# TRANSPORT SYSTEMS OF SHEEP RETICULOCYTES AND THEIR CHANGES DURING CELL MATURATION

By

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# Changes In Transport Systems of Sheep Reticulocytes During Maturation

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## ABSTRACT

Maturation and aging-associated changes in membrane transport of sheep reticulocytes of the high-$K^+$ and low-$K^+$ genotypes were studied. The results indicate that during short term (less than 10 days) in vitro maturation, there is a progressive decrease in ouabain-sensitive $Na^+$,-$K^+$-pump activity ($^{86}Rb^+$ uptake in low-$K^+$ cells) and $Na^+$-ATPase activity (high-$K^+$ cells). However, during long term (several weeks) in vivo maturation in which HbC was used as a marker of newly formed reticulocytes, kinetic changes, as well as a decline in total activity, were observed.

The effect of metabolic depletion on the maturation-associated loss of two membrane transport functions was also studied using in vitro incubation. Both $Na^+$-dependent glycine transport and the $Na^+$,$K^+$-pump, estimated from measurements of the number of $^3H$-ouabain binding sites per cell, were decreased during maturation. ATP also enhanced the decrease in both activities when a 'reconstituted' vesicle system comprised of inside-out vesicles plus cell lysates was incubated at 37°C. Associated with this ATP-dependent loss of activity was an increase in the amount of concomitantly measured ninhydrin-positive material. It is concluded that the loss of certain functions during reticulocyte maturation is retarded by metabolic depletion.

Membrane vesicles of distinct sidedness were prepared from sheep reticulocytes. Using these vesicles the $Na^+$-dependent glycine transport system was found to be symmetrical with respect to: (a) the $Na^+$ dependency of glycine transport, (b) the ability to accumulate glycine against a concentration gradient, (c) the $Na^+$: glycine stoichiometry, i.e. two $Na^+$ ions are transported per molecule of glycine, and (d) the apparent Michaelis-Menten constants for $Na^+$ and glycine.

## RESUME

Les changements dans le transport membranaire associés à la maturation et au vieillissement ont été étudiés chez les réticulocytes de mouton de haute-$K^+$ et basse-$K^+$. Les résultats indiquent que pendant la maturation *in vitro* à court terme (moins de 10 jours), il y a une diminution progressive de l'activité de la pompe-$Na^+,K^+$ sensible à la ouabaine (mesuré par la prise de $^{86}Rb^+$ par des cellules de basse-$K^+$) et également de l'activité $Na^+$-ATPase (étudié chez les cellules de haute-$K^+$). Cependant à long terme (plusieurs semaines), la maturation *in vivo* dans laquelle HbC a été utilisé comme marqueur de réticulocytes nouvellement formés, des changements cinétiques ainsi qu'une baisse de l'activité totale ont été observés.

L'effet d'épuisement métabolique sur la perte de deux fonctions de transport membranaire associeés à la maturation, a aussi été étudié par incubation *in vitro*. Le transport de la glycine dépendant du $Na^+$ ainsi que l'activité de la pompe-$Na^+,K^+$ ont diminués durant la maturation; ceci a été mesuré par le nombre de sites par cellule liant la $^3H$-ouabaine. L'ATP accentue aussi la diminution de ces deux activités lorsqu'un système de vésicules reconstitué, comprenant des vésicules inversées et des lysats de cellules sont incubés à 37°C. Associée à cette perte d'activité dépendante de L'ATP fut une augmentation concommitante de la mesure de matériel ninhydrine-positif. On en conclut que la perte de certaines fonctions durant la maturation de réticulocytes est retardée par l'épuisement métabolique.

