Changes from High Potassium (HK) to Low Potassium (LK) in Bovine Red Cells

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ABSTRACT Red cells of newborn calves contain 105–110 mmole K^+ and 1–5 mmole Na^+ per liter of cells. As the animals age the K^+ content decreases to a value of 25–30 mmole/liter of cells after about 60 days. At approximately the same time, the sodium content reaches a value of 60–70 mmole/liter. The time required for half change ($t_{1/2}$) is 35–37 days for both Na^+ and K^+. The activity of (Na + K)–adenosine triphosphatase (ATPase) and the influx of K^{42} and Rb^{86} into the red cells are high at birth and are reduced to 5 and 15% of their original values, respectively, in mature animals. $t_{1/2}$ for both is of the order of 30–35 days. The membrane Mg-ATPase activity is also high at birth and is reduced with a $t_{1/2}$ of 28–32 days to a final value of about 20% of its activity at birth. Separation of red cells according to their age showed that, in animals at the age of transition, newly formed red cells contain a higher K/Na ratio and a higher active transport capacity than older red cells of the same animal. It is suggested that the changes observed are a reflection of the average age of the red cell population as the animal grows.

INTRODUCTION

Although the concentration of potassium in most mammalian cells is 10–30 times higher than in their extracellular medium, the red cells of some mammals differ in this respect. The adult dog, cat, and most bovine strains have low concentrations of K^+ in their red cells. Sheep can be divided into two groups with respect to the K^+ content of their red cells: high potassium (HK) and low potassium (LK) animals (Evans, 1954). Studies by Evans and coworkers (1956) showed that a single gene locus is responsible for these differences, with dominance of the low potassium gene. Similar studies have been recently reported by Ellory and Tucker (1970), in Jersey cows in which a small proportion of the animals is HK.

Tosteson (1966) and Brewer et al. (1968) reported that newborn lambs have HK red cells, regardless of their potassium genotype. In LK homozygotes and heterozygotes, but not in HK homozygotes, the red cell population

is transformed into the low potassium form as the animal grows. This system has great advantages for the study of the molecular mechanisms that can regulate active ion transport. It is necessary, however, to know the genotype of each animal. This is achieved only after a prolonged study of the parents and their progeny, or after a rather difficult immunological technique (Rasmusen and Hall, 1966). As an alternative model, we have investigated the possibility of such changes also occurring in cattle of the Holstein strain, a relatively common and highly inbred variety of domestic cattle in which all the adult animals have a low K^+ content in the red cells.

METHODS

Holstein cattle of different ages and of both sexes were used. No sex differences in ion transport or red cell electrolytes were observed throughout this study. Sodium and potassium content of red cells and plasma was determined by conventional internal standard flame photometry. These ions were determined in whole blood and in plasma and the results were expressed in terms of millimoles per liter of cells, using the hematocrit values determined after centrifugation in a Clay-Adams microhematocrit centrifuge (Clay-Adams Inc., New York) for 7 min. In a few experiments the cells were washed three times with 0.11 M $MgCl_2$ and Na^+ and K^+ were determined. Very similar results were obtained by these two procedures. The uptake of K^{42} and Rb^{86} (New England Nuclear Corp., Boston, Mass.) by red cells was determined at 37°C in a medium containing 150 mM NaCl, 2.2 mM $CaCl_2$, 1.1 mM $MgSO_4$, 0.2% glucose, 25 mM glycylglycine-$MgCO_3$ buffer pH 7.4, and 5 mM of either KCl or RbCl as described by Bernstein and Israel (1970). When ouabain was used, it was added to a final concentration of 10^{-4} M. The uptake of K^{42} or Rb^{86} was linear with time for at least 1 hr. As a standard procedure, the cells were incubated for 40 min. After this time the contents of the incubation flask were transferred into ice-cold centrifuge tubes and centrifuged for 3 min at 3000 *g*. The red cells were further washed three times with 30 times their volume of ice-cold 0.11 M $MgCl_2$. The radioactivity in the cells and incubation medium was determined in a gamma radiation counter. ATPase activity was determined by measuring P_i liberated by hemoglobin-free ghosts prepared and assayed as previously described (Israel et al., 1970). The incubation medium contained 2 mM tris(hydroxymethyl)amino methane–adenosine triphosphate (Tris-ATP); 100 mM Tris-HCl, pH 7.65; 4 mM $MgCl_2$; 100 mM NaCl; and 20 mM KCl. The (Na + K)–ATPase activity was calculated by subtracting from the total ATPase activity that in the absence of Na^+ and K^+, plus 10^{-3} M ouabain (Mg–ATPase).

