CHANGES IN ERYTHROCYTE MEMBRANE Na^+-ATPase ASSOCIATED WITH CELL MATURATION AND ONTOGENY

ABSTRACT

Age-related changes in the transport ATPase system of mammalian erythrocyte membranes have been investigated. Two developmental processes were followed, maturation of reticulocytes in humans, rabbits and low-potassium sheep, and development of erythrocytes during ontogeny in LK lambs.

Human and rabbit erythrocytes contain similar high potassium concentrations, though human membranes exhibit more alkali cation-dependent ATPase than rabbits. In both species, a decline in this activity is observed with cell maturation.

LK sheep erythrocytes have low potassium content and little Na^+-dependent ATPase. Reticulocytes possess elevated potassium levels and increased ATPase activity. Both parameters decrease during maturation, but the loss of ATPase activity is due mainly to a decrease in the Mg^{++}-dependent component.

LK lamb erythrocytes possess elevated potassium content and ATPase activity. Na^+-ATPase resembles HK rather than LK erythrocytes, both in quantity and K^+-response kinetics. Ontogeny is associated with changes in cell potassium levels and Na^+-ATPase activity to mature LK characteristics.

M.Sc. degree
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Short title

Developmental changes in erythrocyte membrane ATPase.

CHANGES IN ERYTHROCYTE MEMBRANE Na^+-ATPase ASSOCIATED WITH CELL MATURATION AND ONTOGENY

by

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
BSA	bovine serum albumin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)-tetraacetic acid
HK	high-potassium
LK	low-potassium
NAD^+	β-nicotinamide adenine dinucleotide
NADH	reduced β-nicotinamide adenine dinucleotide
PCMBS	parachloromercuribenzene sulfonate
PHR	post-hemolytic residue
Pi	inorganic phosphate
Tris	tris(hydroxymethyl) aminomethane

1. INTRODUCTION

The average life span of mammalian erythrocytes is 120 days. Primordial stem cells in the bone marrow are stimulated by erythropoetin to form hemocytoblasts, the first precursors of erythrocytes, and the hemocytoblasts divide to become basophil erythroblasts which begin the synthesis of hemoglobin. Throughout subsequent divisions, the cell fills up with hemoglobin, and the nucleus gradually shrinks until it disappears, probably by autolysis and absorption. At this point, the new erythrocytes squeeze through the pores of the capillary walls into the circulation. Most red cells entering the blood retain a small amount of basophilic endoplasmic reticulum, and because of this they are called reticulocytes. Reticulocytes continue to synthesize hemoglobin for a period of two to three days until they lose their characteristic reticulum and become adult erythrocytes. After approximately four months in the circulatory system, the senescent red cells are sequestered by the spleen, completing the erythrocyte life cycle.

The aging process in red cells can be followed easily by administering a pulse label of ^{59}Fe and studying the labeled cells in the circulation at various time intervals after introduction of the isotope.(1) Since only the cells actively synthesizing hemoglobin at the time of the pulse will take up the label, a discrete population of young cells will be tagged, and can be observed throughout the maturation period. In this manner, it has been discovered that erythrocytes undergo a wide variety of physical and metabolic changes during their short lifetime. Before the cells ever leave the bone marrow, they lose their nuclei and the ability to synthesize nucleic acids. As the reticulocyte matures, protein and lipid synthesis cease (2), along with the ability to actively transport amino acids into the cell.(3) As the aging process continues, oxygen consumption (2) and, to a lesser extent, the glycolytic rate decline (4,5), the ATP concentration in the cell decreases (5), and the lipid composition of the cell membrane changes (6), incorporating more short chain fatty acids.(7) Accompanying these metabolic changes, the cell shrinks in size (8) and changes shape.(9) The density of the cell increases (8,10), as does the osmotic fragility (11), while the surface charge density decreases.(8,12) These physical changes, especially the increasing density, allow separation of various populations of cells with respect to age, a vital aid in studies of the maturation process.

In addition to the properties already mentioned, many cellular enzymes have been shown to lose activity as the erythrocyte matures. For example, pyruvate kinase shows decreasing maximum velocity and electrophoretic mobility with age, while the K_m for phosphoenolpyruvate and the pH optimum both increase. (13) Likewise, glucose-6-phosphate dehydrogenase (2), 6-phosphogluconic dehydrogenase (2), phosphohexosisomerase (2,5), hexokinase (4,5), aldolase (4,5), and glutamic-oxalacetic transaminase (4) all exhibit age-dependent loss of activity. This deterioration of enzyme activity is probably a result of the cell's inability to synthesize new proteins as the old ones are damaged or destroyed, and has been implicated as the primary cause of erythrocyte destruction.(11) Based on this evidence, it seems logical that similar kinetic changes might be expected of other red cell enzymes. For this reason it was decided to investigate the effects of maturation on the Na^+,K^+-activated ATPase in the membranes of mammalian erythrocytes.

Red blood cells, like all mammalian cells, maintain a constant internal concentration of sodium and potassium ions. Originally it was thought that the cell membrane was impermeable to these cations, since in most erythrocytes, the intracellular concentrations are quite different from those found in the plasma.(14) Permeating ions, such as chloride and bicarbonate, distribute themselves freely across the plasma membrane according to the Donnan equilibrium; but the equilibrium ratios determined for sodium and potassium in human red cells vary greatly from the expected Donnan values.(15) This indicated that there are steep electrochemical gradients for these ions across the cell membrane. Furthermore, it was believed that if the cells were not impermeable to these cations, they would undergo colloid osmotic swelling and lysis due to their high protein concentration with respect to the plasma.

The concept of the cell membrane as a barrier to the alkali metal cations was dispelled, however, when radioactive isotopes of sodium and potassium were shown to permeate the red cell membrane.(16) This discovery posed a dilemma. If sodium and potassium could leak across the membrane along their electrochemical gradients, then there must also be some mechanism available to pump the ions back against the gradients and maintain the steady-state composition of the cell. Since this pumping would be against steep electrochemical gradients, sodium efflux and potassium influx must be coupled in some way to an energy producing system.(17) This concept of a dynamic maintenance of alkali cation levels involving energy expenditure by the cell is now generally accepted, and is termed the "pump-leak" hypothesis.

Although it seemed likely for many years that ATP was the source of energy for the cation pump, providing proof for this theory was difficult. Since ATP does not cross the cell membrane, it could not be added exogenously to test its effects on the transport system. Eventually, however, a method was devised for injecting ATP into a giant squid axon.(18) In addition, red cell ghosts were prepared containing ATP, and were discovered to have the ability to hydrolyze the high-energy phosphate.(19) In 1957, Skou was able to associate this energy-producing system with sodium and potassium transport.(20) He found, in particles from minced crab nerves, an enzyme system which hydrolyzed ATP at a rate which was increased by sodium ions; and in the presence of sodium was further stimulated by potassium. The synergistic effects of sodium and potassium ions suggested that this ATPase activity was related to ion transport, since the simultaneous presence of the two ions is also required for activation of the "sodium pump".(21)

Attempts to correlate the ATPase activity with sodium and potassium transport were continued in red cell ghosts, a more homogeneous system than Skou's crab preparation. (21,22,23,24) It was soon found that in the presence of the other ion, K_m values for sodium (24 mM) and for potassium (1-3 mM) were remarkably similar for the pump and the ATPase. Furthermore, like the cation transport system, the sodium-plus-potassium stimulated component of the ATPase activity was inhibited by low concentrations of cardiac glycosides. In both systems the inhibitory effect could be lessened by increasing the concentration of potassium ions in the incubation medium. Conclusive evidence linking the two systems was obtained using resealed red cell ghosts. (25) Glycoside-sensitive ATPase was shown to require sodium inside and potassium outside, the precise distribution which also activated the pump mechanism.

It is now widely accepted that a membrane-bound ATPase is directly involved in the process of maintaining the alkali cation concentrations in mammalian cells. If this enzyme system were to become defective or inactive with age, the consequent alterations in cation gradients might adversely affect the cell's viability. Indeed, it has been shown that osmotic fragility increases with age (11), and a severe hemolytic anemia has recently been associated with abnormal sodium and potassium transport in red cells.(26) With these observations in mind, it seemed logical to look for alterations in this enzyme system as the cell ages.

The use of red blood cells in the study of maturation changes in the membrane-bound Na^+,K^+-ATPase is dictated by both their availability and their relative homogeneity. In cells such as nerve, muscle and cardiac tissue, where the transmembrane ion fluxes are used to propagate electrical impulses, the activity of this enzyme system is considerably greater than in erythrocytes. Despite their relatively low rate of Na^+,K^+-dependent ATP hydrolysis, however, red cell membranes are easily isolated free of intracellular organelles and connective tissue, and thus present a more suitable system for the study of the membrane-bound enzymes than the more active tissues.

In addition, erythrocytes of different species vary with respect to their cation concentrations and ATPase activities, thereby allowing comparison of ATPase activity under a variety of physiological conditions. Dogs and cats, for example, have erythrocyte sodium and potassium concentrations in the order of 110 mM and 3 mM respectively, while plasma cation concentrations are around 140 mM sodium and 5 mM potassium. In contrast, human red cells contain around 15 mM sodium and 85 mM potassium while surrounded by plasma of composition similar to that of dogs and cats. The transport ATPase requirements of these two types of red cells differ considerably due to the variation in cation gradients that must be maintained.

Sheep provide a unique system for studying these two types of cells. Though their erythrocytes are morphologically indistinguishable, and their plasma cation concentrations identical, some sheep have red cells with sodium and potassium concentrations similar to dogs and cats (LK), while the erythrocytes of others resemble human cells with respect to alkali cation content (HK). This difference is genetically determined, the LK type being dominant.(27) In 1966, Rasmusen and Hall produced a specific antiserum which hemolyzed the red cells of all HK sheep and two out of three LK sheep.(28) Since then another antibody, active only against LK sheep red cells, has been discovered.(29,30) The antigenic sites corresponding to these two antibodies have been designated the M and L antigens respectively. HK sheep possess only the M antigen, while homozygous LK sheep have only L. Heterozygotes have both L and M antigens though they are phenotypically LK.

In addition to the difference in Na/K ratio in the red cells of these two types of sheep, differences in pump rate and ATPase activity were also observed. Both ouabain-sensitive potassium influx, or pump activity (31), and ouabain-sensitive Na^+,K^+-stimulated ATP hydrolysis (32) were found to be 4-6 times greater in HK than in LK erythrocytes. Furthermore, HK cells have approximately six times more ouabain-binding sites than LK cells (33), while the permeability of the plasma membrane to potassium is considerably greater in LK than HK cells. (31) There is evidence that the development of the L and M antigens is in some way related to these differences in activity between HK and LK erythrocytes. Although treatment of HK cells with anti-M serum has no noticeable effect on either the pump or ATPase activity (34,35), incubation of LK cells with anti-L serum produces a 6-8 fold increase in both potassium transport and Na,K-dependent ATPase (29), and a two-fold increase in the number of ouabain-binding sites.(30) This data would appear to suggest that neutralization of the L antigen on LK membranes converts the cells to HK type activity. Dunham and Hoffman have carried this concept a step further.(36) They assume that both pump (active) and leak (passive) fluxes of potassium occur through the same specific sites on the red cell membrane. HK and LK cells have the same total number of these cation-transfer sites, but in HK cells most are active while in

LK cells the majority are passive. The L antigen is responsible for switching the normal pump sites to leak sites in LK cells, and treatment with anti-L serum somehow reverses this effect, making the sites active once more.

Whittington and Blostein, however, have reported a difference in not only the rate, but also the kinetics, of sodium-stimulated ATP hydrolysis by HK and LK erythrocyte membranes.(37) Stimulation of ATPase activity in LK cells by anti-L serum results in only a quantitative increase in ATP hydrolysis, while the kinetics of the ATPase activity remain distinctly LK in character.(38) Similarly, a kinetic difference was also found in the pump activity of these two cell types.(39) Evidently the distinction between LK and HK erythrocyte cation transport is more complex than a simple difference in the number of active pump sites. The possibility that HK and LK characteristics evolve during the maturation of young erythrocytes could not be overlooked. It was hoped that investigation of developmental changes in red cell membrane ATPase might help to elucidate the nature of the gene products responsible for the observed variations in cation regulation by HK and LK sheep erythrocytes.

Two developmental systems have been used in this project for the study of erythrocyte maturation. The method most generally employed in investigations of this kind involves the comparison of enzyme activity in reticulocytes with analogous data from mature red cells. In animals, the reticulocyte count of the blood can be increased easily by either massive hemorrhage or subcutaneous administration of acetylphenylhydrazine. In the study of human reticulocytes, however, experimental material is more difficult to obtain, since the normal reticulocyte count of the blood is only 0.5%. Nevertheless, subjects exhibiting reticulocytosis due to some anemic process are available. Once the concentration of reticulocytes in the blood has been enhanced, these young cells can be isolated by differential density centrifugation and analyzed separately from the mature population.

Attempts were made to study maturation changes of the Na^+,K^+-ATPase in reticulocytes of four mammalian species; dogs and sheep which have low potassium erythrocytes, and humans and rabbits with high potassium red cells. It is of interest that reticulocytes in the two low-potassium (LK) species contain high levels of intracellular potassium which decrease to normal LK concentrations as the cells mature.(40) Several explanations are available to account for this increased potassium content in genetically LK cells. Since only the red blood cells of LK animals display the LK phenotype, these young cells may simply retain potassium acquired from their high-potassium precursors. It is possible that the differentiation process does not allow sufficient time for the cells to lose potassium and gain sodium, becoming LK in character. Another more interesting alternative is that the immature cells are still HK when they enter the circulation, and develop LK-type ATPase and pump activity as they grow older. This latter theory is supported by the work of Lee, Woo, and Tosteson, who measured the potassium transport rate in LK sheep reticulocytes and found it to be quantitatively characteristic of HK type cells.(41) They suggest that the specific LK characteristics of the erythrocyte membrane do not evolve until after the young cell has stopped dividing and left the bone marrow. If the pump activity of the red cell membrane changes during the course of reticulocyte maturation, it follows that the membrane ATPase activity would be similarly affected by the age of the erythrocyte.

The second system used in the investigation of age-related changes in red cell Na^+,K^+-ATPase is one following development of lamb erythrocytes during ontogeny, i.e. from birth until eight weeks of age. Like reticulocytes from LK animals, the red cells of these lambs contain a high intracellular potassium concentration, which falls progressively during the first 7-8 weeks postpartum until normal adult LK potassium levels are attained.(42-44) Similarly, the potassium transport properties of LK lamb erythrocytes resemble those of HK rather than LK adults (36,44,45), and the activity of the ouabain-sensitive ATPase compares quantitatively to mature HK cells.(35,45) Within a period of thirty days after birth, these two parameters also assume values characteristic of normal adult LK erythrocytes.