Des vésicules membranaire inversées des deux façons ont été préparées à partir de réticulocytes de mouton. On a trouvé que le transport de la glycine dépendant du $Na^+$ est symmétrique chez ces deux types de vésicules quant au (a) transport de la glycine dépendant du $Na^+$, (b) l'abilité d'accumuler la glycine contre un gradient de concentration, (c) la stoichiométrie $Na^+$ : glycine i.e. deux ions $Na^+$ sont transportés par molécule de glycine et (d) les constantes Michaelis-Menten apparentes pour $Na^+$ et glycine.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| $A_{259}$ | absorbance at 259 nm |
| $A_{280}$ | absorbance at 280 nm |
| ADP | adenosine diphosphate |
| AMS | anemic mouse serum |
| ATP | adenosine triphosphate |
| Anti-L | anti-LK antibody |
| [$\gamma$ - $^{32}$P]-ATP, ATP$^{32}$ | adenosine triphosphate labelled with $^{32}$P in the gamma position |
| ATPase | adenosine triphosphatase |
| BFU-E | burst forming unit-erythroid |
| BPA | burst promoting activity |
| CCCP | carbonylcyanide-m-chlorophenyl hydrazone |
| CFU-E | colony forming unit-erythroid |
| CFU-GM | colony forming unit-granulocyte, monocyte |
| CFU-L-M | colony forming unit-lymphoid-myeloid |
| CFU-M | colony forming unit-megakaryocyte |
| CFU-S | colony forming unit-spleen |
| CTP | cytosine triphosphate |
| dglc | 2-deoxyglucose |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |

| | |
|---|---|
| DPG | diphosphoglycerate |
| 1,3-DPG | 1,3 diphosphoglycerate |
| 1,3-D$^{32}$PG | 1,3 diphosphoglycerate (with $^{32}$P) |
| DTNB | 5,5-dithiobis-2-nitrobenzoic acid |
| EDTA | ethylenediaminetetracetic acid |
| GAP | glyceraldehyde-3-phosphate |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| Hb | hemoglobin |
| HER | hematopoiesis engendered randomly |
| HIM | hemopoietic inductive microenvironment |
| HK | high potassium |
| IgG | immunoglobulin class G |
| IOV | inside-out vesicle(s) |
| $K_m$ | Michaelis-Menten constant |
| LDH | lactate dehydrogenase |
| LK | low potassium |
| MCHC | mean cell hemoglobin concentration |
| MOPS | 3-(N-morpholinopropanesulfonic acid) |
| $Na_2ATP$ | disodium adenosine triphosphate |
| $Na^+$-ATPase | sodium stimulated adenosine triphosphatase |

| | |
|---|---|
| NAD | nicotinamide adenine dinucleotide (oxidized form) |
| NADH | nicotinamide adenine dinucleotide (reduced form) |
| NBTI | nitrobenzylthioinosine |
| N/C | nucleus to cytoplasm ratio |
| PAGE | polyacrylamide gel electrophoresis |
| 3-PGA | 3-phosphoglycerate |
| PGK | phosphoglycerate kinase |
| $P_i$ | inorganic phosphate |
| RNA | ribonucleic acid |
| ROV | right side-out vesicle(s) |
| SDS | sodium dodecyl sulphate |
| S.E.M. | standard error of the mean |
| SITS | 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate |
| TCA | tricarboxylic acid |
| Tris | tris(hydroxymethyl)aminomethane |
| UMP | uridine monophosphate |
| UTP | uridine triphosphate |
| $V_{max}$ | maximum initial velocity |
| x g | times the force of gravity |

