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Study of Maturation of Membrane Transport Function in Red Blood Cells by X-ray Microanalysis

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Summary. Red blood cells of certain species of animals, such as dogs and cats, contain low potassium and high sodium, whereas the erythropoietic stem cells giving rise to these cells are of high potassium type. This paper examines the sequence of membrane transport changes during erythropoiesis by analyzing the K, Na and Fe in single bone marrow cells, reticulocytes and mature red blood cells with X-ray microanalysis. The relationship between K/Na ratios and Fe/(K + Na) ratios were examined by X-ray microanalysis. The K/Na ratios give a measure of the membrane cation transport function. The Fe/(K + Na), which is analogous to hemoglobin concentration, gives an index of maturation stage. The relationships between K/Na and Fe/(K + Na) in the marrow cells of normal adult dog and those of a phenylhydrazine-injected dog with accelerated erythropoiesis show that the modification of cation composition occurs after the initiation of hemoglobin synthesis but before its completion. Similar relationships in the reticulocytes obtained from phenylhydrazine-injected dogs as well as from newborn dogs show a consistent decrease in K/Na with increased Hb, indicating a drastic change in cation composition during the maturation of the reticulocytes. Therefore the modification in membrane transport function must have occurred before or during the formation of reticulocytes.

Key words X-ray microanalysis · electron probe · maturation · transport · red blood cells · erythropoiesis

Introduction

Red blood cells from various mammalian species contain varying amounts of potassium and sodium ions. The red cells of most mammalian species such as humans, horses, pigs and rabbits have large amounts of potassium and small amounts of sodium. These are known as high potassium (HK) type. A few species such as dogs and cats have red cells of low potassium (LK) type where the potassium concentration is low and sodium concentration is high. Other species such as sheep, cattle, and the Australian opossum have red cells which are either HK or LK depending on the genetic type of the individuals. The erythropoietic stem cells which give rise to the LK red cells are presumably of HK type. This presumption is based on the observation that when the marrow is stressed in anemic state the reticulocytes (immature red cells) released from the marrow into the circulation can have high concentrations of potassium (*see* Parker, *1972*). Therefore, the transformation of the HK erythropoietic stem cells to the LK red cells must occur at some point during the differentiation and maturation of the erythroblastic cells in the bone marrow. In order to understand the sequence of events in the maturation of membrane transport function and hemoglobin production during erythropoiesis, we have by X-ray microanalysis (electron probe microanalysis) analyzed the K, Na and Fe contents of the bone marrow cells in dogs, cats and rabbits, as well as those of mature and immature red blood cells in adult and newborn dogs. Since it is generally accepted that the cation composition in red cells is determined by the pump and leak processes of cation transport across the cell membrane and that a change in K/Na ratio in cells is predetermined by a change in pump and leak parameters (Tosteson & Hoffman, 1960) we assume that the K/Na ratio in single cells may give us an index of the membrane transport function. In general a higher K/Na ratio reflects a higher pump-to-leak ratio. Fe concentration in the cells is taken as a measure of hemoglobin concentration, since in the cells of erythroblastic series almost all the Fe atoms are incorporated in the hemoglobin molecules (Noyes, Hosain & Finch, 1964; Ganzoni, 1969). An increase in Fe concentration is indicative of an increase in hemoglobin concentration, hence an increase in maturity of the erythroblastic cells. By studying the relationship between the K/Na ratio and Fe concentration, we can deduce the time course of the maturation of the membrane transport function in relation to hemoglobin synthesis. Our previous study of the dog bone marrow (Kirk, Lee & Tosteson, 1978) indicated that the modification of membrane transport function from high potassium type to low potassium type probably occurs early in the maturation sequence, before hemoglobin synthesis is complete. The finding of HK

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stress reticulocytes in anemic dogs (Henriques & Orskov, 1936; *also see* Parker, 1972) and in LK sheep after severe bleeding (Blunt & Evans, 1965; Lee, Woo & Tosteson, 1966) suggests that the modification of membrane transport may occur as late as the reticulocyte stage. This paper examines the time course of the maturation of membrane transport function in dog red cells during normal erythropoiesis and during accelerated erythropoiesis. Data for a HK species, the rabbit, and another LK species, the cat, are also presented for the purpose of comparison.