One simple interpretation of these observations is that fetal cells with HK-type membranes are being replaced in the circulation by adult LK erythrocytes during the first six weeks of like. This explanation is feasible since the life span of fetal red blood cells is shorter than that of adult erythrocytes (46), plus the fact that rapid expansion of the circulatory system in the growing animal dilutes the concentration of fetal cells. Data obtained from studies of hemoglobins in young sheep tend to substantiate this theory, although the actual values vary somewhat from report to report.(46-48) On the average, fetal hemoglobin comprises 80-95% of the total hemoglobin concentration at birth, and decreases to 50% in 15-17 days. By the age of two months, all fetal hemoglobin in these lambs has disappeared.

This exchange of adult for fetal cells, however, may not be the sole explanation for the observed changes in pump and ATPase activity during maturation. Ellory and Tucker have followed the development of the L antigen (formerly called m) on LK lamb red cells, and found that the amount of L-reactive material (m score) peaks at about four weeks postpartum, at least two weeks before the cellular potassium level reaches a minimum value.(44) Likewise, Tosteson reports that the potassium pump : leak ratio and the ouabain-sensitive ATPase activity of maturing lambs both show a more rapid decline than the intracellular potassium concentration.(45) These findings seem to suggest that alterations in the transport ATPase of lamb erythrocyte membranes might precede and contribute to the loss of cellular potassium.

On the preceding pages, an attempt has been made to describe the function and importance of the alkali cation transport system in erythrocyte membranes. The effects that aging of the cell might have on this enzyme system and, conversely, the effects that deterioration of the enzyme system might have on the cell have been pointed out. For these reasons, an investigation of the age-dependent changes in the Na^+,K^+-ATPase of mammalian erythrocytes has been carried out using the two developmental systems described, maturation of human, rabbit, and LK sheep reticulocytes and ontogeny of LK lamb red blood cells.

2. MATERIALS AND METHODS

2.1 Source of red blood cells.

a. Human blood.

Blood samples with high reticulocyte counts were obtained from the clinical hematology laboratory of the Royal Victoria Hospital, Montreal, Canada. The samples were drawn from patients for routine hematological procedures, and those with elevated reticulocyte counts were selected for studies of Na^+,K^+-ATPase. Blood was collected in Vacutainers containing EDTA as an anticoagulant. Controls were obtained from normal subjects by venipuncture using a 50 ml disposable syringe containing 100 units of heparin (sodium heparin, British Drug Houses) per milliliter of blood.

b. Rabbit blood.

White New Zealand rabbits of either sex weighing approximately ten pounds were purchased from the Quebec Breeding Farms. Blood was obtained by cardiac puncture using a 20 ml disposable syringe equipped with a 20 gauge needle. Heparin served as anticoagulant. To produce reticulocytosis, 20 milliliters of blood were withdrawn daily from a rabbit while ferrous sulfate was added to the drinking water as an iron supplement.

After one week, reticulocyte counts reached 18-20% and remained at this level for as long as daily bleedings were continued.

c. Sheep and lamb blood.

The sheep used in this project are from a flock of Cheviots, Hampshires and Dorsets. The LK sheep selected for bleeding experiments weighed about 150 pounds. Reticulocytosis was induced by jugular phlebotomy at the rate of one liter of blood per day for three consecutive days. Intramuscular injections of Imferon (Fisons) were administered on each day of bleeding as an iron supplement. On the eighth day after the start of bleeding, 150 milliliters of blood were collected by jugular venipuncture using heparinized 50 ml disposable syringes and 20 gauge needles.

The lambs studied were offspring from sheep in this same flock. All four lambs were LK, although one had an HK mother. All four were sired by the same LK ram. Blood was drawn by jugular venipuncture once a week for up to eight weeks after birth.

d. Treatment of blood samples.

Blood from all sources was chilled on ice as soon as it was collected, and kept cold during all subsequent procedures. Aliquots of whole blood were routinely reserved for determinations of hematocrit, reticulocyte count, and whole blood sodium and potassium levels. The remainder of the blood was centrifuged for five minutes at top speed in an International Equipment Company clinical centrifuge, model CL, and the plasma and buffy coat were removed by suction. Usually an aliquot of plasma was reserved for sodium and potassium analysis. The cells were then thoroughly resuspended and washed three times with 4-5 volumes of cold 154 mM sodium chloride.

2.2 Isolation of reticulocyte-rich fraction.

Based on the observation that reticulocytes are less dense than older red blood cells, several centrifugation methods have been developed to isolate reticulocyte-rich populations from whole blood.(49-53) For human and rabbit bloods, a modification of Shulman's method was chosen, using discontinuous dextran density gradients.(53) The dextran (M.W. 60-90,000, Sigma) was dissolved in a buffered saline solution containing 0.5% bovine serum albumin (BSA fraction V, Sigma). Gradients were prepared by layering 2.5 ml aliquots of dextran solution in cellulose nitrate centrifuge tubes (5/8" x 3", Beckman). In most cases three different concentrations of dextran were used per tube, in the range of 22% to 32% (w/v), the densities being chosen by trial and error to give a good separation of the reticulocyte fraction.

Blood was prepared for the gradients by washing the cells three times with cold BSA-saline, containing 137 mM NaCl, 54 mM KCl, 1.08 mM Na_2HPO_4, 1.10 mM KH_2PO_4, 0.625 mM $MgSO_4$, 6.1 mM dextrose, and 0.5% BSA. Approximately 4 ml of the washed, packed cells (hematocrit ~ 70%) were carefully layered on the dextran gradients. The tubes were centrifuged for 30 minutes at 13,200 x g and 0°C in a Beckman preparative ultracentrifuge, model L, equipped with an SW 36 swinging bucket rotor. After centrifugation, the top layer of cells and dextran was transferred to a 50 ml polythene centrifuge tube using a Pasteur pipette. The cells were suspended in BSA-saline, and spun for 10 minutes at 2000 x g in a Sorvall RC2-B refrigerated centrifuge with an SS-34 fixed angle rotor. After two more similar washings, an aliquot of cells was removed for reticulocyte counts; the remainder was refrigerated in a small amount of BSA-saline.

Dextran gradients were not used in the sheep experiments; instead a method developed by Blunt and Evans was employed. (40) Reticulocyte-rich blood was centrifuged in 12 ml graduated tubes in an International Equipment Company refrigerated centrifuge for one hour at 3000 x g and 15°C. The plasma was removed and set aside. The top 20% of the cells, including the white cells, was removed with a Pasteur pipette and resuspended in plasma. These cells were then transferred to Wintrobe hematocrit tubes (Pfeiffer Glass) and centrifuged as before. The plasma and buffy coat were removed by suction, and the top 25% of the cells were again resuspended in plasma. In one experiment, the bottom-most fraction of cells from the first spin was also kept as a control. The separated cells were stored at 4°C until membranes could be prepared the next day.

2.3 Membrane preparation.

Membranes from human and rabbit blood were prepared by a modification of the method of Warrendorf and Rubinstein.(54) This method is particularly useful for work with reticulocytes because of the apparently complete removal of mitochondria and other intracellular particles from the plasma membrane fraction. Washed, packed red blood cells were lysed in 50 ml polythene centrifuge tubes with 10 volumes of a 0.025 M sucrose solution, and left standing on ice for 30 minutes. The sucrose concentration of the lysate was then adjusted to 0.44 M by the addition of 0.5 ml of 1.27 M sucrose for each ml of lysing solution used. The tubes were centrifuged at 2000 x g for 20 minutes in the

RC2-B Sorvall. After the supernatant was aspirated, the post-hemolytic residue (PHR) was washed eight times; twice with 0.88 M sucrose, once with 2 mM Tris HCl, pH 7.4, twice with 10 mM Tris EDTA, pH 7.4, once with 1 mM Tris EDTA, pH 7.4, and twice more with 2 mM Tris HCl. With each washing the membranes were gently suspended in the appropriate solution and the residual, relatively insoluable, small pellet in the bottom of the tube was discarded. The tubes were centrifuged at 35,000 x g in the Sorvall. After the final wash the membranes were stored at 4°C suspended in a small amount of 2 mM Tris HCl. For assays, the membrane suspension was diluted to a concentration of 1-2 mg protein/ml.

Sheep membranes were prepared somewhat differently. The washed cells were lysed with ten volumes of cold distilled deionized water and left on ice for thirty minutes. The PHR was spun down at 35,000 x g in the Sorvall for 20 minutes, and the supernatant aspirated. The membranes were then washed three times with 1 mM Tris EDTA, pH 7.4, and three times with 2 mM Tris HCl, pH 7.4, and once with 2 mM Tris HCl containing 0.004 mM EDTA. The membranes were left suspended in a small amount of the final wash solution and stored at 4°C. All membranes were used within four days after preparation.

2.4 Protein determinations.

The protein concentration of the membrane suspensions was measured by the method of Lowry (55), using crystalline bovine serum albumin (fraction V, Sigma) as standard.

2.5 Hematocrit measurements.

Microhematocrit determinations were done in duplicate on samples of whole blood or $MgCl_2$-washed cells using Micro-Cal heparinized capillary tubes (75 mm x .5-.6 mm (i.d.), Chase Instruments). The tubes were sealed with Seal-Ease (Clay Adams) and spun five minutes in an Adams Readacrit microhematocrit centrifuge.

2.6 Reticulocyte counts.

Blood for reticulocyte counts was stained for at least ten minutes using the new methylene blue stain described by Brecher. (56) Slides were prepared and examined under the oil-immersion lens of a Zeiss binocular microscope. Reticulocyte percentages were determined by counting the number of reticulocytes present in a sample of at least 1000 cells.

2.7 Alkali metal determinations.

Sodium and potassium concentrations in whole blood and plasma and $MgCl_2$-washed cells were measured with an Instrumentation Laboratories Flame Photometer, model 143, using an internal lithium standard. 0.1 ml aliquots of blood, cells, or plasma were diluted to 20 ml with a standard solution containing 15 mEq lithium per liter. The instrument was calibrated with a standard solution of 140 mEq sodium and 5 mEq potassium per liter, and the standard and unknown samples were read repeatedly to maximize the accuracy. Cellular internal cation concentrations were calculated from the values obtained for the whole blood and plasma cations and the previously determined hematocrits.

2.8 Optical density measurements.

All spectrophotometric determinations were carried out using either 1 ml (Zeiss) or 4 ml (Beckman) quartz cuvettes with a 1 cm light path in a Zeiss PMQ II Spectrophotometer.

2.9 Preparation of γ-^{32}P-ATP.

γ-^{32}P-labeled ATP for use in the ATPase assay was prepared by Mr E.S. Whittington in this laboratory using a modification of the method of Post and Sen (57) as described by Whittington.(58) This enzymic method makes use of the exchange reaction between inorganic phosphate and the terminal phosphate group of ATP that occurs in the presence of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase. $^{32}P_i$ was obtained from New England Nuclear, Boston, Mass. The γ-^{32}P-ATP was separated from the reaction mixture by the method of Glynn and Chappel (59) on a 0.6 x 1 cm Dowex-1-Cl column (200-400 mesh, 2% cross-linked) and neutralized with a known amount of solid Tris base (THAM, Fisher). The ATP concentration in the eluate was determined by measuring the optical density at 259 nm, and an aliquot was removed for determination of radioactivity. Samples were stored at -18^{o}C and thawed just prior to use.

2.10 ATPase assays.

ATPase activities of all membrane preparations were determined by the rate of hydrolysis of $^{32}P_i$ from γ-^{32}P-ATP. The radioactive ATP, prepared as above, was diluted with unlabeled Tris ATP (Sigma) to give the desired ATP concentration and specific activity. $MgCl_2$ was routinely added to the incubation medium in a concentration equal to that of the ATP; though in in cases when very small amounts of ATP (e.g. .002 mM) were used, $MgCl_2$ was present in large excess as indicated in the text. Approximately 30 mM Tris HCl, pH 7.4, was used as buffer for the medium, and in most experiments 0.1 mM EGTA to reduce the level of free calcium ions. Supplementing this basic medium, various quantities of alkali metal cations were included to distinguish the different components of the total ATPase activity. 50 mM KCl was generally used to measure the baseline level of activity referred to as Mg^{++}-ATPase. 50 mM NaCl was added to the basic medium to determine the sodium-stimulated ATPase component, or Na^+-ATPase. To measure the amount of ATPase dependent on both ions, Na^+,K^+-ATPase, 50 mM NaCl and varying quantities of KCl, from 1 to 20 mM, were included in the incubation medium. Ouabain (octahydrate, Sigma) was routinely used at a concentration of 0.2 mM as an ATPase inhibitor.

All assays were carried out in a water bath at 37°C. Prior to incubation, all media and membrane suspensions were kept cold on ice. Membranes were diluted with 2 mM Tris HCl, pH 7.4, to a concentration of 1-2 mg protein/ml and preincubated at 37°C for 10 minutes. The incubation media were prewarmed for 5 minutes at 37°C. Following preincubation, 0.05 ml of the membrane suspension was added to 0.1 ml of medium in 12 ml Pyrex centrifuge tubes and incubated for 2-30 minutes depending on the ATP concentration. To stop the reaction, 1.35 ml of an ice-cold 5% trichloroacetic acid solution containing 2.5 mM ATP (disodium salt) and 5 mM KH_2PO_4 was added to each tube. The tubes were vortexed well and returned to the ice bath. To remove excess ATP from the mixture, 0.75 ml of the Norit-A charcoal suspension (1.5 g/10 ml 5% trichloroacetic acid, Fisher) was added to the trichloroacetic acid supernatant in each tube. The tubes were left on ice for an hour with occasional mixing, and then filtered through Whatman no. 1 filter paper. 0.45 ml of each filtrate was pipetted into scintillation counting vials (Wheaton Glass) and dissolved in 15 ml of a modification of Bray's scintillation fluid containing 700 ml xylene (Mallinckrodt), 700 ml dioxane (Fisher), 420 ml ethanol, 160 g napthalene (Eastman), 1 g 1,4-bis-2-(5-Phenyloxazolyl)-Benzene (Packard), and 10 g 2,5-Diphenyloxazole (Packard). The vials were counted for 10 minutes in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 3003. Each assay was done in duplicate, and the average of the two determinations computed. Duplicate samples of the original basic medium were also counted in order to calculate the percent of the ATP hydrolyzed.