# 1. INTRODUCTION

## 1.1 Morphological Changes in the Erythroid Cell During Differentiation and Maturation

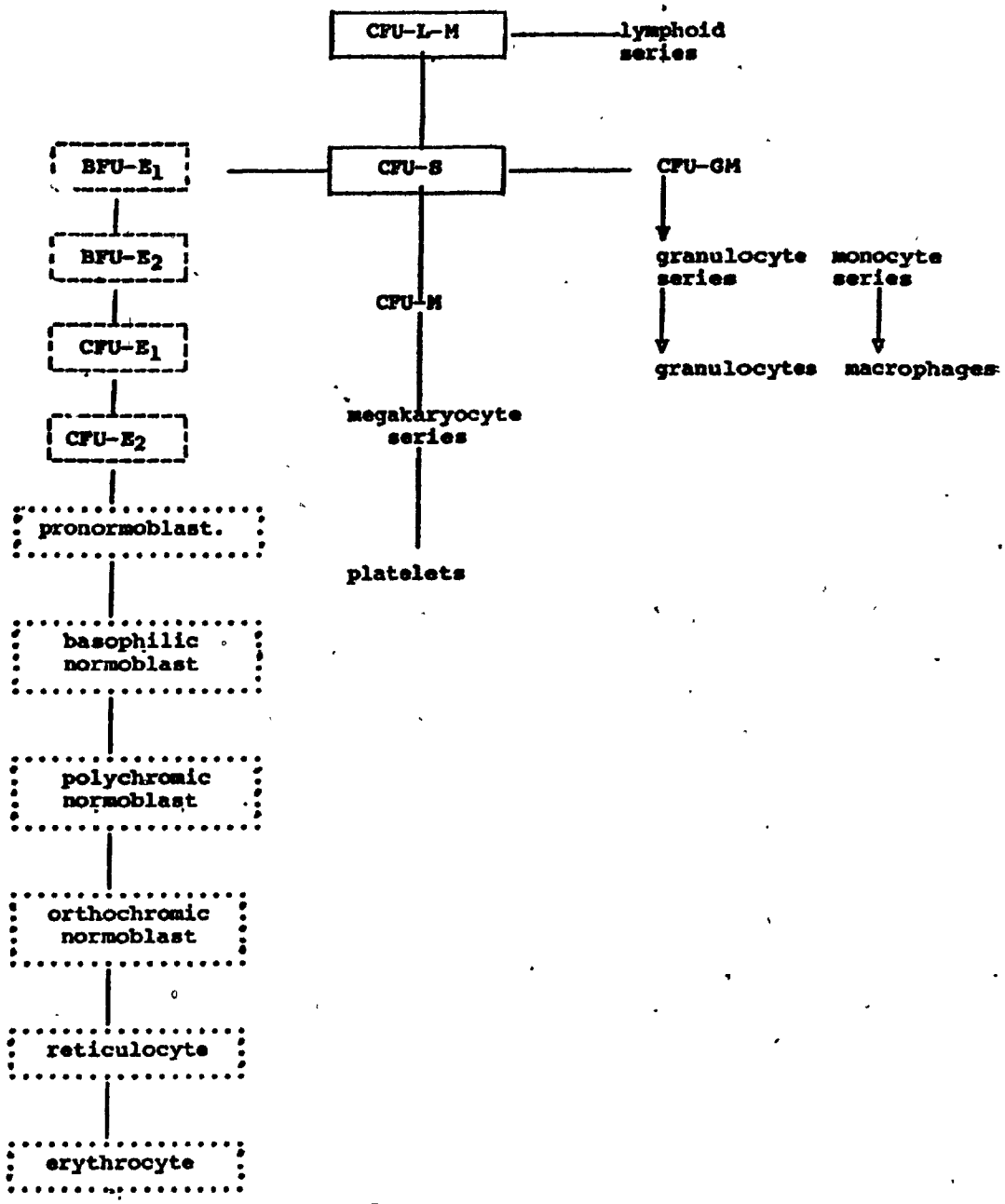
Hematopoiesis is the process of formation and development of all blood cells. Erythropoiesis refers exclusively to the production of erythrocytes (1). In the mammalian embryo erythropoiesis takes place in the yolk sac. During the middle trimester of gestation the main erythropoietic organ is the liver, although a significant quantity of erythrocytes is also produced by the spleen and lymph nodes (2). During the fourth and fifth months of gestation the bone marrow's erythropoietic activity commences and it later becomes the major erythrocyte producer. In man the relative rate of red blood cell production in various bones changes with age (2). In essence, all bones produce red blood cells until the age of 5. By the age of 20 the only bones producing erythrocytes are the vertebra, sternum, ribs and pelvis.

There have been several recent reviews on erythropoiesis (3-9). Most research concerned with elucidating the individual steps and factors controlling erythropoiesis in mammals has been carried out using the mouse as the experimental model. Figure 1 which has been modified slightly from the review by Till and McCulloch (3) depicts a model that summarizes current concepts of hematopoiesis.

**Figure 1**

**Model of Hematopoiesis.**

Rectangles (solid and dashed lines) represent the stem cells. The solid-line rectangles are pluripotent, while the dashed lined rectangles are the unipotent erythroid stem cells. The rectangles (dotted lines) represent the hemoglobin containing cells. For details of erythropoiesis see text. Modified from Till and McCulloch (4).