Materials and Methods

Preparation of Cells

Animals were anesthetized with sodium pentobarbitol (65 mg per kg body weight). Fresh blood samples from dogs, cats and rabbits were drawn into heparinized syringes (10 units heparin per ml blood). To obtain red blood cells the blood samples were centrifuged and the plasma together with the thin layer of buffy coat which lay on top of the packed red cells and which also contained the white blood cells were removed by suction. The long bone from the hind leg, the femur, was used for bone marrow cell harvesting. The marrow cells were obtained by flushing the marrow cavity of the bone with ice-cold NaCl (0.155 M) containing heparin (1 unit/ml). Both blood and bone marrow cells were washed four times with ice-cold $MgCl_2$ (0.12 M) buffered with 10% glycylglycine buffer (3.4 g magnesium carbonate, 5.1 g glycylglycine in 100 ml H_2O, pH 7.4) by repeated centrifugation (500 $\times g$, 5 min each time) and resuspension of the cells in the same buffered $MgCl_2$ solution. After the final wash, the cells were suspended (1 part cells in 10 parts medium by volume) in sucrose solution (0.285 M) with 10% glycylglycine buffer (pH 7.4). The cells were immediately smeared onto several polished pyrolytic blocks ($10 \times 2 \times 2$ mm) cut from a larger graphite disc (1 in diameter; Fullam, Schenectady, N.Y.). The technique of smearing the cells onto the graphite blocks has been described by Kirk, Lee, Duplinsky and Tosteson (1979). This involves: (1) dipping a long (5 cm) cotton-tipped applicator into the cell suspension to obtain a cotton tip well soaked with the cell suspension, (2) inserting the wooden dowel of the applicator in the collet of a motor-driven hand-drill (Dremel, Racine, Wisconsin), (3) rotating the applicator slowly at approximately 300 rpm, and (4) passing a preheated graphite block (70 °C) rapidly over the wet surface of the rotating cotton tip of the applicator. The block was then returned to the platform of a hotplate (70° C). Each block was subsequently examined with a metallurgical microscope equipped with a hot stage (70° C) to determine the proper distribution of the cells. Several warm blocks were anchored to warm scanning electron microscope (SEM) stubs with graphite in isopropanol (Dag-154, Acheson Colloids Co., Port Huron, Michigan) and stored in a desiccator with silica gel until analysis.

When reticulocytes (immature red cells) were studied they were obtained from both newborn and adult dogs. Newborn dogs (1–3 week) have 5–12% reticulocytes in their blood. These reticulocytes can be separated from the main red cell population by centrifuging the whole blood at 25 °C in a nonrefrigerated centrifuge with swinging bucket rotor (30 min at 5000 $\times g$). After centrifugation, the plasma was removed and the buffy coat was carefully moved to the side of the centrifuge tube and removed with a Pasteur pipet. The cells in the layer immediately below the buffy coats were reticulocyte-rich. A layer (2 mm in depth) of these cells was collected with a pipet and suspended in the buffered isotonic $MgCl_2$ solution mentioned previously. Suspensions of red cells with about 70% reticulocytes were obtained from the blood of newborn dogs (1–3 week) in this manner. Adult dog blood contains less than 1% reticulocytes and it is not easy to obtain a reticulocyte-rich suspension from it. To produce adequate reticulocytes for study, healthy adult dogs were injected with phenylhydrazine (10 mg/kg body wt/day) for four consecutive days. On the eighth day after the initial injection the dog was killed and its blood was used for harvesting of reticulocytes as described above. A suspension with 99% reticulocytes can be obtained. Reticulocytes were washed and smeared onto the graphite block in the same manner as the bone marrow cells and red blood cells were handled.

K, Na and Fe contents in each individual cell were determined by means of X-ray microanalysis. The methods have been previously described by Kirk et al. (1978, 1979). About a hundred individual cells of each sample were routinely analyzed. Cells were analyzed one at a time with an electron probe (ETEC Autoscan, Hayward, CA) using a raster sufficiently large to include a cell. Characteristic K_α X-ray lines were measured by two wavelength spectrometers using a lithium fluoride crystal for Fe analysis, a pentaerythriotol crystal for K, and rubidium acid phthalate crystal for Na. An electron beam current of 200 nA and a 15 kV accelerating voltage was used in this study. It was found to be helpful in the visualization of the cells to expose the samples to a 200 nA beam current at a low beam density achieved by reducing magnification for 30 sec to remove the sucrose layer coating the cell. Calibrations were made by comparing the mean internal sodium and potassium concentrations from flame photometry and from the X-ray microanalysis.

It has previously been found that there is a linear relationship between X-ray intensity and cellular elemental content in red blood cells (Kirk et al., 1979). The X-ray microanalysis permits the measurement of the amounts of K, Na and Fe present in each of the cells but not their concentration. The elemental concentration can be computed if the individual cellular water content is known. Our X-ray data do not permit the computation of cellular water content directly, but one may estimate the cellular water indirectly from the total major cations in the cells. Funder and Wieth (1966) have shown that cellular water is directly proportional to the total (K + Na). This is valid when the K and Na ions are bound only to a neglible extent. In the bone marrow cells and the red blood cells, this is most likely to be the case. By dividing the amount of a given element by the total (K + Na), one obtains a number which is essentially proportional to the concentration of that element in the cells. It is this number which has been given as an index for concentration. Since K, Na and Fe shown in the data are amounts in fentonmoles (10^{-15} moles) per cell, the ratios K/(K + Na) Na/(K + Na) and Fe/(K + Na) are dimensionless fractions which are proportional to the K, Na and Fe concentrations.

Results

Adult Dog Bone Marrow Cells

As mentioned in the Introduction the K/Na ratio allows us to have a measurement of the membrane cation transport properties whereas the Fe/(K + Na) ratio gives us an index of the maturational stage of the cells. Figure 1 shows a plot of K/Na *vs.* Fe/(K + Na) in bone marrow cells of normal adult dog. It should be noted that only cells of the erythropoietic line contain hemoglobin, and that a number of cells which were found to contain very low Fe/(K + Na)