2.11 Sodium loading of erythrocytes.

The internal cation concentration of rabbit red cells was altered by a modification of the parachloromercuribenzene sulfonate (PCMBS) method of Garrahan and Rega (60), as described by Hoffman.(61) Blood was freshly drawn from a rabbit by cardiac puncture and spun in an International Equipment Company clinical centrifuge at top speed for 5 minutes. The plasma and buffy coat were aspirated, and the packed cells suspended at a concentration of about 2% in a solution containing 135 mM NaCl, 3 mM sodium phosphate, pH 7.4, 0.2 mM $MgCl_2$, 30 mM sucrose, and 0.02 mM PCMBS (Sigma). The suspension was incubated for 10 hours at 4°C using a Multipurpose Rotator, model 150 TC, (Scientific Instruments Inc.) to keep the cells suspended in the solution. After this incubation, the cells were spun down for five minutes, and resuspended in a similar medium containing 1.1 mM dextrose and 0.5 mM dithiothreitol (DTT, Sigma) instead of the PCMBS. This new suspension was then incubated for 30 minutes at 37°C. The cells were washed three times with a 120 mM $MgCl_2$ solution that had been saturated with $MgCO_3$ and filtered through a 0.8 μ millipore. Hematocrit and alkali metal determinations were done on aliquots of these washed cells. Using this procedure, almost complete reversal of the internal cation concentration was accomplished. Within 10 hours, the potassium ion concentration fell from values of around 95 mM to 5-10 mM, while sodium ions increased from 15 mM to greater than 100 mM. ATP levels remained between 1-2 mM throughout the procedure.

2.12 ATPase assay with reversed gradients.

"High Na" rabbits cells were kept on ice prior to incubation. Media for incubation contained 3 mM sodium phosphate, pH 7.4, 30 mM sucrose, 0.2 mM $MgCl_2$, 5 mM Tris HCl, pH 7.4, and either NaCl or KCl at a concentration of 135 mM. Ouabain, 0.2 mM, was included in half the flasks to determine the ouabain-sensitive component of the ATPase activity. 3 ml aliquots of each incubation medium were preincubated at 37°C in 25 ml Erlenmeyer flasks. One ml of the cold packed cells was added to each flask and incubated at 37°C for up to two hours with continuous gentle shaking. At 0, 30, 60, and 120 minutes of incubation, 0.5 ml aliquots of the suspension were removed into an equal volume of ice-cold 10% trichloroacetic acid. Tubes were thoroughly vortexed and spun in a clinical centrifuge for three minutes. The trichloroacetic acid supernatants were carefully removed with Pasteur pipettes and extracted with diethyl ether to near neutrality. Excess ether was blown off with air and the samples were quickly frozen in acetone and dry ice, then stored at -70°C to await assay of the ATP concentrations. Hematocrits were determined on the remaining suspension in each flask so that ATP values could be expressed per ml packed cells.

2.13 ATP determinations.

Measurement of the intracellular ATP concentration of red cells was accomplished using a modification of the enzymic method of Bucher (62), available as a Biochemica Test Combination from Boehringer Mannheim. Into a 1 ml quartz cuvette was placed 0.17 ml ether-extracted supernatant and 0.8 ml of 0.5 M triethanolamine buffer, pH 7.6, containing 4 mM $MgSO_4$ and 6 mM glycerate-3-phosphate. The solutions were mixed well with a plastic spatula, and 0.02 ml of freshly prepared 10 mM NADH was added to each cuvette. Optical densities were read at 340 nm against a blank containing water instead of supernatant. Finally, to each cuvette was added 0.01 ml of an enzyme mixture containing glyceraldehyde phosphate dehydrogenase (7 mg/ml), phosphoglycerate kinase (1 mg/ml), and a mixture of α-glycerol phosphate dehydrogenase and triose phosphate isomerase (2 mg/ml). The change in optical density was followed at 340 nm for 10-15 minutes after the addition of the enzymes until there was no further decrease. Calculation of the ATP concentration in the supernatant was based on the fact that two moles of NADH are converted to NAD^+ by the enzymes of this reaction sequence for each mole of ATP utilized. Since the conversion of one micromole of NADH to NAD^+ results in an optical density change of 6.2 units, a decrease in optical density of 12.4 units indicates the presence of one micromole of ATP in the assayed sample

2.14 Hemoglobin analysis.

Hemoglobin determinations were performed on LK lamb blood using the following modification of the method described by Briere, Golias and Batsakis.(63) To prepare samples for electrophoresis, five drops of whole blood from each lamb were mixed with 2-3 drops of water in a small test tube and vortexed well. 0.5 ml of carbon tetrachloride was added to each hemolysate, and the tubes were centrifuged for ten minutes at 12,000 x g in the RC2-B Sorvall. This procedure separates the stroma from the hemolysate, allowing a clear hemoglobin solution to be isolated. The membrane-free supernatant was removed from each tube with a Pasteur pipette and stored at 4°C.

Electrophoresis of the hemoglobin samples was carried out on Sepraphore III cellulose acetate strips (Gelman) in a Gelman

SepraTek electrophoresis chamber connected to a D.C. power source. The buffer solution contained 15 g solid Tris base (THAM, Fisher), 1 g EDTA (British Drug Houses), and 3.15 g Boric acid (Anachemia) made up to one liter with water and adjusted to pH 8.6 with Tris. Following a one hour separation at 400 volts, the strips were soaked for 3-5 minutes in Ponceau S stain (1 capsule dissolved in 100 ml aqueous 5% TCA). Excess stain was removed by three successive rinses in 5% acetic acid. The strips were then dehydrated in absolute methanol, cleared in solution of 10% acetic acid and 90% absolute methanol, and stretched over glass slides to dry.

In order to quantitate the fetal hemoglobin component in the hemolysates, each strip was scanned at 540 nm in a Zeiss PMQ II Spectrophotometer equipped with a Vicon linear gel scanner, model 1050 (Brinkmann), and a Photovolt linear/log varicord recorder, model 43. The areas of the individual peaks were extrapolated where necessary, and the peaks traced on a piece of paper. Each tracing was cut out and weighed, and the weight of the hemoglobin F peak was expressed as a percent of the total weight of all the peaks.

3. RESULTS

3.1 Preliminary attempts to produce and isolate reticulocytes.

As with most research projects, many procedures were attempted and abandoned before successful techniques were developed to study maturation changes in erythrocyte membrane Na^+-ATPase. Two of these unsuccessful procedures will be described below in order to point some of the inherent pitfalls in the use of these methods.

a. Production of reticulocytosis with acetylphenylhydrazine.

As in LK sheep, mature red cells of dogs contain little potassium, while the reticulocytes were observed to have a high potassium content (two preliminary experiments). For this reason, the original plan for studying maturation changes in erythrocytes included the use of dog blood. Dogs were injected subcutaneously with an alcoholic solution of acetylphenylhydrazine, a drug which causes massive hemolysis and induces a rapid reticulocyte response. (64) This procedure is used by many investigators to increase reticulocyte counts; and, as expected, within a few days high concentrations of reticulocytes were produced by these dogs. Unfortunately, exposure to phenylhydrazine appeared to damage the red cells, making them unsuitable for membrane preparation. The post-hemolytic residue (PHR) obtained from the blood of phenylhydrazine-treated dogs was a greenish-brown sediment, instead of the usual white, homogeneous membrane suspension. This abnormal PHR could not be uniformly suspended and was not suitable for enzyme assays. Similar alteration of red cell physiology by phenylhydrazine was reported by Allen and Jandl.(65) The results described here should probably be taken into consideration whenever studies of membrane function are considered using phenylhydrazine-induced reticulocytes.

b. Isolation of reticulocytes with phthalate esters.

Danon and Marikovsky have developed a unique red cell separation technique using varying densities of phthalate esters.(49) The method was quite simple to carry out, and consistently gave a good separation of the reticulocytes from whole blood containing elevated reticulocyte counts. Post-hemolytic residues derived from these phthalate-isolated fractions, however, were similar to those obtained with phenylhydrazine-treated cells in their inability to form a uniform, white membrane suspension. The oils apparently altered the cell membranes in some manner, possibly related to an extraction of membrane lipids by the organic esters. Because of this, membranes were not suitable for ATPase analysis, and a new method of reticulocyte isolation was then sought. (see section 2,2)

3.2 Changes in membrane ATPase activity during red cell maturation.

a. Human and rabbit erythrocytes.

1. Humans.

It has previously been shown that the ATPase activity of human erythrocyte membranes is highly sensitive to the presence of the alkali metal cations, and that this dependence is further modified by the concentration of ATP in the assay medium.(66-69) At low levels of ATP (2 μM), membrane ATPase activity is greatly stimulated by the presence of sodium ions, and this stimulation is slightly inhibited by the addition of 10 mM KCl. At high ATP concentrations (~1 mM) on the other hand, sodium alone only slightly stimulates ATPase activity, but sodium and potassium together enhance the rate of hydrolysis several fold. Both the sodium-dependent activity with low ATP, Na^+-ATPase, and the sodium-plus-potassium dependent stimulation with higher ATP, Na^+,K^+-ATPase, are completely inhibited by cardiac glycosides.

These characteristics of human mature red cell membranes are observed in the bottom fraction of reticulocyte-rich blood, depicted in the left-hand columns of Figs. 1 and 2. The activity observed with 50 mM KCl added to the incubation medium to counteract possible activating effects of trace amounts of residual sodium represents the baseline level of unstimulated ATPase activity, and

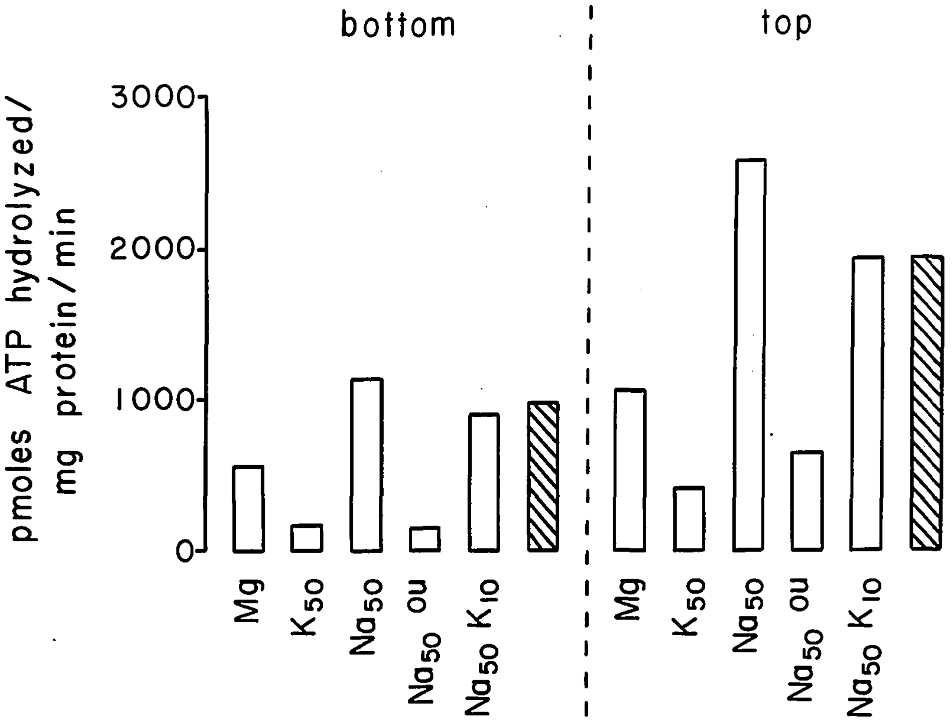

Figure 1. Effects of sodium and potassium on the membrane ATPase of human erythrocytes and reticulocytes measured at 2 µM ATP.

Membranes were prepared from top and bottom fractions of human blood separated on dextran gradients. The top fraction contained 22% reticulocytes and the bottom 5%. Assays were carried out for 2 minutes in medium containing 2 µM Tris ATP, 25 µM $MgCl_2$, 30 mM Tris HCl, pH 7.4, and 0.1 mM EGTA. Sodium, potassium, and ouabain (ou) were added as indicated. Values graphed represent the average of two separate experiments done with the same membrane preparation. Striped bars represent the total ouabain-sensitive Na^+-ATPase.

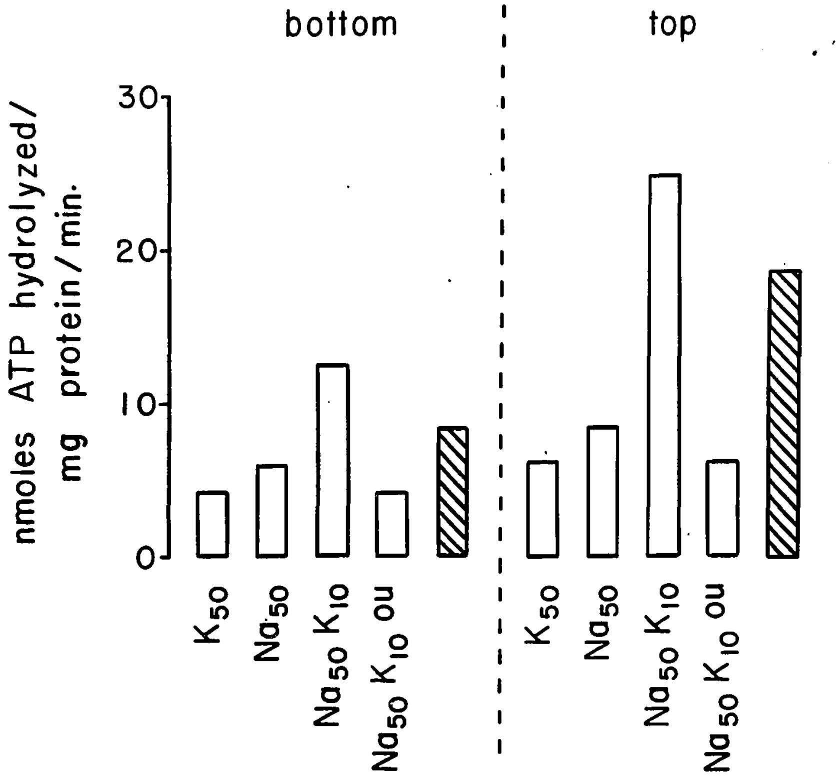

Figure 2. Effects of sodium and potassium on the membrane ATPase of human erythrocytes and reticulocytes measured at 1 mM ATP.

Membranes used in this assay were from the same preparation as used in Fig. 1. The membranes were incubated for 30 minutes in medium containing 1 mM Tris ATP, 1 mM $MgCl_2$, 30 mM Tris HCl, pH 7.4, and 0.1 mM EGTA. Sodium, potassium and ouabain (ou) were added as indicated. Results are the averaged duplicates of one experiment. Striped bars represent the total ouabain-sensitive Na^+,K^+-ATPase.

is referred to as the Mg^{++}-ATPase. With 2 μM ATP, addition of sodium ions to the medium yields a five-fold increase in ATPase activity over this Mg^{++}-ATPase value. When both sodium and potassium are added, the stimulation is slightly less, i.e. about four times the Mg^{++}-ATPase activity. In contrast, with 1 mM ATP, sodium alone increases the Mg^{++}-ATPase activity only 0.4-fold, while the two ions together effect almost a two-fold stimulation. These results are in accord with previous reports on human erythrocyte membrane ATPase, as is the complete ouabain-sensitivity of the alkali cation-stimulated components.(66-69)

When the same experiments are carried out with membranes from reticulocyte-rich blood, (top fraction), significant enhancement of all components of ATPase activity is observed at both ATP concentrations (Figs. 1 and 2). With low ATP, all the values increase more than two-fold; with higher ATP, the increase is approximately 1.5-fold in all cases. Ouabain-sensitive Na^+-ATPase and ouabain-sensitive Na^+,K^+-ATPase (striped bars) are both increased about two-fold in the preparations containing elevated reticulocyte counts. These results show that even with only 22% reticulocytes, there is a significant increase in the mean ATPase activity of the membrane preparation. Further enrichment of the reticulocyte count in the blood would be expected to produce even greater activity.