CFU-L-M
lymphoid series
BFU-E1
CFU-S
CFU-GM
BFU-E2
granulocyte series
monocyte series
CFU-M
CFU-E1
granulocytes
macrophages
megakaryocyte series
CFU-E2
pronormoblast.
platelets
basophilic normoblast
polychromic normoblast
orthochromic normoblast
reticulocyte
erythrocyte

Accordingly, there are two basic types of cells involved in erythropoiesis: (1) those stem cells which are capable of both self renewal and differentiation into erythroid precursors, and (2) the hemoglobin containing cells which are capable of maturation and aging.

The stem cells can be divided into two groups namely, (1) the pluripotent or uncommitted stem cells, and (2) the unipotent or committed erythroid stem (precursor) cells. The former can be further subdivided into two types, the first one being the colony forming unit-lymphoid-myeloid (CFU-L-M), which is theoretically capable of repopulating all the body's myeloid and lymphoid cell lines. The myeloid lineage includes erythrocytes, platelets and leucocytes. The second type is called the colony forming unit-spleen (CFU-S), which is the pluripotent myeloid stem cell. The CFU-S is defined by its ability to form nodules in the spleen of a heavily irradiated mouse injected with bone marrow cells (containing CFU-S) from a syngeneic mouse (10). These nodules, which form 8 to 10 days after injection, are called spleen colonies consisting of erythroid, granulocytic, megakaryocytic and undifferentiated cells as pure populations or in varying mixtures (11-13). The cells that form these colonies are called CFU-S.

The latter group of stem cells are subdivided into two classes of committed erythropoid stem cells, (1) the burst forming unit-erythroid (BFU-E), and (2) the colony forming unit-erythroid (CFU-E). The BFU-E is an early (immature) erythroid progenitor characterized by developing in culture into bursts or multilobulated aggregates containing many cells; the CFU-E is a mature

erythrocyte progenitor which in culture forms small colonies. Each of the two types of committed erythropoietic stem cells has been further divided into immature and mature BFU-E and CFU-E respectively (3,14).

An underlying question in hematopoiesis concerns the differentiation of stem cells along certain specific hematological lineages. There are two stages of cell differentiaton, the first being 'commitment' or 'determination'; during this phase the pluripotent stem cells lose their independence or pluripotentiality and are committed to a specific cell lineage (16). The actual progress along a certain pathway (i.e. erythroid) and the expression of specific markers (i.e. hemoglobin) constitute the second stage, called 'maturation'. The term differentiation refers to both steps, 'commitment' and 'maturation'. Stem cells are morphologically indistinguishable from each other and as mentioned above are classified according to the progeny they produce. The progeny examined are several generations removed from the original precursor. There are two hypotheses concerning the commitment of the stem cell to a particular cell line. The first, called the Hematopoiesis Inductive Microenvironment Model or HIM model (15) assumes that the variation in cellular composition of the resulting in vivo spleen colonies is a direct result of the environment surrounding the progenitor cell. Thus, for example, in experiments with CFU-S, 80% of the colonies on the surface of the spleen are either erythroid or of mixed composition, while most of the megakaryocytic colonies grow beneath the spleen capsule (17,18).