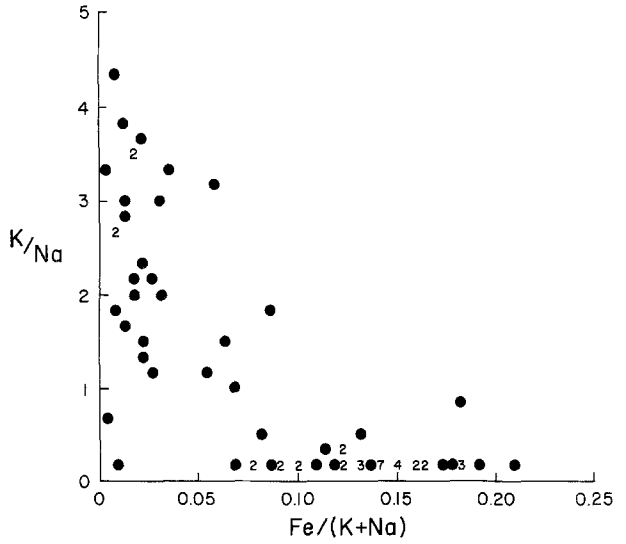

Fig. 1. Relationship between the ratios of K/Na and the ratios of Fe/(K + Na) in single bone marrow cells of normal adult dog. Filled circles locate single cells. When two or more cells have the same value, the number of cells located at a given point is indicated by a number instead of a filled circle

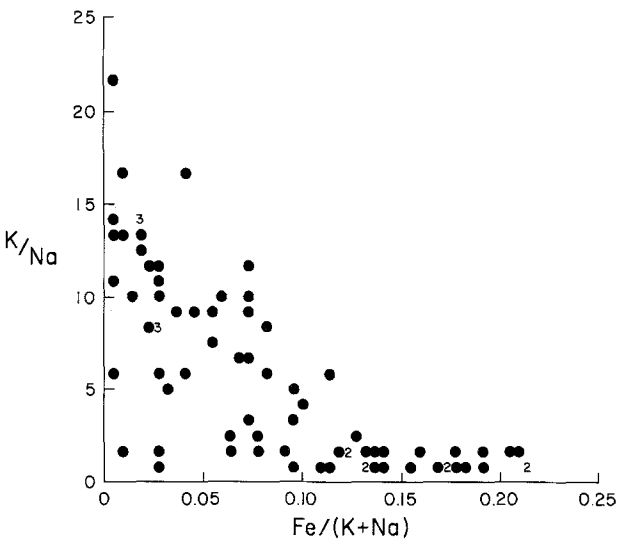

Fig. 2. Relationship between the ratios of K/Na and the ratios of Fe/(K + Na) in single bone marrow cells of anemic dog (induced by phenylhydrazine injection)

may not be involved with the erythropoiesis process. In normal adult dog bone marrow (Fig. 1), the K/Na ratios in the cells varied over a wide range. All the cells which contain high hemoglobin concentrations indicated by the increased Fe/(K + Na) levels have low K/Na ratios, indicating that the more mature red cells have already become low-K. The cells with very low Fe/(K + Na) have varied K/Na ratios. It cannot be determined here which of these cells are the erythropoietic stem cells. The fact that some reticulocytes (the immature red cells) are of high-K type implies that the stem cells should have relatively high K concentration. Therefore some of the high-K cells which are located at low Fe/(K + Na) range must be the erythropoietic stem cells. Many of the cells with low Fe/(K + Na) have low K/Na ratios. We do not know what these cells are. If these cells are of the erythropoietic line, the results would suggest that the membrane transformation from HK to LK type occurs fairly early in the erythropoietic process, before hemoglobin synthesis is appreciable but this occurs only in relatively few cells. Most of the cells have their cation composition change occurring after the cells have accumulated some Fe content, suggesting that modification of membrane transport occurs predominantly after the initiation of hemoglobin synthesis but well before its completion. Therefore it is probable transformation of membrane transport function from HK to LK type occurs primarily after the stem cells have committed to become erythropoietic cells. Hence it seems unlikely that the trigger for the membrane transport transformation and that for the hemoglobin could be the same one.

Anemic Dog Bone Marrow Cells

Figure 2 shows the change of K/Na as a function of Fe/(K + Na) in the marrow cells of a phenylhydrazine-treated dog. As can be noted the pattern seems similar to that found in normal dog shown in Fig. 1. The cells with low Fe/(K + Na) have considerably higher K/Na than those found in normal dog marrow. (Note that the ordinate scale is increased 5×.) The reason for this higher K/Na is not known. Additionally there are many more cells in the medium hemoglobin range with higher K/Na ratio than those found in normal dog bone marrow. It is probable that these cells are the normoblasts, nucleated red cells and reticulocytes. These types of cells are not as numerous under normal situations as they are during rapid erythropoiesis in response to the anemic state produced by phenylhydrazine injection. It is clear from this Figure the major change in cation composition occurs at levels of Fe/(K + Na) ratios between 0.05 and 0.10, suggesting again that the transformation of membrane transport function is preceded in time by hemoglobin synthesis but at a time before hemoglobin synthesis is complete. It is not possible to correlate precisely the hemoglobin concentration of the cells deduced from the X-ray data to the morphological stage of the erythroid cells.