Albumin density gradients were prepared in 0.11 M $MgCl_2$ using a commercially available dialyzed and lyophilized bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.). The technique for preparation of the gradients was that of Leif and Vinograd (1964) as described by Bishop and Prentice (1966). It was found, as reported by the latter authors, that Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) density gradients were unsatisfactory because of a tendency for the cells to clump. Two stock solutions containing 20 and 28% w/w BSA were

prepared by slowly adding the albumin to the preweighed 0.11 M $MgCl_2$ solution at room temperature, under continuous stirring. Complete solubilization was accomplished after about 2–3 hr of stirring. These solutions were stored at −20°C and thawed to room temperature before use. The gradient was prepared by mixing 9.5 ml of 28% w/w BSA with an excess of 20% w/w BSA and pumping the mixture into a 96 × 14.5 mm ultracentrifuge tube to a height of about 80 mm. Red cells (0.75–1.0 ml) previously washed twice in ice-cold 0.11 M $MgCl_2$ and resuspended to a hematocrit of about 50% in this solution were carefully layered on top of the BSA gradient that had been cooled to 0°–2°C. The tubes were centrifuged at 2°C for 1 hr at 33,000 *g* (calculated in the center of the tube) in a swinging-bucket rotor. After centrifugation 1.0 ml fractions were carefully removed proceeding from the top to the bottom of the gradient. The concentration of albumin in each fraction of the gradient was determined by the biuret method (Gornall et al., 1949) after a 10-fold dilution with 0.11 M $MgCl_2$ and removal of the red cells by centrifugation.

Hemoglobin was determined spectrophotometrically in hemolysates at 540 mμ. Fetal and adult hemoglobins were separated by electrophoresis at 375 v and pH 8.6 for 2–3 hr in cellulose acetate gel strips as recommended by the Gelman Instrument Company (Ann Arbor, Mich.). The unstained strips were cut and the bands were eluted for optical density determination at 540 mμ. A preliminary scan at different wavelengths showed that the major absorption peaks were identical for both the fetal and adult hemoglobins.

The polypeptide pattern of the red cell membranes solubilized in 3% sodium dodecyl sulfate (SDS) was determined in 0.1% SDS-polyacrylamide gels and stained with Coomassie brilliant blue, as described by Laemmli (1970). The phospholipid pattern of the erythrocyte membranes was determined by two-dimensional thin layer chromatography of chloroform-methanol extracts (Folch et al., 1957) as described by Rouser et al. (1966).

Reticulocytes were determined after staining with new methylene blue for 15–20 min at room temperature, as described by Dacie and Lewis (1968).

RESULTS

The concentrations of Na^+ and K^+ in red cells was determined in 64 animals varying in age from 12 hr to 10 yr (Fig. 1). As can be observed, erythrocytes of newborn animals contain 105–110 mmole K^+ and 1–5 mmole Na^+ per liter of cells. As the animals age, the K^+ content decreases to a stable value of 25–30 mmole/liter at about 60 days. Erythrocyte sodium, on the other hand, increases after birth and reaches a stable value of 60–70 mmole/liter also after approximately 60 days. The time required for half change ($t_{1/2}$) is 35–37 days for both Na^+ and K^+; this suggests a common process for the changes in intracellular concentrations of these two ions. Two components seem to occur in the curves for Na^+ and K^+: a slow initial one, and a faster component that starts at about 35 days.

Since it is established that human reticulocytes contain higher K^+ concentrations than mature red cells (Bernstein, 1959), it seemed appropriate

to determine if the neonatal changes in electrolyte content of cattle red cells could be attributed to differences in the reticulocyte counts. Table I shows that although the percentage of reticulocytes is indeed higher in newborn animals and decreases with age, the changes in electrolyte concentrations could not be due to differences in reticulocyte proportions unless a K^+ concentration of about 25 M existed in reticulocytes. Furthermore, it is a well-