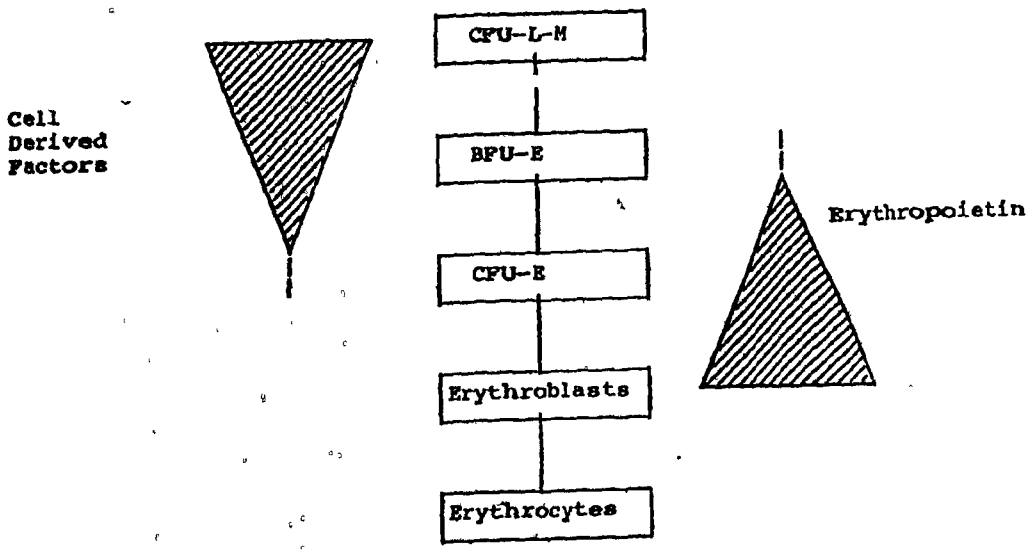
The second model is the Hematopoiesis Engendered Randomly, or HER model (19), in which the cellular composition of the colonies is stochastically determined (20) and only the probabilities of the events can be influenced by environmental factors. The HIM model was developed from histological studies of the composition of spleen colonies (15) which revealed the colony to colony variation. The HER model was also based on the analysis of distributions of various cell classes among spleen colonies; however, developmental assays were used to determine progenitor cell classes (19). Since cell determination (commitment) is an early event in differentiation, and it is the examination of cell types in colonies after several generations which reveals the basic differences in these two models, it is therefore imperative to investigate the original stem cells or their immediate progeny in order to determine the correct model (3).

The exact nature of the determinants of stem cell regulation is not unequivocally known. In a scheme (Figure 2) which is taken from the review by Till and McCulloch (3) two factors which control in vitro differentiation and maturation are depicted. The cell-derived factor which is present in the added serum acts on the less differentiated cells (pluripotent or committed stem cells), with decreasing influence as the cells become more differentiated (CFU-L-M to CFU-E). The active material in this factor is called the burst promoting activity (BPA). According to Iscove's (21) hypothesis BPA acts at the pluripotent stem cell level by allowing self-renewal of this cell population until sensitivity to a specific factor occurs. This factor allows the

## Figure 2

### Model of the Regulation of Erythropoiesis

In culture, erythropoiesis involves two aspects; 1) a decreasing responsiveness of cells to cell-derived factors and 2) an increasing responsiveness to erythropoietin during transition from early to late stages of differentiation (3).


Cell
Derived
Factors
CFU-L-M
BFU-E
CFU-E
Erythroblasts
Erythrocytes
Erythropoietin

stem cell to become committed. The specific erythroid factor is erythropoietin, a glycoprotein synthesized mainly in the kidney; erythropoietin acts on the erythroid-responsive compartment which mainly consists of the BFU-E and CFU-E (7-9) and allows for the induction of hemoglobin synthesis. In addition, but of secondary importance are a number of other factors such as proteases (22), phospholipids (23), and androgens, growth hormone, thyroid hormone, dexamethasone, adrenergic agonists, and prostaglandin $E_2$ (24), which appear to promote the proliferation of erythrocyte-producing cells.

The second major class of cells in the red blood cell lineage is the hemoglobin containing cells (Figure 1); this class is divided into six morphologically distinguishable cell types (25). These six subclasses, in order of their increasing maturity, are: pronormoblasts, basophilic normoblasts, polychromic normoblasts, orthochromic normoblasts, reticulocytes, and mature erythrocytes. Cell size, nuclear maturation and cytoplasmic differentiation are the three criteria used to differentiate one cell type from another during red blood cell maturation. The distinguishing characteristics of each of the cell types are seen using the basophilic and eosinophilic dye, Wright's stain (25). Using electron micrographs (26) Heynen and Verwilghen observed that many of the cytoplasmic organelles are either lost or dramatically reduced in number during maturation. In this study Heynen and Verwilghen (26) illustrated that the only difference between the early and intermediate erythroblasts as classified by Rose and Trotter (27) is a decrease in the surface area of the

rough endoplasmic reticulum. In addition, the late erythroblasts which appear after the last mitotic division reveal reductions in rough endoplasmic reticulum, mitochondria, golgi apparatus, and in the number of ribosomes. At the reticulocyte level the number of mitochondria and ribosomes are further reduced.