Reticulocytes

To determine if the hemoglobin concentrations in reticulocytes do indeed have a similar value as those cells located in the medium range of Fe/(K + Na)

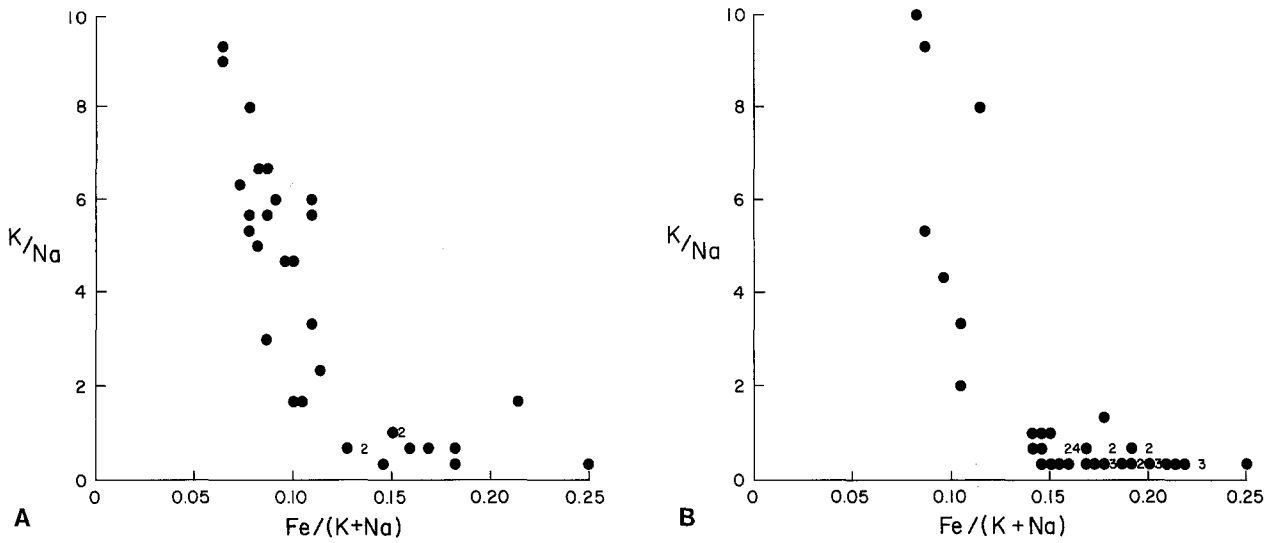

Fig. 3. Relationship between the ratios of K/Na and the ratios of Fe/(K + Na) in single blood cells of dog injected with phenylhydrazine. (*A*) Reticulocyte population enriched by centrifugation. (*B*) Unseparated peripheral blood cells

values shown in Fig. 2, it is important to examine an enriched population of reticulocytes. An examination of the stress reticulocytes obtained by a centrifugation isolation procedure from a phenylhydrazine-injected dog (Fig. 3) shows that these cells contain Fe/(K + Na) ratios similar to the intermediate values for bone marrow cells shown in Fig. 2. The highest ratios of K/Na in these reticulocytes also have intermediate values comparable to those in the marrow cells with approximately the same Fe/(K + Na) content (Fig. 2). The K/Na ratios show a consistent decrease with increase in Fe/(K + Na). The K/Na values change from a high value of 9 to a low value of 0.2 indicating that a drastic change in cation composition is occurring during the maturation of reticulocytes. This result also suggests that during accelerated erythropoiesis the membrane transport modification which affects the change in cation composition must have been initiated before or during the formation of reticulocytes. It may be as late as the time of denucleation of the normoblast, which then yields the reticulocytes. The K/Na also shows a slight decrease with increase in Fe/(K + Na) in the unseparated red blood cells of phenylhydrazine-injected dog (Fig. 3*B*). This is to be expected because the whole population contains 20–30% reticulocytes. In normal dog red cells the K/Na values are always very low. In phenylhydrazine-induced reticulocytes, the K concentration decreases with increased Na concentration (Figs. 4*A* and 4*B*).

Newborn Dog Marrow Cells and Reticulocytes

In newborn dogs a high rate of erythropoiesis also occurs and their blood contains 5–12% reticulocytes. We felt it would be of interest to compare the marrow cells, reticulocytes as well as the mature red blood cells of the newborn dogs with those of adult dogs. Therefore we have examined these three types of cells obtained from newborn dogs. As shown in Figs. 5*A* and 5*B* the K/Na ratios decrease with increase in Fe/(K + Na) both in marrow cells and in reticulocytes of 10-day-old dog. The patterns of change are similar to those observed in those of phenylhydrazine-injected dog (Figs. 2 and 3*A*). The highest K/Na ratios of the newborn dog reticulocytes, however, are considerably less than those of adult dog stress reticulocytes. The red blood cells in newborn dogs are also of LK type although their K concentrations are higher than those of adult dog red cells (K/Na ratio in newborn red cells is less than 0.5). These results seem to indicate that the newborn dog reticulocytes entering the circulation have undergone more high potassium to low potassium transformation relative to the changes observed in the stress reticulocytes in adult dog. This also may suggest that the extent to which a dog reticulocyte has changed to a low potassium cell depends on the length of time the reticulocyte remains in the marrow before it is released to the circulation. The transit time of a reticulocyte in the marrow may depend on the rate of erythropoiesis. In phenylhydrazine-induced anemic dog, the erythropoiesis is very rapid and the reticulocytes are presumably released into the circulation sooner than what occurs in normal erythropoiesis and many of these cells still retain very high K/Na ratios. In newborn dogs the erythropoietic rate is high but does not seem to be as high as those seen in phenylhydrazine-induced anemic adult dog judging from the lower reticulocyte counts (5–12% in newborn dogs *vs.* 20–30% or more in phenylhydrazine-induced anemic adult dog). In normal adult dog the erythropoiesis is low