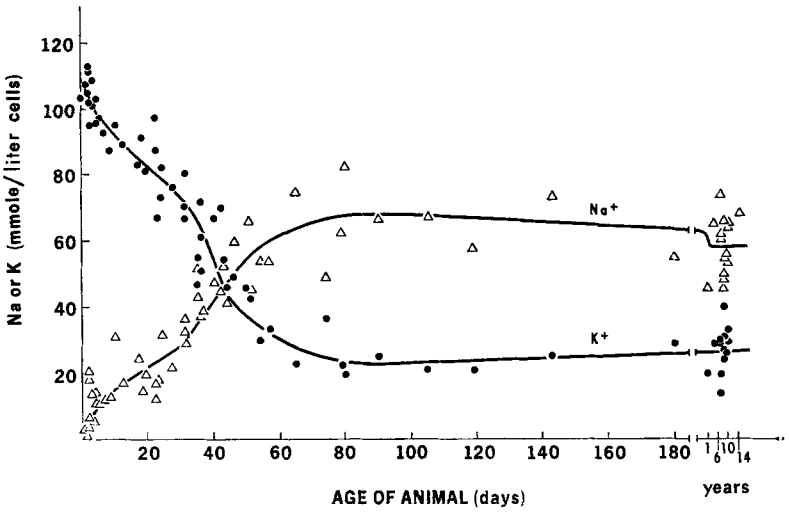

FIGURE 1. Sodium and potassium content of bovine red cells as a function of animal age. ● = K^+; △ = Na^+. Each point represents a different animal.

TABLE I

PER CENT RETICULOCYTES IN BLOOD OF ANIMALS OF DIFFERENT AGES

Age of animal	Reticulocytes
	%
0–5 days (5)	0.33 (0.0–0.6)
10–30 days (4)	0.08 (0.0–0.2)
40–60 days (5)	0.04 (0.0–0.2)
0.5–9 yr (4)	0.0 (0.0–0.0)

Numbers in parentheses represent numbers of animals tested.

known fact that bovines have an extremely low reticulocyte count. Maximum values of 1–1.4% have been reported in newborn calves but these virtually disappear two days after birth (Schalm, 1965).

The plasma concentrations of Na^+ and K^+, as a function of age, were also determined in some animals (Fig. 2). The data presented indicate that the minor changes obtained in plasma are not likely to be responsible for those observed in the red cells. It is interesting to note that while the concentration

of (Na + K) per liter of plasma remained constant, the concentration per liter of red cells decreased from 115 mmole at birth to about 88 mmole in mature animals. This would suggest that the water content in the erythrocytes of newborn calves is higher than in older animals (see Discussion).

The active (ouabain-sensitive) and passive (ouabain-insensitive) transports of K^{42} and Rb^{86} were determined in red cells of animals of different ages.

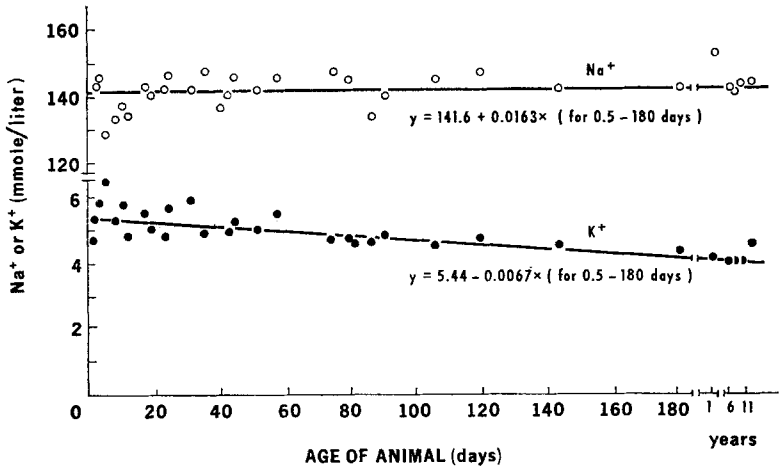

FIGURE 2. Sodium and potassium concentration of bovine plasma as a function of animal age. ● = K^+; ○ = Na^+. Each point represents a different animal.

TABLE II

COMPARISON BETWEEN K^{42} AND Rb^{86} INFLUX IN BOVINE RED CELLS

	Influx (μmole/liter cells × hr)					
	K^{42}			Rb^{86}		
Age of animal	Total	Ouabain sensitive	Ouabain insensitive	Total	Ouabain sensitive	Ouabain insensitive
11 days	629	542	87	630	473	157
34 days	638	391	247	661	302	359
43 days	558	246	312	629	205	424
13 yr	294	174	120	244	102	142

Previous studies (Bernstein and Israel, 1970) had shown that Rb^{86} could be used as an analogue of K^+ in human erythrocytes. A comparison between the active and passive transports of K^{42} and Rb^{86} in bovine red cells is shown in Table II. At 5 mM K^+ or Rb^+, the cells showed a slightly higher active transport of K^{42} than of Rb^{86}. For both tracers, however, it could be seen that the active transport diminished progressively as the age of the animal increased. The passive transport of both K^{42} and Rb^{86} showed an initial increase followed by a decrease. In all cases, the passive influx of Rb^{86} was somewhat

higher than that of K^{42}, a phenomenon that is perhaps a reflection of the smaller hydrated ionic radius of the former. Fig. 3 shows the active and passive fluxes of K^{42} and Rb^{86} in a larger population in which the two ions were not studied in the same animals. The $t_{1/2}$ for the change in active (ouabain-sensitive) fluxes was of the order of 30–35 days.