2. Rabbits.

In contrast to humans, rabbit erythrocyte membranes do not have a very active Na^+,K^+-stimulated ATPase.(70-72) This finding was surprising in light of the fact that rabbit red cells do have high intracellular potassium and low intracellular sodium levels, and must therefore have an active pump to maintain these gradients. This anomaly will be discussed in more detail later.

Like humans, however, rabbit membrane ATPase does depend both quantitatively and qualitatively on the concentration of ATP present as shown in the left-hand columns of Figs. 3 and 4. Though the changes are small, there is an increase in activity when sodium is added to the system at low ATP levels (Fig. 3). With higher ATP concentrations (Fig. 4), sodium ions stimulate the ATPase a little, and addition of potassium enhances activity somewhat further. These alkali cation components of the ATPase comprise only a small fraction of the total activity, but they are nevertheless completely inhibited by ouabain (striped bars representing paired differences).

A comparison of the ATPase activity in human and rabbit mature red cells clearly demonstrates the difference between these

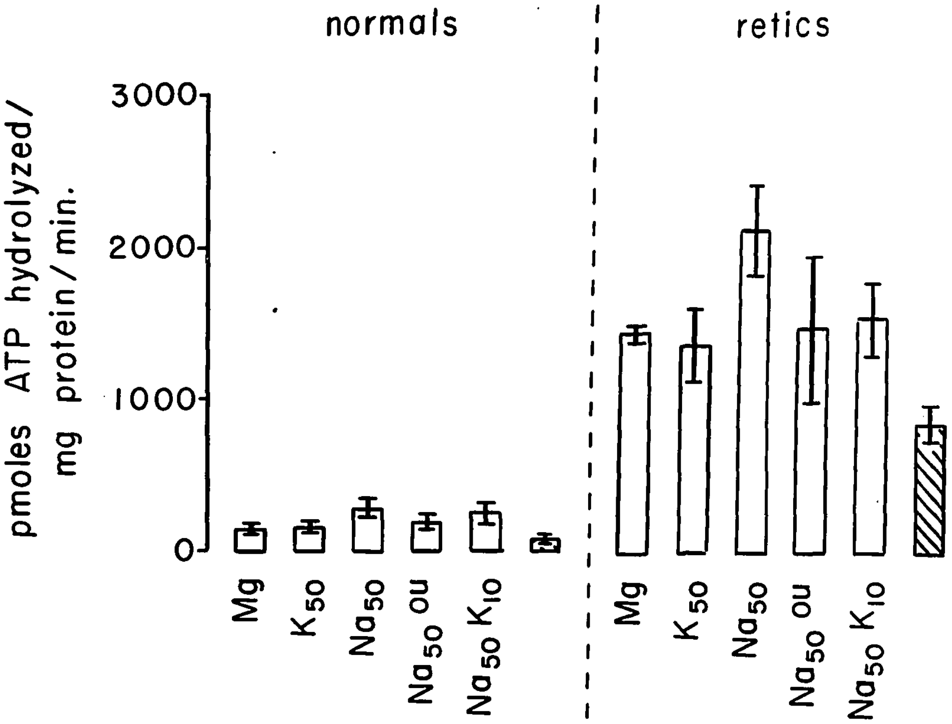

Figure 3. Effects of sodium and potassium on the membrane ATPase of rabbit erythrocytes and reticulocytes measured at 2 μM ATP.

Assays were carried out as described in Fig. 1. Normal membranes were prepared from the blood of rabbits not previously bled. Results are the average of 7 experiments. Reticulocyte results are the average of three experiments all done on the same membrane preparation. Blood for this preparation was from the top fraction of a dextran gradient and contained 24% reticulocytes. Standard errors of the means are indicated. Striped bars represent the ouabain-sensitive Na^+-ATPase activity, ie. the mean of paired differences of activities measured with and without ouabain.

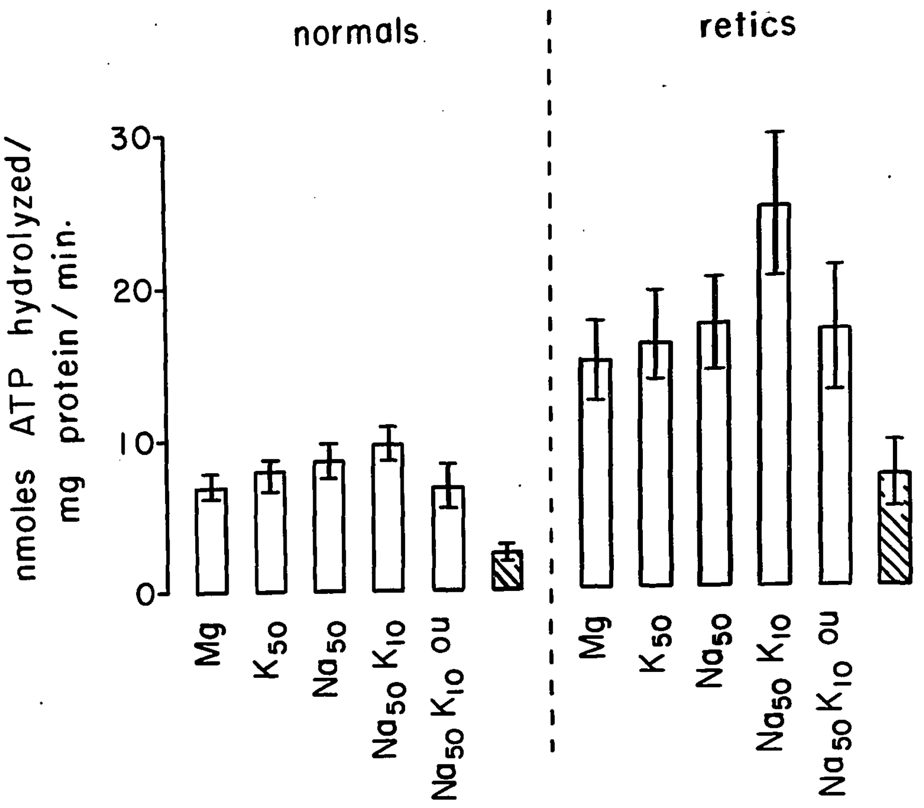

Figure 4. Effects of sodium and potassium on the membrane ATPase of rabbit erythrocytes and reticulocytes measured at 1 mM ATP.

Assays were carried out as described in Fig. 2. Normal membranes were obtained from blood of previously unbled rabbits. The average of 15 experiments is shown. Reticulocyte membranes were made from whole blood or the top fraction of dextran gradients with reticulocyte counts varying from 7% to 40% with a mean of 17% ± 3.2. The average of 11 experiments is graphed. Standard errors of all the means are indicated. Striped bars represent the ouabain-sensitive Na^+,K^+-ATPase components.

two species (Fig. 5). Ouabain-sensitive Na^+,K^+-ATPase in rabbit membranes is only 2.44 nmoles/mg protein/minute as compared to 8.3 nmoles/mg/minute in the human erythrocytes. Furthermore, this ouabain-sensitive component represents only 30% of the total ATPase activity in rabbit cells, in contrast to 67% with humans. At first, the unusually low level of Na^+,K^+-dependent ATPase activity in mature rabbit membranes was considered quite interesting. Perhaps this was a species in which ATPase stimulation by sodium and potassium was lost as the red cell matured. Studies of the ATPase activity in rabbit reticulocytes were initiated to investigate this possibility.

As observed with human preparations, rabbit reticulocyte membranes also display greatly elevated ATPase activity with respect to the mature red cells (Figs. 3 and 4). It appears that both Mg^{++}-ATPase and the alkali cation-stimulated components decrease with age in the red cells of rabbits even more markedly than in human erythrocytes. Membranes of reticulocyte-rich rabbit red cells have 8-10 times the total ATPase activity of mature cells at low ATP levels, and at least twice as much activity at high ATP concentrations. The ouabain-sensitive Na^+-ATPase was similarly ten-fold higher in reticulocyte-rich preparations and the Na^+,K^+-ATPase showed a three-fold greater activity, as compared to the

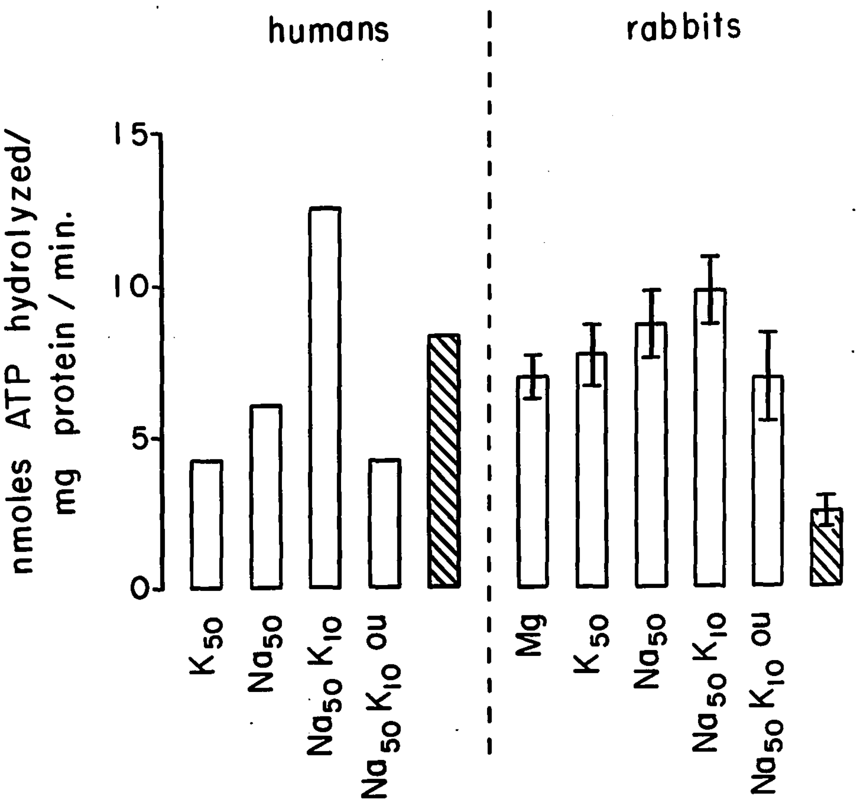

Figure 5. Comparison of human and rabbit erythrocyte membrane ATPase; 1 mM ATP.

Data taken from Figs. 2 and 4.

two-fold increase of both of these components in human reticulocyte-rich membranes. The higher ratio of reticulocyte : mature erythrocyte activity observed in rabbits at low as compared to high ATP concentrations is notable, and may, in part, be due to the slightly greater percentage of reticulocytes in the preparation assayed at 2 µM ATP (24% at 2 µM ATP, average 17% at 1 mM ATP). This explanation cannot account for the difference between a ten-fold increase of Na^+-ATPase activity in rabbits and the two-fold increase in humans, since both membrane preparations contained 22-24% reticulocytes.

Though the ouabain-sensitive components of rabbit red cell membrane ATPase show a marked increase in activity in reticulocytes, they remain only 30% of the total ATPase activity. Even with the elevated ATPase activity, the alkali cation-stimulated component is small when compared to human values. These difference between human and rabbit membranes are demonstrated in Table I. Though rabbit reticulocytes possess a large amount of ATPase activity, at no time do the ouabain-sensitive, cation-dependent components of rabbit ATPase ever resemble quantitatively the values obtained with human red cell membranes. Although the data is based on the membrane protein concentration, this comparison is considered valid, since both human and rabbit

TABLE I. Comparison of ATPase activity in membranes from human and rabbit erythrocytes and reticulocytes

2 μM ATP	pmoles Pi/mg protein/minute			
	Human		Rabbit	
Addition	normal	retic	normal	retic
a) 50 mM KCl	186	406	156	1370
b) 50 mM NaCl	1145	2579	283	2127
c) 50 mM NaCl + 0.2 mM ouabain	157	642	192	1480
d) Ouabain-sensitive Na$^+$-ATPase (b-c)	988	1937	85	855

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1 mM ATP	nmoles Pi/mg protein/minute			
a) 50 mM KCl	4.2	6.2	7.7	16.1
b) 50 mM NaCl	6.0	8.5	8.6	17.4
c) 50 mM NaCl + 10 mM KCl	12.5	24.8	9.8	25.0
d) 50 mM NaCl + 10 mM KCl + 0.2 mM ouabain	4.2	6.2	6.9	17.0
e) Ouabain-sensitive Na$^+$,K$^+$-ATPase (c-d)	8.3	18.6	2.4	7.4

Data taken from Figs. 1-4. Ouabain-sensitive components are the averages of paired differences from individual experiments.

blood yield about 2-3 mg protein per ml packed cells and both samples of blood contained similar reticulocyte counts. The reason that rabbit erythrocytes demonstrate such a small component of alkali cation-dependent ATPase activity is as yet unclear, but it does not seem to be a result of an aging process, at least not at a stage of maturation subsequent to entering the circulation.

3. Effects of alanine on reticulocyte membrane ATPase activity.

There have been suggestions that transport of organic molecules into cells is dependent on the membrane-bound Na^+,K^+-ATPase activity.(73) Since rabbit reticulocytes are known to retain their ability to transport amino acids (74), it was decided to test the effects of alanine on the ATPase activity of these cells. It was reasoned that if alanine transport requires activation of the sodium transport system, addition of alanine to the assay system might affect the activity of the Na^+-ATPase. As shown in Table II, this was not the case with either human or rabbit reticulocyte membranes. Moreover, alanine consistently inhibited the ATPase activity under all conditions, albeit to a small and probably insignificant extent. Although these experiments failed to show an effect of alanine, it must be pointed

TABLE II. Effects of alanine on ATPase activity in human and rabbit reticulocyte membranes

	pmoles Pi/mg protein/minute			
	Human		Rabbit	
Addition	-alanine	+alanine	-alanine	+alanine
None	1107	1009	1493	1397
50 mM KCl	372	326	1183	1097
50 mM NaCl	2465	2237	1967	1707
50 mM NaCl + 10 mM KCl	1990	1883	1357	1250

Membranes were made from the top fractions of human and rabbit reticulocyte-rich blood which contained 22% and 24% reticulocytes respectively. Assays were carried out for 2 minutes in medium containing 2 μM Tris ATP, 25 μM $MgCl_2$ 30 mM Tris HCl and 0.1 mM EGTA. Sodium, potassium and 1.0 mM alanine were added as indicated. Results shown are the averaged duplicates of one typical experiment for each species. Ten experiments with rabbit blood and four with human blood were carried out with similar results.

out that this ATPase assay system uses broken membrane preparations which have lost the sidedness of intact cells. This lack of membrane asymmetry could account for the absence of alanine effects, since an asymmetric distribution of sodium and potassium is required for transport of organic compounds across the cell membrane.(75) With loss of the inside and outside compartments of the cell, it is possible that the system would not be operational.

b. LK sheep erythrocytes.