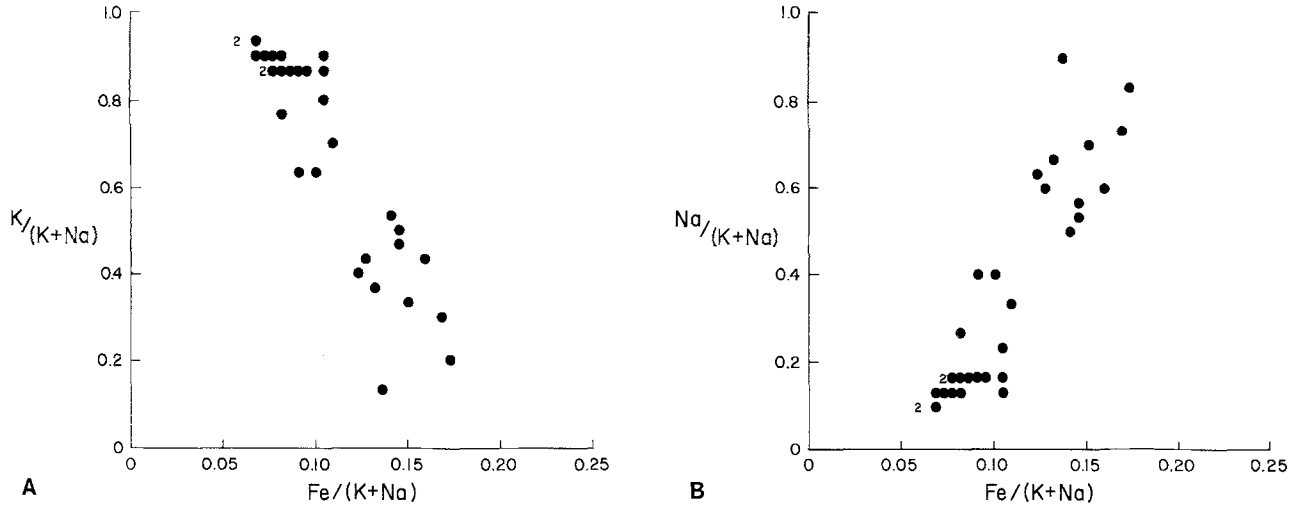

Fig. 4. Relationship between the ratios of (*A*) K/(K + Na), (*B*) Na/(K + Na) and Fe/(K + Na) in single reticulocytes produced in anemic dogs after phenylhydrazine injection

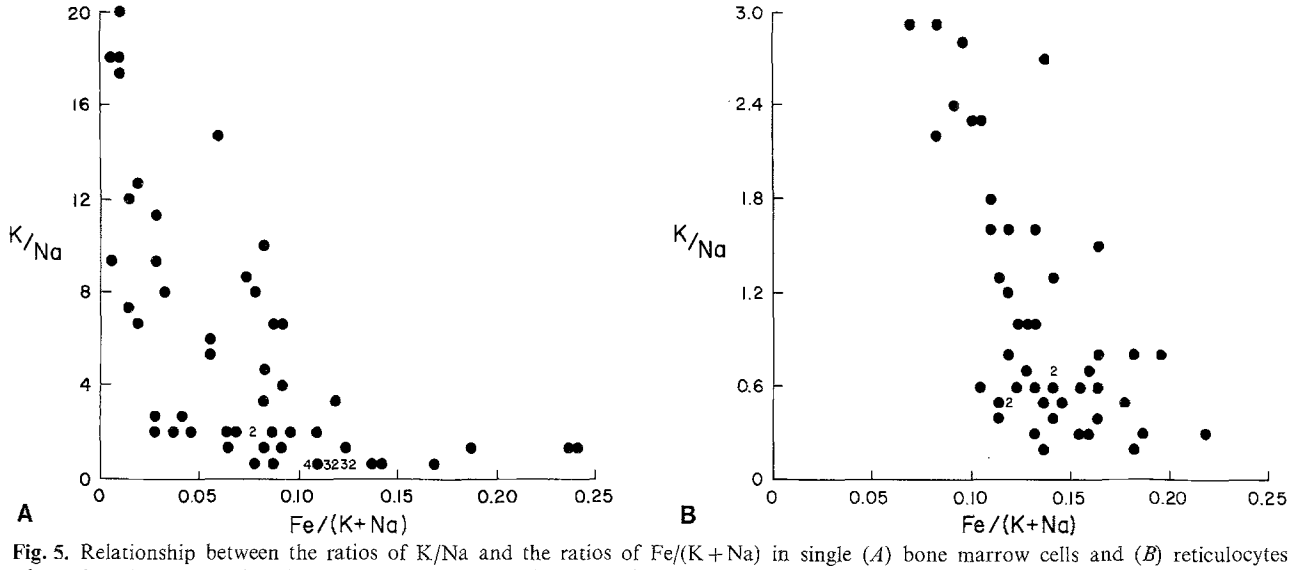

Fig. 5. Relationship between the ratios of K/Na and the ratios of Fe/(K + Na) in single (*A*) bone marrow cells and (*B*) reticulocytes of ten-day-old dog. In (*A*) the lowest ordinate point is 0.5. A few cells that have lower K/Na values than this are all plotted as 0.5