The membrane-bound (Na + K)–activated adenosine triphosphatase [(Na + K)–ATPase], an enzyme believed to represent the carrier mechanism for the active transport of Na^+ and K^+ (Skou, 1965; Whittam and Wheeler, 1970), and Mg-ATPase, an enzyme also present in the membrane and for which no physiological role is known at present, were also determined in

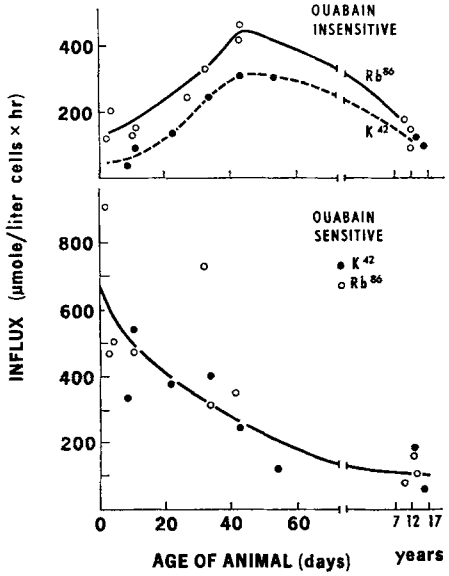

FIGURE 3. Influx of K^{42} and Rb^{86} in bovine red cells. ● = K^{42}; ○ = Rb^{86}.

washed red cell ghosts as a function of animal age. The activity of both ATPases diminished with age. The (Na + K)–ATPase activity was of the order of 100 mμmole/mg protein × hr at birth and was reduced to 5 mμmole/mg protein × hr in adult animals. Under the same conditions, the Mg-ATPase was also reduced from a value of about 180 to 40 mμmole/mg protein × hr. The time for half decay was 30–35 days for (Na + K)–ATPase and 28–32 days for Mg-ATPase (Fig. 4).

It is known that mammals are born with fetal hemoglobin (HbF) in their red cells and that as the animal ages, there is a change to adult hemoglobin (HbA). The time of transformation of the cells from HbF to HbA was studied in these animals (Fig. 5). A curve with two components was obtained: a slow initial one followed by a fast one after about 30 days. The time for half

change was of the order of 35–37 days. Initially, we interpreted these results as an indication that changes in the type of hemoglobin coincide with changes in the active transport capacity of the cells.

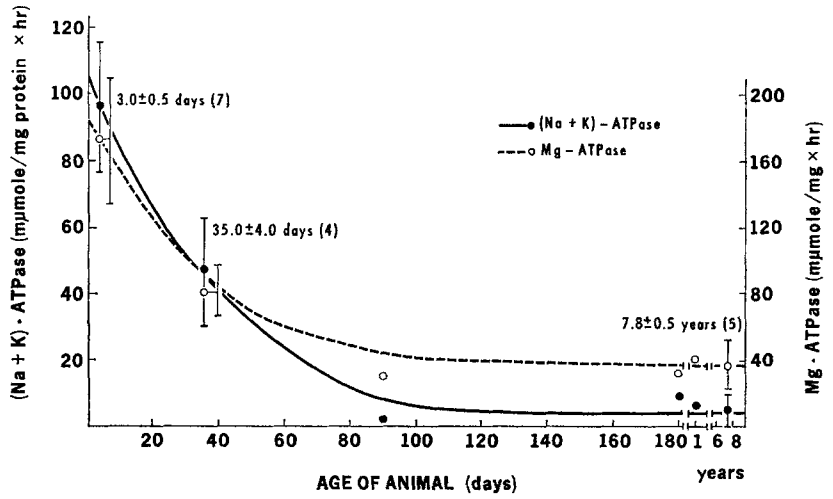

FIGURE 4. ATPase activity of bovine red cell membranes as a function of animal age. Deviations from the mean represent standard errors of the mean. ●——● = (Na + K)-activated ATPase; o----o = Mg-ATPase (activity in the absence of Na^+ or K^+).