In contrast to humans and rabbits, the sheep used in these experiments has erythrocytes with a very low internal potassium concentration. Moreover, the mature red cells of such a low-potassium (LK) sheep exhibit very little active Na^+,K^+-transport (31) or Na^+,K^+-dependent ATPase activity.(32) Teleologically, because there is little alkali cation gradient across the red cell membrane, there is virtually no need for a pump to maintain the gradient. In addition to the relative lack of ATPase activity found in LK erythrocytes with respect to their high-potassium (HK) analogues, the Na^+,K^+-ATPase of these two types of cells may also be distinguished on the basis of kinetics. These kinetic differences were first described by Whittington and Blostein using HK and LK sheep (37), and are illustrated in an experiment similar to ones carried out by these authors (Fig. 6). The Na^+-stimulated ATPase of LK membranes assayed with 0.2 μM ATP is highly sensitive to inhibition by potassium, 3 mM KCl producing 50% inhibition of activity. Na^+-ATPase in HK membranes, on the other hand, is stimulated by low concentrations of potassium, and requires greater than 20 mM KCl to reach the 50% level of inhibition. Fig. 6 demonstrates both the quantitative and qualitative differences between HK and LK types of Na^+-dependent ATPase activity.

It is of interest to note that all other cells in animals with LK erythrocytes display normal HK cation concentrations.(76) The genetic factors which produce LK characteristics appear to be manifested solely in the red blood cell population. It is conceivable that young red cells are originally HK in character, having not only a high intracellular potassium concentration and active potassium transport (41), but also an active, potassium-stimulated Na^+-ATPase. Once these cells are released into the circulation, the ATPase enzyme system is somehow altered to produce LK-type activity; the cation pumping rate similarly declines, and the alkali cation gradients across the cell membrane are gradually lost.

In an effort to elucidate the nature of this HK-to-LK change,

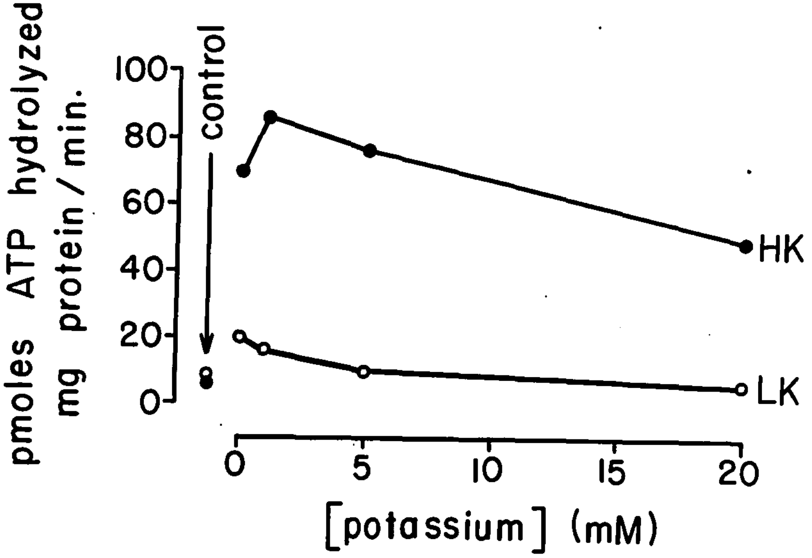

Figure 6. Effects of potassium on total ATPase activity in membranes of HK and LK sheep erythrocytes.

Membranes were prepared from the blood of an HK sheep (72Y) and an LK sheep (284). Assays were carried out for 2 minutes in a medium containing 0.2 μM Tris ATP, 12 μM $MgCl_2$, 30 mM Tris HCl, pH 7.4, 50 mM NaCl, and KCl as indicated. Points labeled control contained 50 mM KCl instead of sodium. Values plotted are the averaged duplicates of a single representative experiment.

reticulocytes were produced in an LK sheep by massive bleeding. The results shown in Table III confirm observations by Blunt and Evans (40) that the average intracellular potassium concentration increases with reticulocytosis. With only 24% reticulocytes, the mean cell potassium level increases from 18 to 55 mEq/l. This indicates that reticulocytes and young red cells do indeed have HK-type cell potassium values. This observation serves to substantiate the concept that erythrocytes begin life as HK-type cells and proceed through some unknown mechanism to become LK adult erythrocytes.

Examination of the ATPase data in Table III, however, seems to contradict this theory. Though reticulocyte-rich membrane preparations show a distinct increase in the amount of total ATPase activity and a slightly elevated Na^+-stimulated component, the K^+-inhibition profile remains distinctly LK in nature. This fact is seen more clearly in Figs. 7 and 8. The total ATPase activity level is elevated to values characteristic of HK membranes in the reticulocyte-rich membranes (Fig. 7), but the effects of adding potassium still resemble those of the mature LK cells. When the Mg^{++}-ATPase values (controls) are subtracted from each set of figures to give the Na^+-stimulated component (Fig. 8), the activity curves for reticulocytes and mature LK membranes almost coincide.

TABLE III. ATPase activity and cell potassium levels in LK sheep reticulocytes

Experiment no.	Sheep type	Fraction	% Reticulocytes	Cell K (mEq/l)	pmoles Pi/mg/minute				
					Mg^{++}-ATPase	Na^+-ATPase			
						K_0	K_1	K_5	K_{20}
1	LK	W	-	18	8	12	9	2	-1
3	LK	B	8	45	51	15	2	-2	-3
2	LK	W	9	36	60	13	13	3	1
2	LK	T	15	39	52	10	10	8	3
3	LK	W	15	53	94	24	-12	-7	-17
3	LK	T	24	55	78	18	4	-4	-7
1	HK	W	-	85	7	63	79	70	43

Blood was obtained from an LK sheep subjected to massive bleeding. Reticulocyte counts and cell potassium values were determined and membranes were assayed as described in Fig. 6. Normal HK and LK values are included for comparison. Fractions indicated are top (T), bottom (B), and whole blood (W).

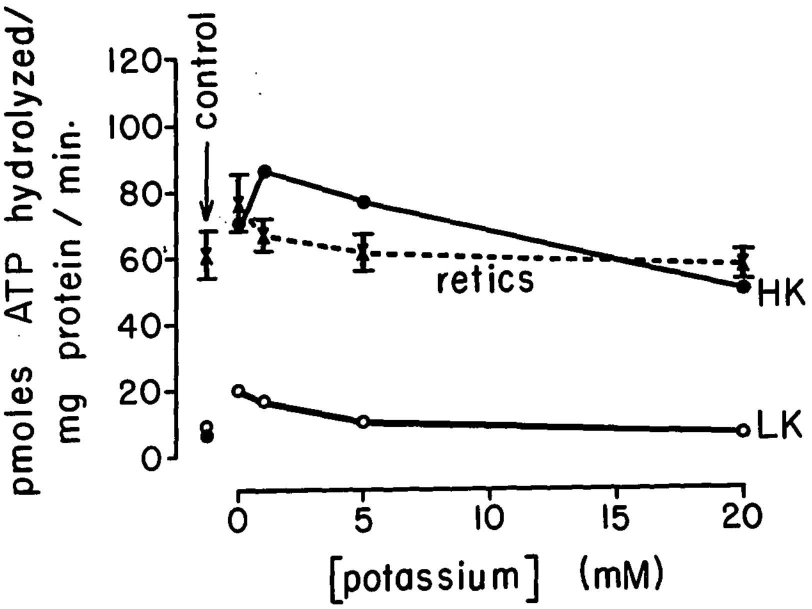

Figure 7. Effect of potassium on total ATPase activity in membranes of LK sheep reticulocytes.

Membranes were prepared from sheep blood containing between 8% and 24% reticulocytes, mean 14% ± 3.1, and assayed as described in Fig. 6. Results are the average of 5 experiments, and standard errors of the means are indicated. LK and HK curves from Fig. 6 are included for comparison.

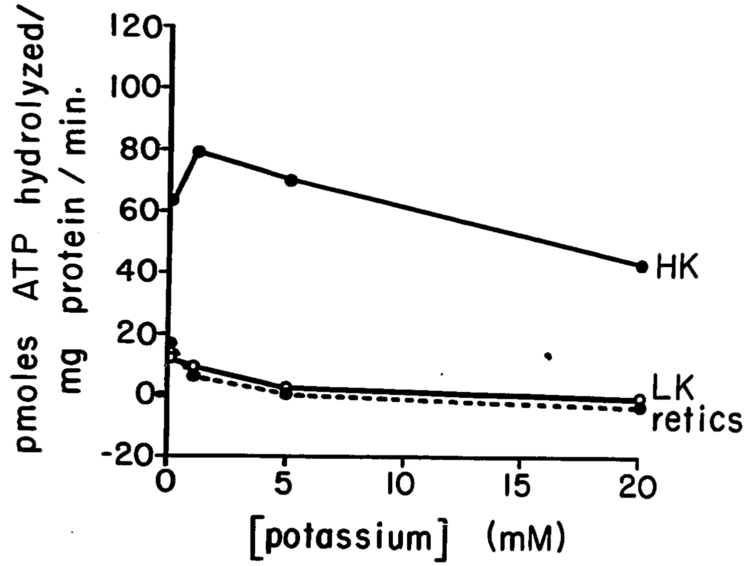

Figure 8. Effect of potassium on Na$^+$-ATPase activity in membranes of sheep erythrocytes and reticulocytes.

Data taken from Fig. 7 with control values subtracted to give the sodium-stimulated component of ATPase activity.

These results seem to indicate that reticulocyte membranes have a large component of alkali cation-insensitive ATPase activity which is lost during maturation, while the transport ATPase remains characteristic of mature LK erythrocyte throughout the cell's lifespan.

It might be argued that the appearance of low Na^+-ATPase values and LK-type kinetics in the reticulocyte-rich membranes is simply a result of a large number of mature cells with LK characteristics masking the effects of the few new cells that are HK in character. Evidence that the apparent LK kinetics of reticulocytes are real is provided when these results are compared to those obtained using LK lamb erythrocytes (see section 3.4). When lamb red cells with elevated potassium concentrations and levels of Na^+-ATPase activity similar to LK reticulocytes are assayed, the low activity displays HK-type K^+-inhibition kinetics. These results contrast with those found for LK reticulocytes, and support the proposal that reticulocytes have LK-type alkali cation-dependent ATPase activity.

3.3 ATPase activity of sodium-labeled rabbit erythrocytes.

As mentioned previously, rabbit erythrocyte membranes have an active Mg^{++}-ATPase component, even greater than that of human red cells, but there is little further stimulation of activity by the alkali metal cations. The high-potassium red cells of rabbits would be expected to have an active cation transport system, and for this reason the low level of alkali cation-dependent ATPase activity was found puzzling. Gardner and Lapey also observed this lack of Na^+,K^+-ATPase in rabbit erythrocyte membranes, and suggested that the absence of stimulation by alkali cations is due to bound sodium and potassium which cannot be washed free of the membranes.(70) This explanation would also account for the large amount of ATPase activity observed without either sodium or potassium added to the incubation medium.

An alternative possibility is that the membrane-bound ATPase of rabbit erythrocytes is highly sensitive to inhibition by cations on the "wrong" side of the membrane. In a broken membrane preparation, the sidedness of the system is lost, and all ions are equally available to both faces of the cell membrane. Inhibition by potassium on the inside or sodium on the outside might mask any stimulatory effects of these ions on their "proper" surfaces. To test this hypothesis, intact rabbit erythrocytes were loaded with sodium and then incubated in high-potassium medium. In this way, ATP hydrolysis could be observed under presumably optimal conditions.

The alkali cation content of the rabbit cells was altered using a modification of the PCMBS method of Garrahan and Rega. (60) Fig. 9 shows the changes in sodium and potassium content of the cells over the course of the PCMBS incubation. After ten hours, the normal cation ratio was found to be almost completely reversed, giving ideal conditions for measuring ATPase activity. The ATP level in the cells, as measured by enzyme assay, remained between 1-2 mM throughout the PCMBS incubation.

The ATPase activity of these cells was determined as described under Materials and Methods. In Fig. 10 the results of these assays are compared to experiments done with broken membranes under similar conditions. Ouabain-insensitive ATPase activity was greatly reduced in intact cells, and there was no ouabain-sensitive activity when sodium alone was present on both sides of the cell membrane. However, ouabain-sensitive Na^+,K^+-ATPase still comprised only 30% of the total activity, comparable to the value found with broken membrane preparations. Even when paired values of ouabain sensitive and insensitive activity are considered (Fig. 10, striped bars and Table IV), the intact cells do not show a very active Na^+,K^+-ATPase. Certainly there is no quantitative similarity between the values

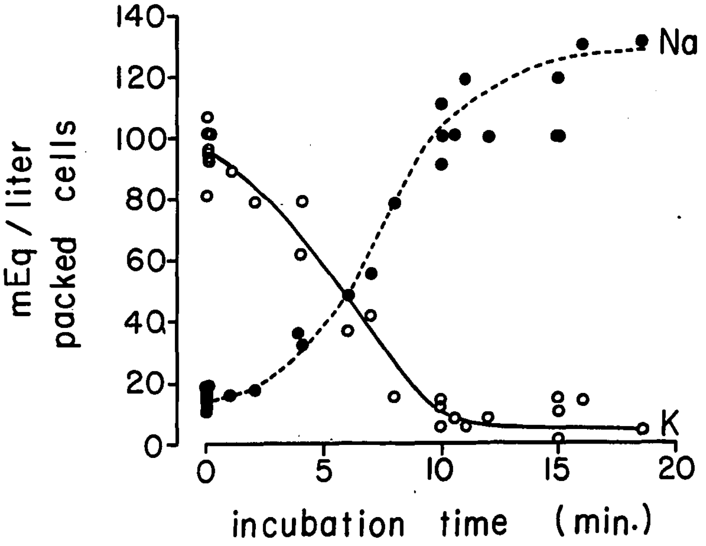

Figure 9. Rabbit red cell cation changes during PCMBS incubation.

Rabbit erythrocytes were made leaky to alkali cations by treatment with PCMBS as described in the text. At various time intervals, aliquots of cells were removed, resealed with DTT, and washed with isotonic $MgCl_2$. The cellular concentrations of sodium and potassium were determined by flame photometry. Results plotted represent 11 experiments.