Thus, the pronormoblast is 14 to 19 microns in diameter. It contains a round or slightly oval, central or slightly eccentric nucleus with one or two very faintly stained nucleoli surrounded by fine reticular chromatin and sparse indistinct parachromatin. The homogeneous basophilic (deep blue) cytoplasm is scanty and opaque with a nucleus to cytoplasm (N/C) ratio of 8. The basophilic normoblast is 10 to 15 microns in diamater with a round eccentric nucleus containing one or no nucleoli and coarse, dark staining chromatin with sparse but distinct parachromatin. The opaque cytoplasm is more abundant (N/C = 6) than the pronormoblast but not as deeply basophilic. The polychromic normoblast is 8 to 12 microns in diamater with a round eccentric nucleus which lacks a nucleolus and contains coarse, dark staining chromatin and distinct parachromatin. For the first time the cytoplasm (N/C = 4) contains recognizable pink or orange hemoglobin patches. The orthochromic normoblast is 7 to 10 microns in diameter with a small shrunken nucleus which may be round or have a bizarre shape and no parachromatin. The cytoplasm (N/C = 0.5) has characteristics of a mature erythrocyte being orange to red in colour. The reticulocyte, which under normal conditions is just slightly larger than the mature erythrocyte (7 microns) may be up to twice as large (28) during severe anemic stress. The distin-

guishing feature of the reticulocyte which lacks a nucleus, is the blue staining reticulum revealed after staining with new methylene blue dye (25). Reticulocytes can be classified according to the Heilmeyer criteria (29), which separates the reticulocytes into 4 groups (I - IV) with the vast majority (approximately 90%) in groups III and IV. In group I, the reticulum appears to be in a dense clump; in group II it then appears as a wreath. The distinguishing feature of group III is that the wreath has disintegrated, while group IV features only a few scattered granules. The mature erythrocyte has a homogeneous orange-red acidophilic cytoplasm and no blue staining reticulum.

## 1.2 Structural and Functional Changes in the Erythroid Cell During Maturation and Aging

As the committed erythroid stem cells (BFU-E, CFU-E) produce their first morphologically distinguishable red blood cells, they are synthesizing and organizing specific cellular components. These will enable the mature erythrocyte to fulfill its specific functions, the main one being the transport of oxygen to and carbon dioxide away from the tissues in the body. Thus 70% to 90% of the cell's protein synthesis is comprised of globin (30). In the mature cell, hemoglobin comprises over 90% of the cell's cytoplasmic protein and the predominant membrane functional component is the anion transport system (31).

The red blood cell, from proerythroblast to senescent erythrocyte, undergoes complex and enigmatic changes until its death (120 days in humans and 62 to 150 days in sheep (32)). Under normal conditions, 2 to 5 days are required to progress from the pronormoblast stage to the last normoblast division in the bone marrow (33). The reticulocyte spends about 36 to 44 hours in the bone marrow and approximately one day in circulation (34). Studies concerning the gradual functional and structural changes which the pronormoblast undergoes during its transition to a mature erythrocyte have been focused primarily on the reticulocyte-to-mature erythrocyte stage. Maturation and aging of the red blood cell can be arbitrarily divided into two separate entities, namely, (1) cytosolic or internal aging, and (2) the (cell) membrane or external aging (35). It is important to bear in mind that the latter process depends on the former (35). Cytosolic aging encompasses changes, usually losses, in the following components and cellular functions: (1) nucleic acid, purine and pyrimidine metabolism, (2) enzymes associated with both aerobic and anaerobic metabolism, (3) enzymes associated with protein and lipid metabolism, and (4) other cytoplasmic components. Membrane aging involves changes in the structural (carbohydrate, lipid and protein) and functional (receptors and transport systems) components.

### 1.2.A Changes in Cytoplasmic Components

Among the most extensively studied cells in animals with respect to the differentiation and maturation process is the erythrocyte since it offers unique advantages for studying many aspects of mammalian cell structure, function, and aging. This is especially true

for studying membrane function, since red blood cells are easily obtainable as a single cell suspension, and membranes derived from the cells, even at the reticulocyte stage, have few interferring organelles. In addition, membranes which are either resealed right side-out ghosts or inside-out (IOV),or right side-out vesicles (ROV) can be prepared from erythrocytes (36); the ionic composition of both the intact cell (37) as well as these cytoplasmic-free vesicle preparations can be readily altered.

Maturation and aging of the erythroctye include changes and losses in many cellular components and metabolic functions as summarized below.