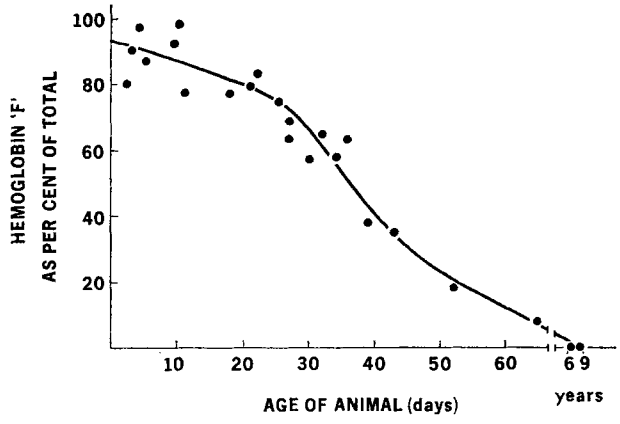

FIGURE 5. Hemoglobin F in bovine red cells as a function of animal age. [HbF + HbA = 100%].

To determine more conclusively if the reduction in K^+ content and in active transport was due to the appearance of a new LK type of population that would "dilute" the existing HK population, it was deemed necessary to separate the red cells on the basis of their ages. This separation has been successfully accomplished by the use of albumin density gradient centrifuga-

tion for erythrocytes of adult man (Leif and Vinograd, 1964), rabbit (Bishop and Prentice, 1966), and sheep (Lee et al., 1966). To our knowledge, however, this has not been reported for bovine red cells. Consequently, we had to ascertain if such a separation was possible for this species particularly at the age at which electrolyte changes are occurring. Fig. 6 indicates the typical distribution of red cells within an albumin density gradient of an animal in the transition age. A small pellet comprising 3–12% of the red cells was always obtained. Increasing the slope of the gradient in order to avoid this pellet resulted in a disruption of the distribution pattern of the rest of the cells.

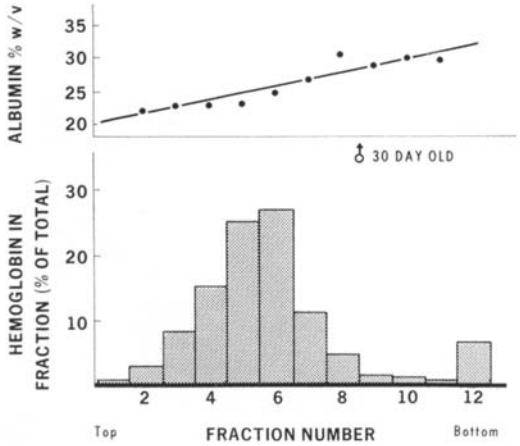

FIGURE 6. Typical distribution of red cells of a young calf separated by albumin density gradient centrifugation

To determine if the lighter cell fractions correspond to the younger red cells, a 24 day animal was injected intravenously with 0.5 mCi of Fe^{59} citrate and the distribution of radioactivity in the cells equilibrated in the density gradient was followed at different times after the injection. As can be seen (Fig. 7), the radioactivity was initially localized in the lighter fractions (younger cells) and moved to the heavier fractions with time, confirming, in fact, that younger cells are lighter than older cells. The relative proportion of HbA and HbF being synthesized at this age (24 days) was measured 2 days after the Fe^{59} injection. HbA and HbF were separated by electrophoresis and the radioactivity of the bands obtained was determined. The relative proportions were 85% HbA and 15% HbF, thus indicating that already at this age the greater proportion of hemoglobin synthesized corresponds to the adult type.

The active ion transport and the K/Na ratio were studied in erythrocytes separated by density gradient centrifugation. Table III shows one such

experiment in which Rb^{86} transport was determined in three approximately equal fractions of red cells of different densities: fraction 1 (lighter), fraction 2 (middle), and fraction 3 (heavier). As can be seen, the younger cell fractions show more active transport than the older fractions. On the other hand, the passive transport was higher in the older fractions. It was not evident why the passive transport of the cells subjected to albumin density gradient centrifugation was always higher than that of the control cells