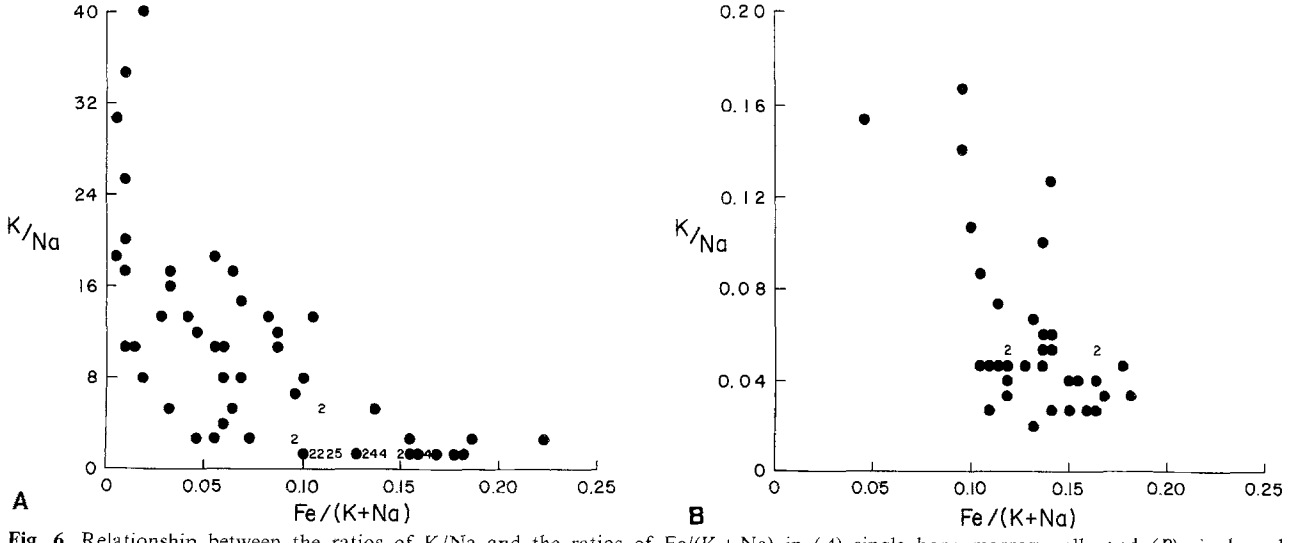

Fig. 6. Relationship between the ratios of K/Na and the ratios of Fe/(K + Na) in (*A*) single bone marrow cells and (*B*) single red blood cells of an adult cat

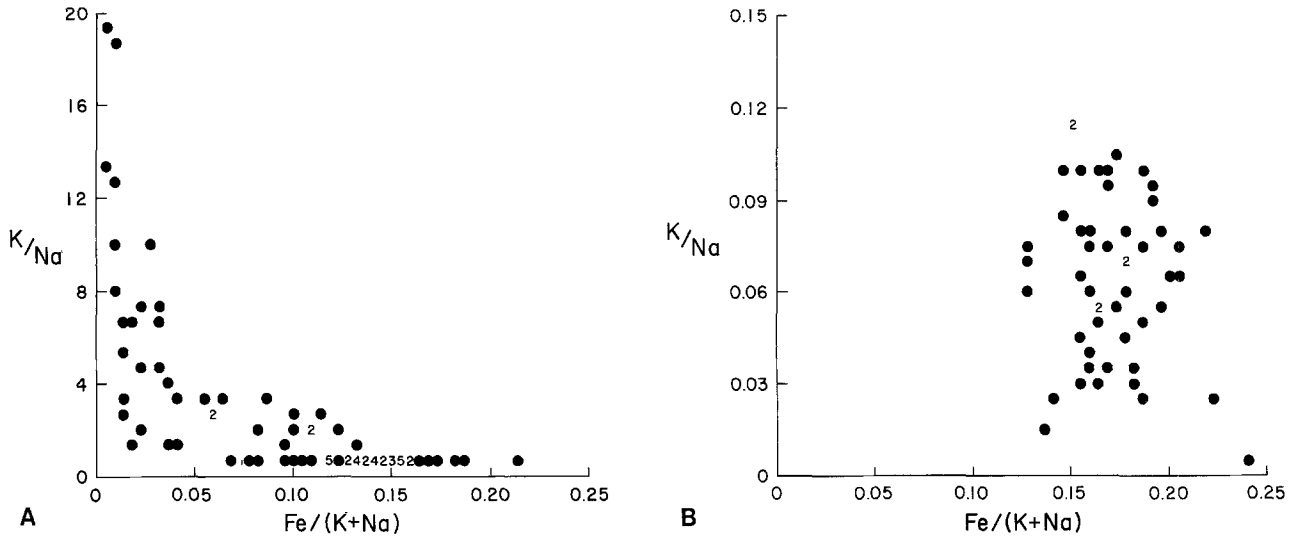

Fig. 7. Relationship between the ratios of K/Na and the ratios of Fe/(K+Na) in (*A*) single bone marrow cells and (*B*) single red blood cells of a newborn cat

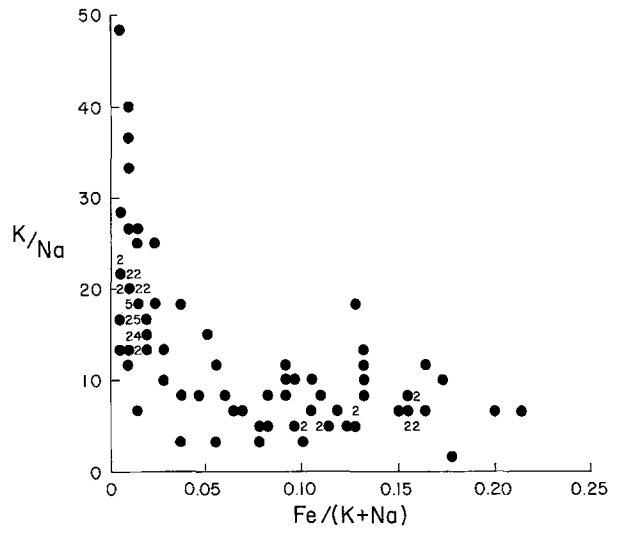

Fig. 8. Relationship between the ratios of K/Na and the ratios of Fe/(K+Na) in single bone marrow cells of rabbit

(generally thought to be about 1% of red cell mass per day) and the reticulocytes probably have K/Na value very close to that of mature red blood cells.