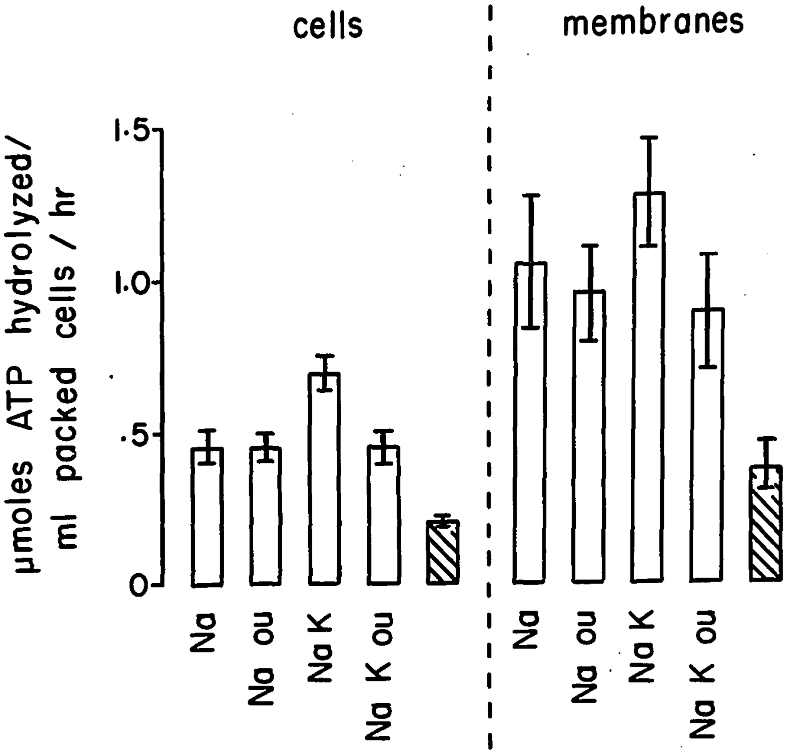

Figure 10. Comparison of ATPase activity in sodium-loaded rabbit red cells and rabbit erythrocyte membranes; 1-2 mM ATP.

Sodium-loaded rabbit red cells were incubated for periods of 1/2 to 2 hours in media containing 3 mM sodium phosphate, pH 7.4, 30 mM sucrose, 5 mM Tris HCl, and either 135 mM NaCl or 135 mM KCl as indicated. 0.2 mM ouabain was added to half the flasks. Values are the averaged results of either 4 (Na-Na) or 7 (Na-K) experiments.

Membranes were assayed as described in Fig. 2. Values are the averaged results of 8 experiments. Calculations of activity per ml packed cells are based on estimation that 2.5 mg membrane protein is obtained from 1 ml packed red cells.

Standard errors of the means are indicated. Striped bars represent the total ouabain-sensitive Na^+,K^+-ATPase.

TABLE IV. Na^+,K^+-dependent ATPase activity in rabbit and human erythrocytes

Intact, high Na cells	µmoles ATP hydrolyzed/ml cells/hour								
Experiment number	1	2	3	4	5	6	7		Mean ± S.E.
Total activity	.57	.84	.49	.70	.66	.92	.70		.70 ± .06
Ouabain-sensitive	.20	.25	.15	.14	.26	.24	.21		.21 ± .02
Broken membranes									
Experiment number	1	2	3	4	5	6	7	8	Mean ± S.E.
Total activity	.42	1.32	.84	1.34	1.24	1.58	1.41	2.13	1.29 ± .18
Ouabain-sensitive	.22	.90	.44	.16	.30	.38	.36	.33	.39 .08
Human membranes									
Total activity	3.72								
Ouabain-sensitive	2.79								

Details of experiments from which this data is taken are given in Figs. 2 and 10. Each pair of values represents the results of a single experiment conducted with 1 mM ATP, with and without ouabain present. Calculations of activity per ml cells are based on the approximation that 2.5 mg of membrane protein is equivalent to 1 ml packed rabbit red cells.

obtained for rabbits and those for humans which are almost ten times higher. The explanation for this lack of ouabain-sensitive, alkali cation-dependent ATPase in rabbit red cell membranes is thus still undetermined, but it does not appear to depend on the asymmetry of the membrane system.

3.4 Changes in red cell membrane ATPase during ontogeny in LK lambs.

The erythrocytes of lambs, both HK and LK, contain a very high concentration of potassium ions, often even greater than mature HK cells. As LK lambs grow older, the average potassium concentration in the blood falls steadily until, after 7-8 weeks, normal LK values are achieved. The data from four lambs, presented in Fig. 11, is in close agreement with results obtained by other investigators.(44,45).

Accompanying and preceding this loss of internal potassium is a decline in alkali cation-stimulated ATPase activity at both high and low ATP concentrations (Table V). In Figs. 12 and 13, the age-related loss of Na^+-ATPase and ouabain-sensitive Na^+,K^+-ATPase is demonstrated. Ouabain-sensitive activity at 1 mM ATP disappears almost completely in these young lambs by about 30 days after birth. A component of Na^+-ATPase activity at 0.2 μM

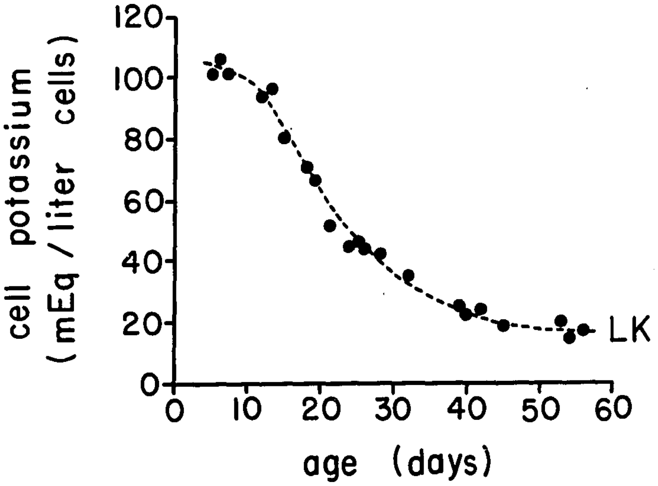

Figure 11. Loss of red cell potassium during maturation of LK lambs.

Lamb blood was drawn fresh each day, and whole blood and plasma potassium concentrations measured by flame photometry. Cell potassium levels were calculated from these values and the hematocrits.

TABLE V. Loss of red cell potassium and ATPase activity during LK lamb maturation

Lamb no.	Age (days)	Cell K (mEq/l)	pmoles Pi/mg/minute					Mg^{++}-ATPase	Na^{+},K^{+}-ATPase
			Mg^{++}-ATPase	Na^{+}-ATPase					
				K_0	K_1	K_5	K_{20}		
05	4	101	39	125	175	162	125	3.4	2.44
06	5	106	47	133	156	159	124	2.7	2.69
07	7	101	61	109	130	116	91	3.6	1.68
05	12	94	37	59	67	83	53	1.3	1.14
06	13	96	45	72	85	72	67	1.7	1.07
07	15	80	52	41	46	40	26	2.0	.72
05	18	71	32	21	25	28	16	2.1	.52
06	19	67	29	21	29	33	21	1.7	.62
07	21	52	20	17	21	17	10	.9	.36
71	24	45	35	29	42	36	29		
05	25	46	46	11	13	15	7	1.2	.15
06	26	44	34	14	9	10	4	.7	.18
07	28	42	49	20	18	11	0	1.2	.18
71	32	35	12	13	13	11	5		
05	39	25	17	3	2	4	-2	2.3	0
06	40	23	10	5	3	4	1	1.8	0
07	42	24	30	5	1	4	-5	2.9	.90
71	45	19	2	3	2	0	0		
05	53	20	9	1	1	-2	-3		
06	54	15	3	3	3	2	0		
07	56	17	8	4	5	3	1		

Membranes prepared from blood of four LK lambs were assayed as described in Figs. 13 and 14.

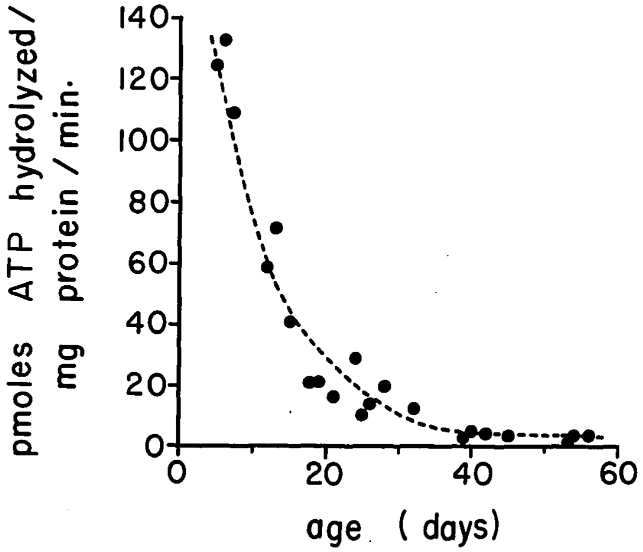

Figure 12. Loss of Na$^+$-dependent ATPase activity in LK lamb erythrocyte membranes during maturation.

Membranes prepared from lamb blood were incubated for 2 minutes in medium containing 0.2 μM Tris ATP, 13 μM $MgCl_2$, 35 mM Tris HCl, pH 7.4, and either 50 mM NaCl or 50 mM KCl. Values obtained with KCl in the assay medium were subtracted from those obtained with NaCl to give the Na$^+$-dependent component of ATPase which is plotted here. Each point represents the averaged duplicates of a single experiment.

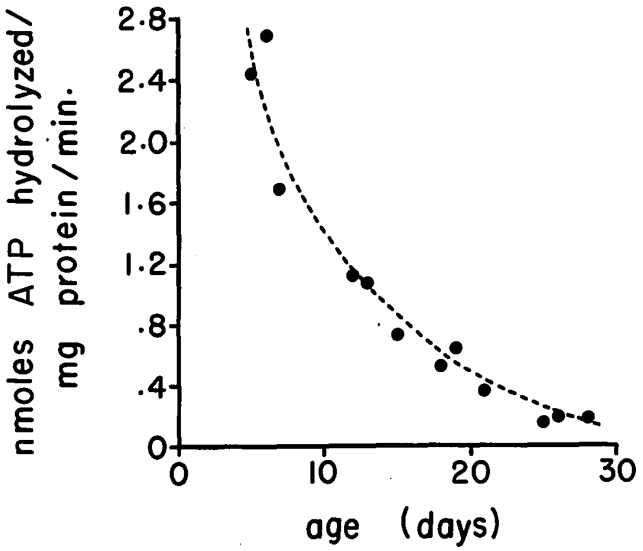

Figure 13. Loss of ouabain-sensitive Na^+,K^+-ATPase activity in LK lamb erythrocyte membranes during maturation.

Membranes prepared from lamb blood were incubated for 30 minutes in medium containing 1 mM Tris ATP, 1 mM $MgCl_2$, 40 mM Tris HCl, pH 7.4, 50 mM NaCl and 10 mM KCl. 0.2 mM ouabain was added to half the tubes, and the difference in ATPase activity between tubes with and without ouabain is plotted here as the ouabain-sensitive component. Each point represents the averaged duplicates of a single assay.

ATP persists in the red cell membranes throughout the period of investigations, but by approximately five weeks after birth the level of activity is even lower than in mature LK erythrocytes. At first, these results seem comparable to the changes already described for LK reticulocytes. With respect to these two parameters, loss of cell potassium and loss of ATPase activity, maturation changes in LK reticulocytes and changes in lamb erythrocytes during ontogeny appear to be similar.

When the kinetics of the Na^+-ATPase activity are considered with respect to the potassium response patterns, however, similarity between the two types of cell populations is no longer apparent. While LK reticulocytes possessing elevated potassium levels have been shown to have normal LK-type Na^+-ATPase kinetics, erythrocytes from LK lambs with similar high potassium concentrations and low rates of ATP hydrolysis display distinctly HK-type Na^+-ATPase activity. These differences are illustrated in Fig. 14. As the lamb matures, there is both a decline in the amount of Na^+-stimulated ATPase activity in the red cell membranes, and a switch from HK to LK-type K^+-inhibition kinetics. Fig. 15 demonstrates these changes over a period of three weeks in the red cells from a single lamb. It is of interest that the HK kinetics persist even when the ATPase activity is decreased to

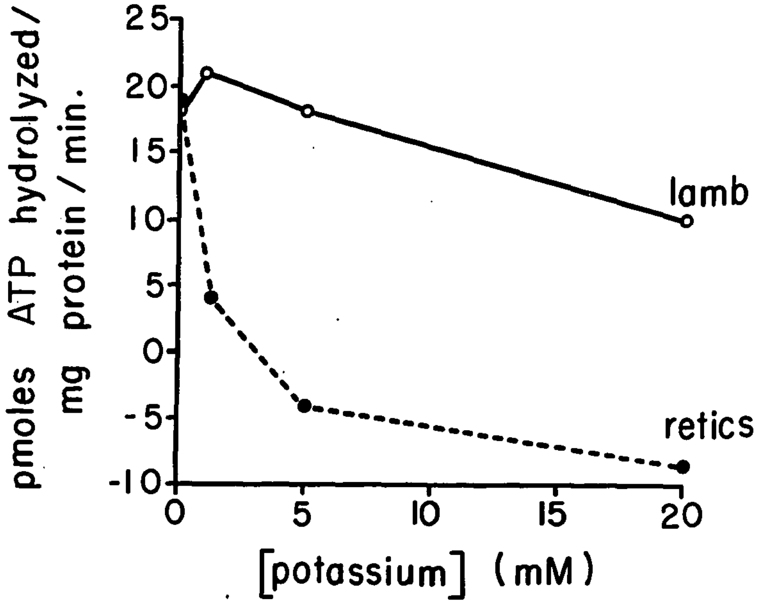

Figure 14. Comparison of Na^+-ATPase activity in membranes from LK reticulocytes and LK lamb erythrocytes.

Membranes were prepared from the top fraction of LK sheep blood containing 24% reticulocytes, and from the blood of a 21 day old LK lamb. Average red cell potassium levels were 55 mEq/l and 52 mEq/l respectively. Membranes were incubated as described in Fig. 12 with the addition of KCl as indicated. Control values (50 mM KCl) were subtracted in each case to give the Na^+-stimulated component.

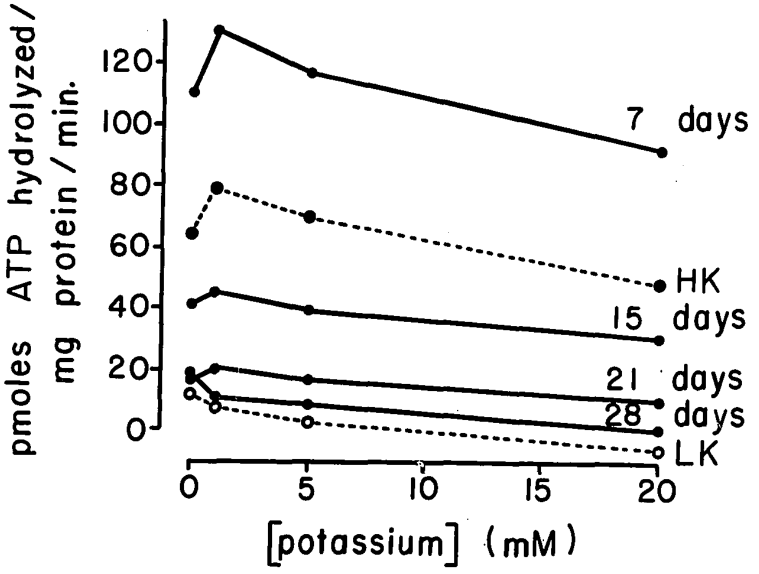

Figure 15. Changes in Na^+-ATPase activity of LK lamb erythrocyte membranes during maturation.