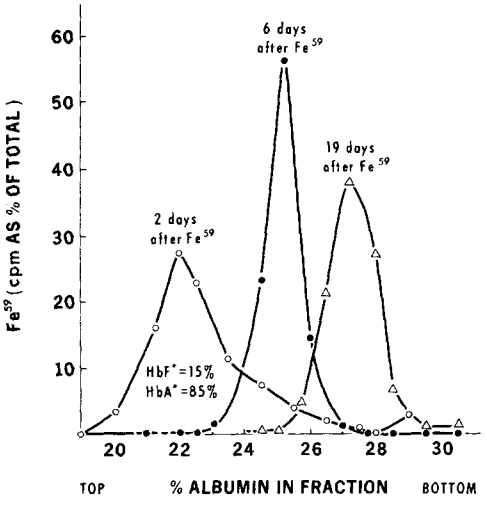

FIGURE 7

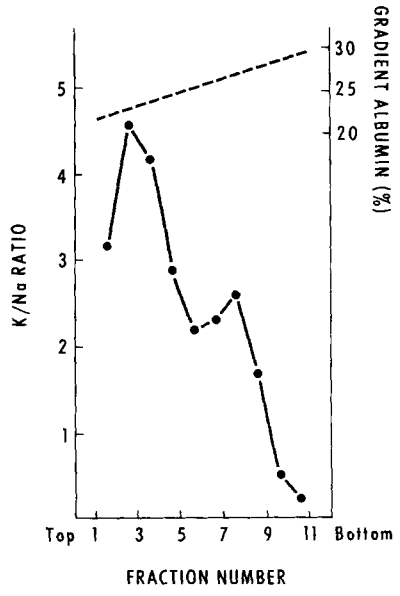

FIGURE 8

FIGURE 7. Distribution of radioactivity in red cells separated by density gradient centrifugation at different times after injection of Fe^{59} (0.5 mCi) into the animal (24 day old male calf).

FIGURE 8. K/Na ratio in red cells separated by albumin density gradient centrifugation. A 30 day old male calf was used.

(which were spun at only 3000 *g* in the absence of albumin). This might indicate that this type of gradient and centrifugation procedure is not entirely harmless to the cells. Fig. 8 shows the K/Na ratio of erythrocytes obtained after density centrifugation. It can be observed that the younger fractions showed a higher K/Na ratio than the older fractions, thus once more suggesting that a more vigorous active transport of Na^+ and K^+ occurs in the younger than in the older fractions. In some experiments, as indicated in Fig. 8, the K/Na ratio of the first fraction containing red cells was slightly lower than that of the second fraction. The reason for this did not become apparent.

Since the data presented show that younger red cells have a greater active

transport capacity than older cells, it follows that if one bleeds an animal to increase the relative proportion of young cells, one should expect an increase in mean cell K^+ and a reduction in mean cell Na^+ of a magnitude that repre-

TABLE III

INFLUX OF Rb^{86} INTO CALF RED CELL FRACTIONS SEPARATED BY ALBUMIN DENSITY GRADIENT CENTRIFUGATION

Cells	Rb^{86} influx (μmole/liter cells × hr)	
	Ouabain sensitive (active)	Ouabain insensitive ("passive")
Whole blood	341	304
Fraction 1	631	409
Fraction 2	310	476
Fraction 3	171	658

Fraction 1 = lighter, fraction 2 = middle, fraction 3 = heavier. The cell fractions were separated by albumin density centrifugation, as described in the text. Blood of a 36 day old calf was used.

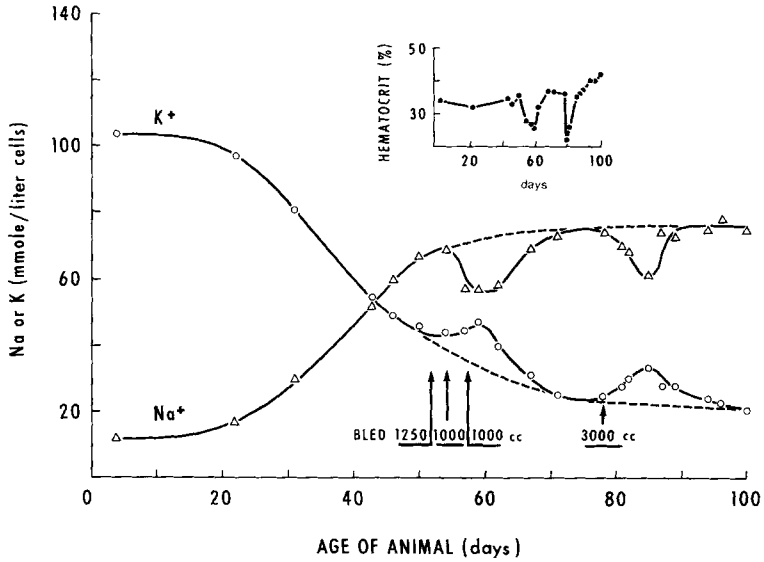

FIGURE 9. Effect of bleeding on the sodium and potassium content of red cells of one male calf at different ages.

sents a compromise between the percentage of new red cells formed and the speed of "aging" of the active transport mechanism. Fig. 9 shows that bleeding did, in fact, tend to increase the K^+ content and reduce the Na^+ content of the red cell population.