Adult and Newborn Cat Marrow Cells and Red Blood Cells

We have also examined the bone marrow and red blood cells of another species having LK red blood cells, the cat and the kitten. Figures 6*A* and 6*B* show the results for the changes in K/Na ratios with changes in Fe/(K+Na) in the adult cat bone marrow cells and the red blood cells, respectively. Figures 7*A* and 7*B* show the results for the newborn kitten bone marrow cells and red blood cells, respectively. These results are very similar to those found for newborn dogs. Here again, we note that the red cells released into the circulation have already become characteristic low potassium type. The K/Na ratios in red cells of both cat and kittens are considerably lower than those found in adult and newborn dogs.

Rabbit Marrow Cells

To examine the relationship between K/Na ratios and Fe/(K+Na) in the marrow cells of a high potassium type animal we have chosen the rabbit. Even in this species there is a cluster of marrow cells with high K/Na ratios at very low Fe/(K+Na) values (Fig. 8). There is, however, a distinct difference between the change in K/Na ratio in rabbit marrow cells and that in adult dog marrow cells. In the dog, the K/Na values drop from a high to a very low value (0.2) with increase in Fe/(K+Na), whereas in the rabbit marrow cells the K/Na ratios decline from a high value to a relatively constant level (K/Na = 5–6) which is distinctly higher than those seen in dog or cat. These results seem to suggest that even in a HK animal such as rabbit the stem cells may have somewhat higher K/Na than mature red blood cells.

Discussion

Our X-ray microanalytical determinations of single bone marrow cells in dog suggest that modification of membrane transport function occurs after the initiation of hemoglobin synthesis but long before its completion. From a study of the stress reticulocytes it is clear that during accelerated erythropoiesis the membrane transport change may occur as late as the reticulocyte stage. The many high potassium cells

found in the reticulocyte populations obtained from newborn puppies and from phenylhydrazine-induced anemic dog indicate that the change in membrane transport properties must have begun to occur immediately before or during the formation of reticulocytes. A major morphological alteration in the cell during the formation of a reticulocyte from the normablast is the loss of nucleus. It is tempting to relate the membrane transport change to the drastic event of denucleation. Aside from the fact that the time course for the two events seems to coincide quite well, there is no evidence to support the idea that the trigger for the two events are the same or that one is the consequence of the other. Whatever the reason, the effect of the change in the membrane transport properties continues to affect the composition of the cells during the maturation of the reticulocytes. As seen in Figs. 3*A* and 5*B* the reticulocytes continued to lose K and gain Na with an increase in hemoglobin concentration. The accelerated erythropoiesis in phenylhydrazine-induced anemic dog is unquestionably an abnormal state. It is unlikely that in normal erythropoiesis of an adult dog the reticulocytes in circulation will have as high a potassium concentration as that shown in stress reticulocytes in the circulation. Analysis of normal dog red cells in circulation (Kirk et al., 1978) shows that all red cells have low K/Na ratios. Even though the adult dog blood contains less than 1% reticulocytes the chance of encountering a reticulocyte in a red blood cell sample is small. If the potassium concentration has been distinctly high we should come across at least a few reticulocytes with high K/Na in all the samples of adult dog red cells we have analyzed. Thus far, all red cell populations from adult dog have shown their characteristic low potassium value, indicating that under normal conditions the reticulocytes and red cells released into the circulation have already undergone the cation composition change to their characteristic low potassium value.

It is to be noted that the criterion for determination of reticulocytes was the presence of darkly stained reticula in the cells when stained with new methylene blue. There is a question as to whether all these red cells which have stained reticula are actually reticulocytes. In the presence of an oxidative chemical such as phenylhydrazine, the hemoglobin molecules in the red cells tend to transform into cocoid hemoglobin, giving rise to what is known as Heinz bodies (Bunn, 1972). These Heinz bodies upon being stained with new methylene blue give the appearance of darkly stained bodies which are not easily distinguishable from the reticula of reticulocytes. Orringer and Parker (1977) have shown that red cells exposed to acetaphenylhydrazine have increased permeability to sodium and potassium. These exposed cells will lose K and gain Na faster than normal red cells when suspended in high sodium-low potassium medium. There are two pieces of data which indicate that it is unlikely the low K/Na and high Fe/(K + Na) cells in the plot for stress reticulocytes are mature red cells which contain Heinz bodies resulting from exposure to phenylhydrazine. First, the reticulocyte suspensions obtained from newborn puppies also show the same decrease in K/Na with increase in the Fe/(K + Na) ratio (Fig. 5*B*). These puppies have no previous exposure to phenylhydrazine and therefore it is not likely that the reticulocyte suspension contains an appreciable number of Heinz-body cells. Secondly, if the low K/Na and high Fe/(K + Na) cells in the stress reticulocyte suspension are in fact mature red cells containing Heinz bodies, the cells should have as high a hemoglobin concentration as that of a mature red blood cell. The reticulocytes which have been examined have in general considerably lower Fe/(K + Na) ratios than mature red cells (Fig. 3*A*). Thus it is more likely that the decrease in K/Na ratios with increase in hemoglobin concentration indicates a true change in the cation composition with maturation of the reticulocytes.