Nucleic Acid Metabolism

The loss of DNA synthesis signifies the beginning of the reticulocyte stage according to Rapoport and co-workers (38). In 1962 Marks, Burka and Schlessinger (39) showed that reticulocytes are unable to synthesize RNA *in vitro*; in the following year, using radioautography, Pinheiro, Leblond and Droz (40) showed that synthesis of RNA ceases after the nucleus is extruded from the rat erythrocyte. The RNA content of the reticulocyte varies considerably, presumably dependent on the relative age of the cell. Thus, the reticulocyte RNA content may be as much as 35-fold higher than the RNA content in mature red blood cells (38,41,42).

Since DNA and RNA synthesis ceases at the polychromic and orthochromic normoblast stages respectively (43), the metabolism of nucleic acids is comprised only of catabolism by nucleases which are

located in bone marrow cells and reticulocytes (38). Moreover, the DNase is active in rabbit reticulocytes and has an optimal pH of 5.0, but is undetected in mature cells (44). Two clearly defined ribonucleases have been described in rabbit reticulocytes (38,45-47). One is a pyrimidine-specific 25 kilodalton endonuclease with an optimal pH of 7.5. This endonuclease has some properties in common with type I pancreatic ribonuclease (38). The other ribonuclease, which has a pH optimum of 6.5, is a base non-specific phosphodiesterase which produces 3'-nucleotides. Both of these nucleases show a pronounced decrease during maturation (38,44).

Pyrimidines are synthesized from aspartic acid and carbamoyl-phosphate through a series of enzymatic steps resulting in the production of UMP. UMP is converted to UTP and CTP through the action of kinases and CTP synthetase, respectively. Although Marks, Johnson and Hirschberg (48) showed that erythrocytes have an overall reduced capacity for pyrimidine nucleotide generation, at least two enzymes, (aspartate carbamoyltransferase, E.C.2.1.3.2. and dihydro-orotase, E.C.3.5.2.3), which initiate reactions in the sequence of steps involved in pyrimidine biosynthesis, are still active in mature human as well as duck erythrocytes (44).

The loss of purine generation is also associated with the loss of at least one enzyme involved in purine biosynthesis, phosphoribosyl pyrophosphate amido transferase (E.C.2.4.2.14), in mature erythrocytes of both man and mouse (44). In mature human red blood cells, purine nucleoside phosphorylase which cleaves

inosine has 7 isozymes but only 4 or 5 in reticulocytes (48). In addition the total activity of this enzyme is increased in the younger cells. The catabolism of purine and pyrimidine nucleotides involves dephosphorylation, deamination and cleavage of glycosidic bonds (49). One enzyme, adenosine deaminase (E.C.3.5.4.4), shows a two-fold increase in activity in rabbit reticulocytes (44).

Aerobic and Anaerobic Metabolism

Maturation of reticulocytes to mature red blood cells is associated with the disappearance of the mitochondria (50) and a marked decrease or loss of most of the enzyme activities associated with the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation pathway (51,52). Thus, the cell undergoes a marked change in energy metabolism, namely from producing ATP via the TCA cycle and oxidative phosphorylation, to glycolytic metabolism, with most of ATP production occuring via the Embden-Myerhof pathway in the mature cell.

During maturation the activity of many of the enzymes (38,52-60) in the glycolytic pathway also decreases substantially. In human red blood cells, for example, pyruvate kinase activity decreases about 3-to 4-fold (44); in the rabbit red cell, phosphofructokinase activity shows a 5-fold decrease (44). Besides a loss in activity, some enzymes which exhibit multiple molecular forms (38,61,62) tend to have a predominance of one or more forms at different stages of maturation. In man, for example, lactate dehydrogenase (LDH) has

five molecular forms (62), of which LDH isozyme 5 is found exclusively in young cells, while isozyme 1 is increased in relative proportion in older cells.

## Protein and Lipid Metabolism

Protein synthesis, which is largely confined to hemoglobin production (30,63), is markedly reduced at the reticulocyte stage and further declines with the age of the cell (64-66). At this stage of maturation protein catabolism is readily apparent. There are many different types of proteases which have been identified and isolated from the red blood cell, including those which differ in pH optimum (62-70), those which are soluble or membrane bound (71), as well as an ATP-dependent proteolytic system (72-74). Many of these proteases decline in activity with increasing cell age (75-78). According to McKay, Daniels and Hipkiss (75) the younger cells have 5 to 10 times the proteolytic activity of the most mature cells. The factor(s) determining which proteins are susceptible to degradation is at present under investigation.