To further characterize the changes that occur in the red cell membrane

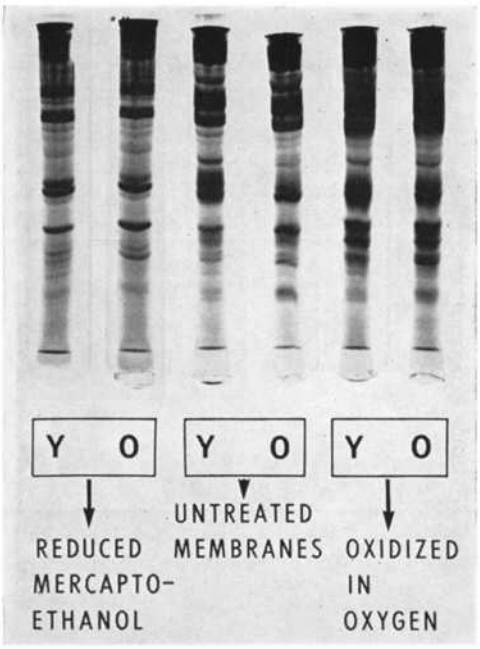

FIGURE 10. Polypeptide pattern of hemoglobin-free red cell membranes after electrophoresis in 10% SDS-polyacrylamide gels and staining. *Y* = young female calf (6 days old); *O* = cow (6 yr old).

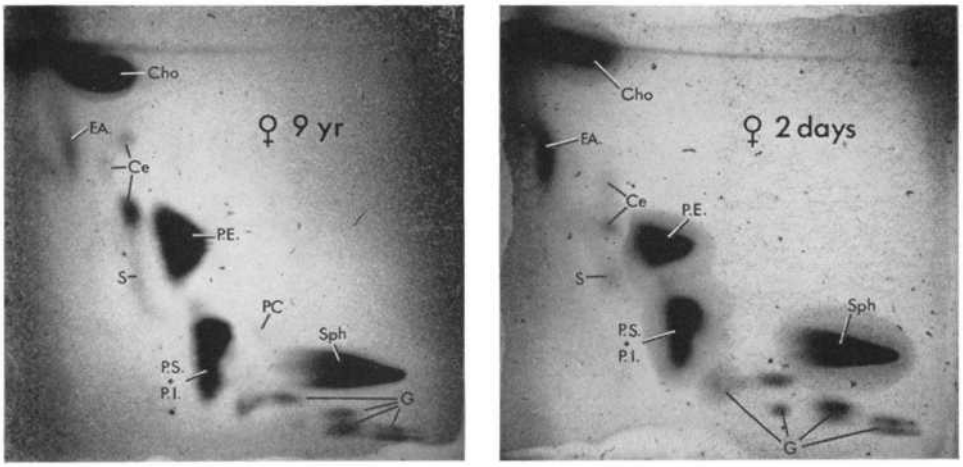

FIGURE 11. Phospholipid pattern of hemoglobin-free red cell membranes. The lipid pattern was determined by two-dimensional thin layer chromatography and sulfuric acid charing. *G*, gangliosides; *Sph*, sphingomyelin; *PC*, lecithin; *P.S.*, phosphatidylserine; *P.I.*, phosphatidylinositol; *S*, sulfatides; *P.E.*, phosphatidylethanolamine; *Ce*, cerebrosides; *F.A.*, fatty acids; *Cho*, cholesterol.

as the animal ages, we determined the membrane polypeptide pattern using polyacrylamide gel electrophoresis in sodium dodecyl sulfate. When the erythrocyte membranes of young and old animals were dissolved in SDS and run without further treatment, the polypeptide patterns obtained were somewhat different. These differences disappeared, however, when the solubilized proteins were fully reduced with mercaptoethanol or were oxidized before the electrophoresis, thereby indicating that apart from some differences in the redox state of some polypeptide chains, the membrane protein composition is very similar in the red cells of young and adult animals. At least 33 different bands could be clearly identified and reproduced in every experiment (Fig. 10). The qualitative phospholipid composition of the membranes was also studied (Fig. 11). No qualitative differences were observed between the red cells of young and old animals. In some, but not in all experiments, a faint spot identified as lecithin appears in the chromatograms of the old animals. A spot corresponding to cerebrosides was found to be consistently increased in the membranes of the old animals.