Na^+-ATPase activity was assayed as described in Fig. 14. The four solid curves represent the changes seen in one lamb (07) over a three week period. Normal HK and LK curves from Fig. 6 are included for comparison.

values approaching those of mature LK red cells.

As mentioned previously, several factors influence the development of these changes during lamb ontogeny. The most evident of these processes involves the death of fetal cells and replacement by adult LK erythrocytes in the circulation. The progress of this transition can be followed by observing the rate of disappearance of fetal hemoglobin from lamb blood with time. Fig. 16 shows the electrophoresis patterns of hemoglobin from three lambs at various ages, illustrating this loss of hemoglobin F.

Domestic sheep fall into three categories with respect to their hemoglobin type; those with just hemoglobin A, those with just hemoglobin B, and those with both A and B.(77) The hemoglobin type of these sheep is controlled by simple Mendelian genetics, two alleles producing two specific hemoglobins.(78) The blood of young lambs contains a third type of hemoglobin found only in fetal cells, hemoglobin F. These three molecules all contain the same α chain, but differ in their non-α chains and electrophoretic mobilities.(79) During electrophoresis at pH 8.6, the order of increasing mobility is $B<F<A$.

As shown in Fig. 16, lamb 05 has only hemoglobins B and F, while the other two lambs are heterozygotes. With aging, the

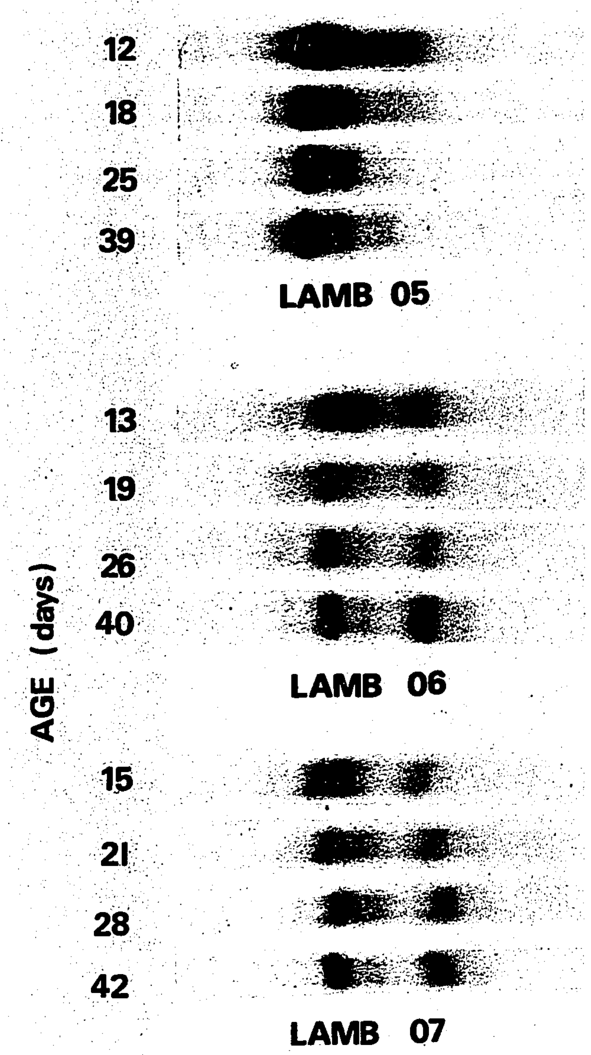

Figure 16. Changes in hemoglobin electrophoresis patterns of three lambs during maturation.

fetal hemoglobin bands slowly disappear as the normal adult hemoglobins become more prominent. To quantitate the percentage of the hemoglobin present as hemoglobin F, the electrophoresis strips were scanned in a spectrophotometer. A series of four tracings are shown in Fig. 17, depicting the changes in hemoglobin content in the blood of one of the lambs over a four week period. The dotted lines indicate the slopes of each individual peak extrapolated to zero absorbance. The ratio of fetal hemoglobin in each sample was determined as described in Materials and Methods. The results of this analysis are shown in Table VI and compare favorably with previously reported data.(46-48,79)

In Fig. 18, the rate of fetal hemoglobin disappearance is compared to the age-related changes in cell potassium level and Na^+-ATPase activity. The curves indicate that loss of Na^+-ATPase activity closely parallels the loss of fetal hemoglobin. The fall in cell potassium occurs later than the decrease in the other two parameters, but seems to show a similar rate of decline. This data is in accord with Tosteson's findings that both the potassium pump:leak ratio and the ouabain-sensitive ATPase in erythrocytes of developing LK lambs declined more rapidly than the intracellular potassium content.(45) The implications of these ontogenetic changes will be discussed.

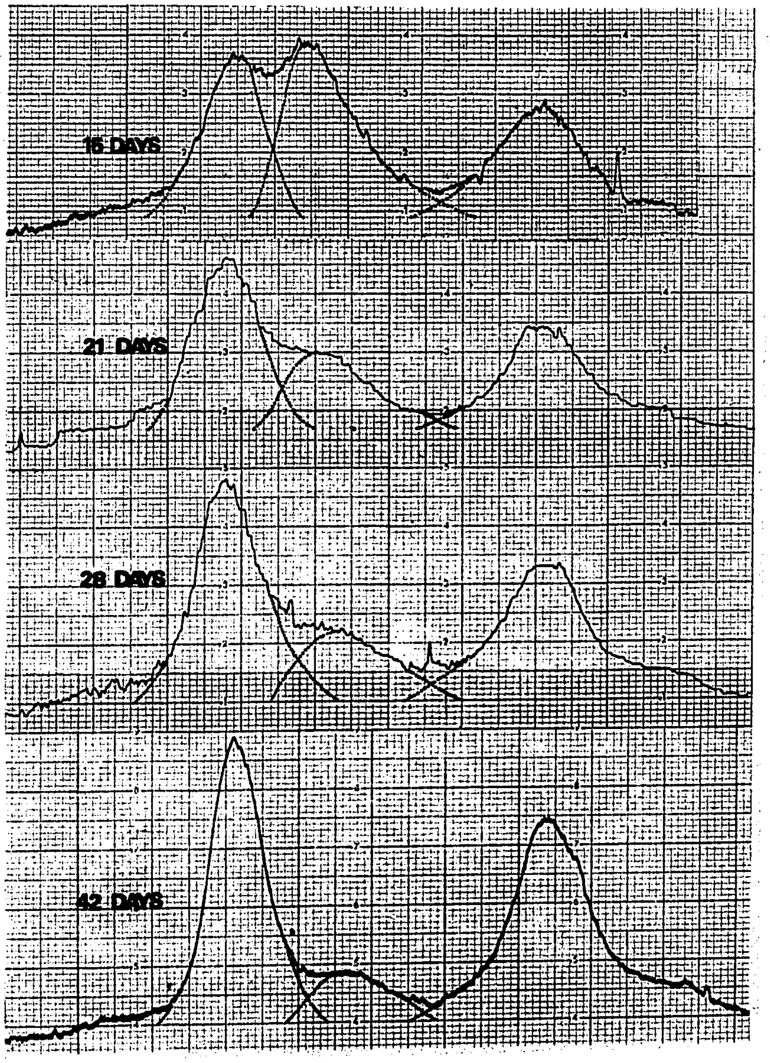

Figure 17. Loss of fetal hemoglobin in lamb blood during maturation.

Hemoglobin electrophoresis patterns were scanned as described in the text. Four tracings obtained from a single lamb (07) at various ages are shown here. Dotted lines delineate the approximate limits of the individual peaks. From left to right, the three major peaks represent hemoglobin B, hemoglobin F and hemoglobin A. Calculations of % fetal hemoglobin are described in the text.

TABLE VI: Loss of fetal hemoglobin with age in lamb erythrocytes

Lamb	Age	Cell K	% Hb F
05	12	94	78
05	18	71	30
05	25	46	18
05	39	25	13
06	13	96	46
06	19	67	28
06	26	44	20
06	40	23	12
07	15	80	38
07	21	52	23
07	28	42	16
07	42	24	9

Calculations of fetal hemoglobin concentration are described in the text.

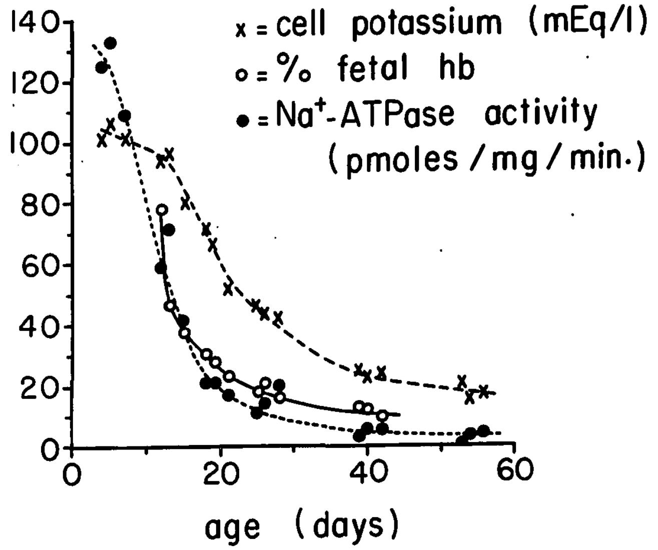

Figure 18. Ontogenetic changes in LK lamb erythrocytes. Data taken from Figs. 11 and 12 and Table VI.

4. DISCUSSION

The results of this investigation show that the rate of ATP hydrolysis by erythrocyte membrane preparations decreases markedly during red cell maturation. This discovery is similar to results reported for many other red cell enzymes, indicating that the loss of enzyme activity is a non-specific process characteristic of red cell aging. The mechanism responsible for this enzymatic deterioration undoubtedly lies in the cell's inability to synthesize new proteins to replace those that have become damaged or destroyed during aging of the cell. Loss of the protein synthetic system in erythrocytes is probably the determining factor in the relatively short lifespan of these cells.

Of the three species studied, human erythrocytes possess the most active membrane-bound transport ATPase system, though this activity is still relatively small when compared to other tissues such as nerve and muscle. It appears that the low level of alkali cation-dependent ATPase activity in human red cells is at least partly a result of deterioration of the enzyme system duing maturation. When membranes are prepared from blood containing a high concentration of reticulocytes, the rate of ATP hydrolysis is significantly increased. With a reticulocyte count of only 24%, the ouabain-sensitive ATPase activity of the membrane preparation was twice that of mature erythrocytes. Extrapolating this data to a theoretical population of cells composed solely of reticulocytes, it can be calculated that the membranes of these young cells must possess approximately five times the alkali cation-stimulated activity of mature red cells.

It is of interest to note that, though the ATPase activity is considerably elevated in reticulocyte membranes, the degree of dependence on sodium and potassium is remarkably similar in young and old cells. The ratios of alkali cation-stimulated activity to the Mg^{++}-ATPase level in reticulocytes remain comparable to those in mature red cell membranes at both high and low ATP concentrations. Addition of sodium ions to the incubation medium containing 2 µM ATP produced a six-fold stimulation of activity with both mature cells and reticulocytes. Likewise, addition of sodium and potassium together with 1 mM ATP enhanced the activity of both preparations 3-4 fold. The ouabain-sensitive component of the ATPase activity changes little with maturation, comprising 86% of the total activity in mature red cell membranes and 75% in reticulocytes with low ATP, and 66% and 75% respectively at high ATP concentrations. These results are consistent with those obtained by Whaun and Oski in experiments on 13 patients with reticulocytosis.(80) They, too, found that reticulocyte-rich blood had more ATPase activity than normal, but that the ratio of ouabain-sensitive to ouabain-insensitive activity was essentially the same in both fractions.

It may be a coincidence that the Mg^{++}-ATPase in red cell membranes seems to deteriorate at the same rate as the alkali cation-dependent activity, but it is also possible that the Mg^{++}-dependent component bears some relationship to the transport ATPase system. In either case, the preservation of constant activity ratios throughout maturation seems to indicate a loss of active pump sites with age, rather than an alteration in the sensitivity of the red cell membrane ATPase to stimulation by sodium and potassium. Thus it would appear that the only change in human erythrocyte ATPase activity as the cell matures is a quantitative one, probably a decrease in the number of active pump sites per cell.

In comparison to humans, the membranes of mature rabbit erythrocytes seem to have very little alkali cation-dependent ATPase activity. The ouabain-sensitive Na^+-ATPase of rabbit red cells (assayed at 2 µM ATP) is less than 1/10 that of human membranes, and the Na^+,K^+-ATPase (at 1 mM ATP) is 3.5 times more active in humans than in rabbits. Similar results have been reported frequently for rabbit erythrocyte membrane Na^+,K^+-ATPase (70-72), and pose a serious question concerning the transport ATPase system. Like humans, rabbit red cells maintain steep electrochemical gradients of both potassium and sodium across the cell membrane. A considerable amount of work has been done establishing a connection between the maintenance of these alkali cation gradients and a membrane-bound system for hydrolysis of ATP. The lack of correlation between the two systems in rabbit red cells is puzzling, and somewhat disturbing.

It is possible that rabbit erythrocytes have evolved some alternative energy source for the cation pump, but this explanation is rather unlikely. On the other hand, rabbit red cell membranes might be highly impermeable to sodium and potassium, and need little pump activity to maintain the normal gradients. This too is unlikely, especially in light of reports by Villamil and co-authors that the rate of ouabain-sensitive sodium efflux from rabbit cells along with coupled potassium influx is quantitatively similar to results obtained with humans.(81,82) Thus it seems that the cation pump of rabbit membranes is working actively, but the alkali cation-sensitive ATPase system, which should supply the energy for the pump, is not.

With this apparent anomaly in mind, a series of experiments was designed to test whether the assay system used could be responsible for the observed lack of alkali cation-dependent ATPase activity. It was considered possible that the absence of membrane sidedness in assays done with broken membrane preparations might account for a loss of stimulation by sodium and potassium on the ATPase system. Since the membrane ATPase of intact cells is stimulated by sodium on the inside and potassium on the outside, it was thought that equal concentrations of both ions on both sides of the membrane might counteract the expected stimulatory effects. The results of these experiments were similar to those with broken membranes, however, and contributed little to the understanding of the basis for this rabbit ATPase dilemma. Ouabain-sensitive Na^+,K^+-ATPase in intact rabbit red cells loaded with sodium and incubated in high-potassium medium was still only about 1/10 the value found with human membranes. Thus, the lack of correlation between the rate of cation pumping and the cation-stimulated ATPase activity of rabbit erythrocytes does not appear to stem from a loss of membrane asymmetry.