As mentioned in the introduction K/Na ratio may be regarded as an index for membrane transport. In maturing red cells where K and Na compositions are changing with time, the X-ray microanalytic data on cellular K/Na ratios only give the cation compositions of the cells at the instant of harvesting of the cells and may not reflect the membrane transport properties of the cells at that instant since the cells are not in a steady-state situation. Any observed change in cation composition is due to previous changes in membrane transport function. Since rapid changes of cation composition are observed in circulating reticulocytes there is no question that the membrane transport modification occurs during or before reticulocyte formation. This is at least true at the time of rapid erythropoiesis after phenylhydrazine injection. Whether or not under normal rate of erythropoiesis the change in membrane function occurs as late as reticulocyte stage is still uncertain. Our data do not permit us to make the same kind of conclusion as we have for stress reticulocytes. In normal dog marrow the reticulocyte and normoblast populations in the marrow are not as large as those found in anemic dog or newborn puppies. In a hundred bone marrow cells analyzed, the chance of picking enough of these cells to yield a plot with sufficient medium range hemoglobin concentrations is small. Therefore it is possible that some of the cells with high K/Na and medium range Fe/(K + Na) ratios in normal dog bone marrow may have been missed during analysis,

giving the impression that during normal erythropoiesis the most drastic change in K/Na occurs at a time when hemoglobin concentration is very low. It can also be argued that the membrane maturation may indeed occur during the formation of reticulocytes in normal dog. The observation in stress reticulocytes is due to the shortened interval that the reticulocytes remain in the marrow during rapid erythropoiesis. The stress reticulocytes produced in anemic dog induced by phenylhydrazine injection have much higher K/Na ratios than the reticulocytes obtained from puppies. From the reticulocyte counts (20–30% in anemic adult dogs *vs.* 5–12% in newborn dogs) it is clear that the erythropoiesis in the anemic adult dogs is much more rapid than that in newborn dogs. There is evidence that stress reticulocytes produced in anemic animals show a reduced marrow iron transit time, a measurement of the maturation time of the marrow reticulocytes (Ganzoni, Hillman & Finch, 1969). The time during which reticulocytes remain in the marrow is shortened. Conceivably the change in membrane transport function preceding the reticulocyte stage will require some time for its full effect to be felt. It must certainly take a few hours for the cell to lose K and gain Na. In LK sheep stress reticulocytes, the loss of the high K content was shown to be preceded by the change in membrane transport (Lee et al., 1966). In an *in vitro* culture of the stress HK reticulocytes from anemic LK sheep, Kim, Theg and Lauf (1980), showed that the K − Na pump decreased in a few days; however the K content remains relatively unchanged. It is generally known during *in vivo* maturation of the reticulocytes the reticulum will be lost in 24–72 h (*see* Bessis, 1973). If one accepts three days to be the maturation time course for reticulocytes in circulation, then from Fig. 3 which shows that the HK reticulocytes can become very low K/Na cells with increased hemoglobin content, it is obvious that it takes three days or less for the reticulocytes to attain a low K/Na ratio which is characteristic of that of mature dog red cells. It should be pointed out that all high-K cells probably maintain their cation composition by the pump-leak mechanism as proposed by Tosteson and Hoffman (1960), but in the LK dog red cells the active K − Na pump cannot be demonstrated by conventional methods using transport inhibitors such as ouabain (*see* Miles & Lee, 1972; Parker, 1973). Whatever changes in membrane transport function were to occur during the maturation of the reticulocytes, the pump and leak would have to coordinate so that cell volume would not alter significantly to produce hemolysis of the cells.

In the marrow of HK animals such as rabbit, there are numerous cells with low Fe/(K + Na) content and high K/Na ratio. A few of these cells have unusually high K/Na values. This is probably due to overestimation of the ratio by the technique. The greatest error in the determination of the K/Na lies in the quantitation of sodium in these large high potassium cells because of (1) the low X-ray intensities due to low Na content, and (2) higher than average absorption of low energy sodium X-rays due to the larger than average cell thickness. In spite of this difficulty, there is no question that these cells are of high potassium type and that the marrow blast cells and membrane modifications occurring during the maturation of the erythropoietic cells do affect the cation composition. One major difference in the cation composition change between marrow cells of HK rabbit and marrow cells of LK dogs and cats is that the decrease in K/Na ratio levels off at a much higher value. Studies on newborn lamb red cells have shown that they have higher K/Na than mature sheep red cells (Evans & Blunt, 1960), suggesting that the stem cells can have a much higher K/Na value than those of mature HK sheep red blood cells. Without identifying the actual stem cells our present study does not allow for an accurate estimate of K/Na ratio in these predecessors of all the mature red cells. It is plausible that the modification of membrane transport function during erythropoiesis is a general phenomenon associated with cell division during the maturation process. The potassium concentration which the cells finally attain depends solely on the magnitude of change in the pump-leak relationship.

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