DISCUSSION

The Na^+ and K^+ content, the ouabain-sensitive uptake of K^{42} and Rb^{86}, and the activity of the (Na + K)–ATPase change drastically, in a parallel fashion, in the red cells of a calf after the animal's birth. These observations are additional evidence for the involvement of the (Na + K)–ATPase in the active transport of Na^+ and K^+ and in the maintenance of a concentration gradient of these ions across the cell membrane (see Skou, 1965, and Whittam and Wheeler, 1970, for general references).

The activity of Mg-ATPase in the erythrocyte membrane is also reduced after the animal's birth. The significance of this activity is not known, although the changes observed in this enzyme in this system could be used as a starting point in the study of its physiological role.

An interesting finding is the fact that in animals up to 35–40 days old the ouabain-insensitive transport increases while the ouabain-sensitive transport (and also both ATPases) decrease. Up to that age, the correlation between active transport disappearance and "passive" transport increase is 0.92, thus suggesting a 1:1 interconversion of active into "passive" transport. Regardless of the mechanism, the increase in passive transport superimposed on a reduction in active transport might conceivably explain the apparently biphasic curve obtained for Na^+ and K^+ content in the cells.

The changes found in the content of fetal hemoglobin in the red cells after the animal's birth parallel almost perfectly the changes in Na^+ and K^+ content. This might be interpreted as an indication that cells containing HbF would be HK and that a population of cells containing HbA would be LK and would dilute the former HK population. However, data obtained

by use of albumin density gradient centrifugation clearly show that in animals of ages close to $t_{1/2}$ time, in which the greater proportion of the new cells produced contain hemoglobin A (85% of the hemoglobin synthesized in a 24 day old animal corresponds to HbA), the young cells are still HK rather than LK. Thus, the Na^+ and K^+ content and the active ion transport of the red cells depend on the average age of the cell population. The existence of a superimposed change in the genetic phenotype cannot, however, be ruled out. Also, it is not inconceivable that an active transport "aging factor" might exist and that the production of this factor could change in the early stages of neonatal development, thereby influencing the rate of transformation of the cells from HK to LK. An aging factor transmitted as a single Mendelian gene could conceivably explain the existence of genetically determined HK and LK red cells in sheep. Work by Blunt and Evans (1963) and by Lee et al. (1966) with LK sheep has shown that an increased production of red cells after massive bleeding leads to the appearance of HK cells in the LK animal. Thus, while under normal circumstances the active transport in LK cells would "age" while the cell is still in the marrow, under conditions of increased hematopoiesis the aging would take place when the cell is in the circulation.

If the changes we have observed after birth in cattle are primarily a function of the average age of the red cell population, it follows that, in the younger animals, the cell population should be younger than in the older animals. Several indications suggest that this is the case.

(a) The percentage of reticulocytes (immature red cells), is usually considered to be a reflection of the age of the red cell population (Schalm, 1965). We have found that in newborn calves the percentage of reticulocytes is of the order of 0.33% and decreases progressively as the animals age.

(*b*) We have found that the sum of (Na + K) per liter of cells is high in newborn animals and diminishes as the animals age. This would indicate that the population of red cells of younger animals must contain more water than that of older animals, in order to maintain isosmolar conditions. It is a well-established fact that young red cell populations contain more water per cell than older ones (Bernstein, 1959; Prankerd, 1961; Lee et al., 1966).

(*c*) A younger population of red cells is also to be expected in a rapidly growing animal that is expanding its red cell pool. Calves of the Holstein strain can double their weight by 3 months after birth.

An increased susceptibility to lysis by mechanical and osmotic stress has also been described for red cells of newborn mammals (Goldbloom and Gottlieb, 1929; Mollison, 1948; Goldbloom et al., 1953). If this occurs in bovines it would also lead to a younger red cell population.

The financial support of the Alcoholism and Drug Addiction Research Foundation (Ontario, Canada) and of the Foundations' Fund for Research in Psychiatry (United States) is greatly appreciated.

We also wish to thank Professor Harold Kalant for his valuable criticism and continuous encouragement and the Department of Hematology of the Toronto General Hospital for their help and advice for the reticulocyte determination and hemoglobin electrophoresis.

Received for publication 22 July 1971.

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