transport properties are accelerated but the changes in K and Na are lagging behind the membrane changes. In order to demonstrate this possibility it would be necessary to have kinetic measurements of cation transport of the erythroid cells. This is not presently available in this study. It may be significant that hemoglobin production and the HK to LK transition approximately maintain a normal temporal relationship in the anemic dog despite a dramatic alteration in the cell type and tissue in which this change occurs.

Although most of the cells from both normal and phenylhydrazine-treated animals had the characteristics discussed, there were a few nonconforming cells in most experiments. For example, in normal marrow most of the early erythroid cells were of HK type, whereas the late erythroid cells were predominantly LK. However, a small number of

early erythroid cells having low potassium concentrations were found, as were a few late erythroid cells having high potassium. The nonconforming LK type cells are not thought to be damaged cells because of their low chloride levels. These observations indicate that there may be some diversity in the erythropoietic process, in that certain steps may be frequently but not obligatorily coupled, or there may be alternative developmental pathways which operate simultaneously.

The occurrence among the minor populations of a few morphologically early, iron-free cells with low cytoplasmic potassium (Fig. 3) is interesting because it suggests that hemoglobin synthesis may proceed at low cellular potassium concentration in these cells. This is contrary to the findings of Cahn and Lubin (1978) who reported that the completion of globin chains is dependent on a high cellular potassium concentration. It is not known whether this independence of hemoglobin synthesis on high potassium concentration is a general phenomenon, or whether it occurs only in erythroid cells of dog and other LK species such as cats, LK sheep, LK cattle and Australian oppossum. The change from HK to LK state in dog erythroid cells occurs more as a result of the loss of potassium than as the consequence of a gain in Na (Fig. 6). It appears that the cells have attained their predetermined Na level early, and that the loss of potassium with the resultant reduction in cellular water and volume caused an increase in the cellular sodium concentration. In the HK stress reticulocytes produced in LK sheep, a similar type of change in cellular potassium without significant gain in cellular sodium has also been reported (Lauf, 1981; Lauf & Valet, 1981). In addition, in pig reticulocyte maturation potassium plays the predominant role in controlling the cell volume, since as volume is decreased potassium is reduced to a much greater extent than is sodium (Zeidler & Kim, 1982). Thus, in the transition from the HK to LK stage, modification in potassium transport processes is the determining factor in the observed change in both Na and K concentrations.

This work was supported by National Institutes of Health grants AM-21391, AM-17433, HL-30514 and HL-13237.

References

Andrews, S.B., Kirk, R.G., Mazurkiewicz, J.E. 1983. The distribution of intracellular ions in the avian salt gland. *J. Cell. Biol.* **96**:1389–1399

Cahn, F., Lubin, M. 1978. Inhibition of elongation steps of protein synthesis at reduced potassium concentrations in reticulocytes and reticulocyte lysate. *J. Biol. Chem.* **253**:7798–7803

Dörge, A., Rick, R., Gehring, K., Thurau, K. 1978. Preparation of freeze-dried cryosections for quantitative X-ray microanalysis of electrolytes in biological soft tissues. *Pfluegers Arch.* **373**:85–97

Funder, J., Wieth, J.O. 1966. Potassium, sodium, and water in normal human red blood cells. *Scand. J. Clin. Lab. Invest.* **18**:167–180

Kirk, R.G., Lee, P. 1981. Elemental concentration determination in single erythrocytes. *In:* Microprobe Analysis of Biological Systems. T.E. Hutchinson and A.P. Somlyo, editors. pp. 367–376. Academic Press, New York

Kirk, R.G., Lee, P., Duplinsky, T.G., Tosteson, D.C. 1979. X-ray microanalysis of red blood cells. *In:* Biological X-ray Microanalysis. C. Lechene and R. Warner, editors. pp. 299–316. Academic Press, New York

Lauf, P.K. 1981. Chloride-activated, ouabain-insensitive K^+ flux in reticulocytes and mature red cells of anemic LK sheep. *Fed. Proc.* **40**:483

Lauf, P.K., Valet, G. 1981. K^+ pump fluxes and passive K^+ permeabilities in large and small erythrocyte volume populations of anemic high and low K^+ sheep. *Biophys. J.* **33**:3a

Lee, P., Kirk, R.G. 1982. A study of maturation of membrane transport function in red blood cells by X-ray microanalysis. *J. Membrane Biol.* **67**:103–111

Schamber, F.H. 1977. A modification of the linear least-squares fitting method which provides continuum suppression. *In:* X-ray Fluorescence Analysis of Environmental Samples. T.G. Dzubay, editor. pp. 241–257. Ann Arbor Science Publishers, Ann Arbor, Michigan

Shuman, H., Somlyo, A.V., Somlyo, A.P. 1976. Quantitative electron probe microanalysis of biological thin sections: Method and validity. *Ultramicroscopy* **1**:317–339

Somlyo, A.V., Shuman, H., Somlyo, A.P. 1977. Elemental distribution in striated muscle and the effects of hypertonicity. Electron probe analysis of cryosections. *J. Cell Biol.* **74**:828–857

Zeidler, R.B., Kim, H.D. 1982. Pig reticulocytes. IV. In vitro maturation of naturally occurring reticulocytes with permeability loss to glucose. *J. Cell. Physiol.* **112**:360–366

Received 6 January 1983; revised 22 April 1983