Gardner and Lapey have proposed that the absence of stimulation by alkali metal cations is due to bound sodium and potassium which cannot be washed off the membranes.(70) This explanation would also account for the rather large amount of ATPase activity observed without either sodium or potassium added to the assay medium. Although such a phenomenon may play a part in decreasing apparent ATPase sensitivity to the alkali cations, it does not seem sufficient to completely explain the marked difference in ouabain-sensitive ATPase activity between human and rabbit red cell membranes, nor the low activity observed with sodium-loaded intact cells. The problem of deficient Na^+,K^+-ATPase in rabbit erythrocyte membranes still awaits an adequate solution.

Reticulocytes in rabbits, like humans, show a significant increase in all components of ATPase activity compared to mature red cells. The results reported here confirm those of Yunis and Arimura (83) and Wise and Archdeacon (84), and suggest that the unusually low level of cation-stimulated ATPase in adult erythrocytes might result from a loss of activity during maturation of the cell. However, both ouabain-sensitive Na^+-ATPase and ouabain-sensitive Na^+,K^+-ATPase in the membranes prepared from reticulocyte-rich rabbit blood still demonstrated less activity than human mature red cell membranes. Moreover, ouabain-sensitive ATPase in reticulocytes still represented only 30% of the total activity, similar to the ratio found with mature cells. As seen in human membranes, the alkali cation-stimulated ATPase seems to deteriorate at a rate proportional to the loss of the Mg^{++}-dependent component. The amount of Na^{+}-ATPase in rabbit red cell membranes decreases with age, but the fraction of the total activity that is sensitive to the alkali metal cations does not. As mentioned before, the consistency in the percentage of total activity inhibited by ouabain and stimulated by sodium and potassium during maturation may be simply a coincidence. It does suggest, however, that rabbit red cells, like human cells, undergo a loss of active cation transport sites with aging, rather than a change in sensitivity to sodium and potassium.

LK sheep, the third species in which reticulocyte maturation was studied, have even lower erythrocyte membrane Na^{+}-ATPase activity than rabbits. In this case, however, the lack of alkali cation-dependent ATPase activity is understandable, since LK sheep have very little sodium or potassium gradients across their red cell membranes. LK sheep reticulocytes, on the other hand, have elevated internal potassium concentrations (40) and alkali cation pump activity (41), and might be expected to show an increased rate of alkali cation-stimulated ATP hydrolysis. Indeed, reticulocytes in these animals do possess significantly greater total ATPase activity than mature red cells. The increase in ATPase activity, though, appears to be mostly in the Mg^{++}-dependent fraction. Unlike rabbits and humans, the ratio of Na^+-stimulated ATPase to Mg^{++}-dependent ATPase activity is not constant with age. There is only a small difference between the amount of Na^+-ATPase seen in preparations from reticulocyte-rich LK blood containing between 36-55 mEq K/l and that found in mature LK cells with less than 20 mEq K/l.

The normal range of Na^+-ATPase in LK membranes assayed at 0.2 μM ATP is 10 ± .81 pmoles/mg protein/minute (23 preparations)[1]. The reticulocyte preparations assayed here under identical conditions displayed Na^+-ATPase activities ranging from 10-24 pmoles/mg/minutes with an average of 16 ± 2.4 pmoles/mg/minute. Since the reticulocyte count was only between 8% and 24%, it is probable that young cells do have a slightly elevated Na^+-ATPase activity. The fact that preparations with the highest cell potassium levels showed the greatest increase in Na^+-ATPase tends to support this idea. The difference between young and old cells, however, is very small, and can hardly be compared to the changes seen in human and rabbit erythrocytes during maturation. The Na^+-ATPase of LK reticulocytes is definitely not comparable to values found for mature HK sheep cells as the elevated potassium levels and active potassium

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[1] Blostein, R. and E.S. Whittington, unpublished observations.

pump rate might tend to suggest.

The reason for the large component of Mg^{++}-dependent ATPase observed in reticulocytes might be due to the procedure used in preparing the membranes. In order to preserve as much similarity as possible between the reticulocyte-rich preparations and the mature cell membranes, reticulocyte-rich blood was lysed with water. All of the control membrane preparations had been made using water lysis, and this procedure was continued to insure comparability. This is in contrast to reticulocytes from rabbits and humans which were lysed and washed with a sucrose solution. Centrifugation in sucrose is reported to remove the mitochondria and other intracellular membranes from the plasma membrane fraction. (54) It is possible that the reticulocyte membrane preparations from LK sheep were contaminated with mitochondrial membranes or vestigial reticulum, either of which might possess an active Mg^{++}-dependent ATPase. These contaminants would not be found in membranes prepared from mature red cells, and the Mg^{++}-ATPase component would therefore be relatively small.

In addition to the quantitative aspects of ATPase activity in LK sheep reticulocytes, the qualitative aspects, or potassium-inhibition kinetics, were also investigated. In this case too, the membranes of LK reticulocytes were found to respond in a manner similar to LK rather than HK mature erythrocytes. The Na^+-ATPase activity of young LK red cells is highly sensitive to inhibition by low concentrations of potassium ions. Thus, both in quantity and kinetic response to potassium, the Na^+-ATPase system of LK reticulocytes resembles that of mature LK cells, suggesting that the LK characteristics of red cell membranes are fully developed by the time the young cells enter the circulation.

It is interesting to compare the activity of these immature LK cells to results obtained with anti-L treatment of mature cells from LK sheep.(38) In these experiments, also, membranes display an increase in Na^+-ATPase activity but retain LK-type K^+-inhibition kinetics. This alteration in enzyme activity is attributed to masking of the L antigen on the surface of the red cell by a specific antibody. It could be that the elevated Na^+-ATPase activity in LK reticulocytes is due to a delay in the development of the L antigen. Possibly because these young erythrocytes have not yet acquired the antigen, they react in a manner similar to those cells in which the antigen has been blocked.

The low level of alkali cation-stimulated ATPase activity in LK reticulocytes, and the LK-type potassium response patterns of this ATPase appear inconsistent with the data reported for cation transport in these young LK cells. Lee, Woo, and Tosteson found that the potassium pump rate in LK reticulocytes was similar to HK rather than LK cells, and changed progressively to LK-type activity as the cells matured.(41) Since pump activity and alkali cation-dependent ATPase are assumed to be linked, the ATPase activity of these cells should also be typical of HK sheep erythrocytes. This was not found to be the case, and at first it proved rather disturbing. However, the kinetics of potassium transport in LK reticulocytes have not been determined, opening the possibility that the pump is kinetically LK but retains a relatively high rate of ion flux. If this were true, pump activity in reticulocytes would resemble Na^+-ATPase in the respect that both have increased rates of activity with normal LK kinetics. Once again the reticulocytes can be compared to anti-L treated LK erythrocytes, which also show an increased rate of potassium pump activity. It would appear that the quantitative aspects of potassium transport and cation-dependent ATP hydrolysis by LK membranes are related to the development of the L antigen, while the kinetics of these two properties develop at an earlier stage of cell differentiation. Further studies comparing both potassium pump and Na^+-ATPase activity in LK sheep reticulocytes to the development of the L antigen will be necessary to clarify the relationships among these three parameters in the erythrocyte membrane.

The unusually high potassium concentration found in LK sheep reticulocytes is probably a reflection of the normal high potassium level present in the stem cells which give rise to the new erythrocytes. Massive hemorrhage forces reticulocytes prematurely into the circulation before the potassium content has fallen to normal LK values and the residual active potassium transport of these young cells helps to maintain the ion concentration at an elevated level. As the low potassium pump rate characteristic of LK membranes develops, the cell potassium level gradually decreases due to slow passive efflux. This, of course, is speculation, and pump-leak experiments with potassium-loaded reticulocytes would be necessary to test this possibility.

In light of the interesting changes in alkali cation-dependent ATPase activity observed during reticulocyte maturation, it was decided to study this enzyme system during ontogeny in LK lambs. Lambs were selected as the experimental animal for several reasons. A considerable amount of work has been done characterizing the Na^+-ATPase in both HK and LK sheep red cells, thus providing a basis of comparison for results obtained with the lambs. Secondly, any changes noted in LK lamb cells during ontogeny could be compared to the observations made while following LK sheep reticulocyte maturation. Furthermore, LK lamb erythrocytes have been shown to possess intracellular potassium content (42-44), alkali cation transport (36,44,45) and ouabain-sensitive ATPase activity (35,45) all quantitatively similar to HK mature red cells. With increasing age, these three factors all decrease to values typical of LK cells. It was hoped that by investigating the course of these changes during ontogeny, some further insight might be gained into the nature of the genetic difference between HK and LK sheep erythrocyte membranes.

The results obtained here indicate that fetal cells in LK lambs possess not only the quantitative but also the qualitative, or kinetic, characteristics of HK erythrocytes. In this respect, the cells of neonates differ from LK reticulocytes, since the reticulocytes retain LK kinetics even when cell potassium, pump rate, and Na^+-ATPase activity are elevated. A comparison of Na^+-ATPase activity in membranes from LK lambs and reticulocyte-rich LK blood with similar cellular potassium content leaves little doubt that the fetal cells have HK-type characteristics. It appears that the gene(s) responsible for the LK phenotype does not become activated until sometime after birth.

The alkali cation dependent components of the membrane ATPase in lamb blood fall rapidly in the first few weeks postpartum. It was observed that the loss of Na^+-ATPase activity closely paralleled the loss of fetal hemoglobin in the blood, suggesting that the changes seen during ontogeny in these lambs are due to replacement of HK-type fetal cells by adult cells with LK membrane characteristics. This theory is supported by the investigations of Ellory and Tucker showing that development of the L antigen in young lamb blood closely follows loss of potassium transport activity.(44) In addition, Tosteson discovered that ouabain-sensitive ATPase and potassium pump:leak ratio decreased progressively with aging.(45) Dunham and Hoffman also observed a loss of ouabain-binding sites on the red cells of maturing lambs, coupled with a decrease in active potassium influx.(36)

Israel and co-workers have reported similar alterations in alkali cation-stimulated ATPase activity and potassium transport in newborn calves, another species which switches from HK to LK during the early post-natal period.(85) These investigations suggest, however, that the changes during ontogeny reflect the average age of the whole cell population, rather than simply a substitution of one cell type for another. Not only are HK fetal cells dying and being replaced by LK erythrocytes, but changes are also taking place in each of these two cell populations as a result of aging. The apparently simple developmental changes seen during ontogeny are the resultant of a complex series of interrelated variations in the total red cell mass.

Upon closer scrutiny of the data obtained during LK sheep ontogeny, it becomes evident that similar complexity can be found in this developmental system as well. The decline in intracellular potassium level in the blood of LK lambs does not follow the same time course as the loss of fetal hemoglobin or the changes in Na^+-ATPase activity as shown in this study, nor is it consistent with the decrease in potassium transport (36) or the development of the L antigen.(44) The curve depicting cell potassium content during the first eight weeks after birth is biphasic, first a slow decline and then a more rapid drop until normal mature LK values are reached. Many factors are responsible for the shape of this curve and its lack of correlation with the other four parameters.

In the first place, the circulatory system of young lambs is expanding rapidly during the first few weeks after birth. After the initial phase of presumably decreased erythropoeisis due to the change from an anoxic environment, young LK cells begin entering the circulation. Like LK reticulocytes, these cells probably have elevated potassium levels retained from their high-potassium precursors; thus, the high blood potassium level originally due to the HK fetal cells is maintained for a while by the advent of these LK-type cells with elevated potassium content. As LK pump and leak rates become predominant, the cell potassium values gradually begin to fall. The new LK cells probably have slightly elevated Na^+-ATPase activity and potassium pump rate also, which would decline quickly with age and affect the curves for these two systems.

The delayed decline of cell potassium level may be further influenced by the entrance of new fetal-type cells into the circulation. Israel et al. found that 15% of the hemoglobin synthesized in calves at 24 days postpartum was hemoglobin F (85), indicating that the change from production of fetal to adult cells does not occur abruptly at the time of birth. Addition of new HK, as well as LK, cells to the total population would obviously have an effect on all the parameters assayed.

Thirdly, the HK fetal cells probably undergo maturation changes even after they have entered the circulation. Like young cells in humans and rabbits, these high-potassium cells would be expected to lose ATPase and probably pump activity as they mature, even though they remain HK in character. Thus, at least four factors contribute to the ontogenetic changes observed in the erythrocytes of young lambs; loss of enzyme activity by HK fetal cells, loss of potassium content from young LK-type cells, loss of enzyme activity by the LK cells, and changes in the rate of production of these two cell types.

It is of interest that the kinetics of the Na^+-ATPase activity in lamb blood remain HK in character, i.e. stimulated by low concentrations of potassium, even when the quantitative aspects approach typical LK values. This fact provides evidence that the fetal cells retain their HK characteristics whether or not they lose activity as a result of aging. Thus, the switch from an HK to an LK kinetic profile in the Na^+-ATPase of lamb blood is probably due to cell replacement rather than a change in the membranes of the fetal cells. Another factor which may influence the persistence of the HK kinetics in these developing lambs is the very low level of activity contributed by the membranes of the mature LK cells. When all the fetal hemoglobin had disappeared from the blood, the level of Na^+-ATPase was found to be even lower than normal LK membranes. For some reason the first LK-type cells produced by these lambs appear to be "super LK", at least with respect to the Na^+-ATPase activity. The small amount of activity which they contribute to the mean ATPase level of the assayed membranes would not be sufficient to mask the HK characteristics of the fetal cells.

In summary, the reticulocytes of all three species studied show an increased level of membrane-bound, alkali-cation-dependent

ATPase activity with respect to the mature red cells. The increase is more marked in humans and rabbits with high-potassium cells than in LK sheep; but in all three, the pattern of activation by the alkali metal cations is the same in reticulocytes and mature cells. The mechanism responsible for this loss of activity during erythrocyte maturation is probably a deterioration of membrane proteins or lipids which cannot be replaced by the adult red cells.

Developmental changes in the blood of newborn lambs appear to be more complex, but dependent in part upon the changes observed during reticulocyte maturation. Fetal cells in LK sheep have HK characteristics, indicating that the genetic machinery controlling the LK phenotype is not activated until after birth. Furthermore, young LK cells display relatively high rates of transport ATPase activity with typical LK kinetics. This picture is also seen with anti-L treated LK cells, suggesting that the L antigen controls the rate of cation pumping while some other factor controls the kinetics. This unknown factor appears to develop before the LK cells enter the circulation. Thus the study of reticulocyte maturation and LK lamb red cell ontogeny has provided a small increment of insight into the genetic differences between HK and LK erythrocytes.

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