In contrast to other cellular constituents, the metabolism of lipids in red blood cells has attracted less attention. It is known that reticulocytes and young erythrocytes from man are able to synthesize lipids, while mature red blood cells lack this capacity (79). Comparing rabbit reticulocytes and erythrocytes, the ratio of $^{14}C$-glycerol incorporation into glycerides and glycerophosphatides is approximately 70 to 1 (44). Catabolism of lipids by phospholipases in erythrocytes has attracted relatively little attention (38). In man,

lysophospholipase (E.C. 3.1.1.5) exhibits about 1.5 times the activity in reticulocytes compared to mature cells (44).

Other Cytoplasmic Components

In addition to the decline in the activity of enzymes which control the metabolism of nucleic acids, proteins, lipids and carbohydrates, several other enzymes and cellular components decrease during red blood cell maturation (for review see reference 38, 44.). Examples of other enzyme activities which decline during maturation are; protein kinases which are either cyclic nucleotide-independent or dependent (80-83) and superoxide dismutase, which is believed to protect the cell from oxidation (84,85). Many cellular constituents and metabolites also decrease markedly during maturation, e.g. ATP (44), amino acids (86) and creatine (87).

## 1.2.B Changes in Membrane Composition

### Lipids

Current concepts of mammalian cell membrane structure have developed from studies of the red blood cell. Over fifty years ago Gorter and Grendel (88) obtained evidence indicating that the amount of lipid extracted from red blood cells was twice as much as needed for a membrane lipid monolayer. This finding led to the lipid bilayer hypothesis, which was formulated ten years later by Danielli and Davson (89). The important feature of their lipid protein bilayer hypothesis was that membranes are composed of a lipid

bilayer to which globular proteins were adsorbed at both surfaces. In the following years Robertson adapted the Davson-Danielli concept to the unit-membrane hypothesis (90) which envisages a membrane comprised of a bilayer of mixed polar lipids with their hydrocarbon chains oriented inward and hydrophilic head groups oriented outward. The lipid bilayer was thought to be covered by a monolayer of protein on each side. In 1972 Singer and Nicholson (91) updated the unit-membrane model to the fluid mosaic membrane model. Briefly, according to this model the membrane consists of phospholipids arranged in a bilayer to form a liquid-crystalline core. This arrangement allows the lipids to move laterally and confers fluidity and flexibility to the bilayer. The extent to which protein molecules are embedded in the bilayer depends on the amino acid sequence and the location of non-polar amino acids side chains. This mosaic is not static, as the proteins are able to move laterally in two dimensions.

From their studies with human red blood cells, Dodge, Mitchell and Hanahan showed that lipids comprise 35 to 45 percent of the dry weight of the red blood cell membrane (92). The distribution of the individual phospholipids and cholesterol in the lipid bilayer is asymmetrical (93-97). The internal lipid monolayer contains amino lipids and unsaturated fatty acids, while the external monolayer is comprised of cholesterol, glycolipids, sphingolipids, choline phospholipids, and saturated fatty acids with longer carbon chain lengths.

In his comparative studies of young and old red blood cells carried out years ago, Prankerd (98)

showed that young human red blood cell membranes have more phospholipid and unesterified cholesterol than old red blood cells. This observation was confirmed by other investigators (99-104), but the decrease in phospholipid was paralleled by the drop in cholesterol so that the ratio of phospholipid to cholesteral remained constant (101). Some investigators who studied the individual phospholipids obtained conflicting results (101,103,104), some finding increases, others, decreases or no significant differences in the phospholipids between young and old cells. One group (105) examined the fatty acid content of all the phospholipids, and found that 4 out of 5 fatty acids with carbon chain lengths of 18 or less increased with cell age, while all 19 fatty acids with chain lengths of 20 or more decreased with cell age.

In recent studies, membrane fluidity changes in cell aging have been examined using fluorescent probes (106) and electron spin labels (107,108). The results have revealed a decrease in membrane lipid fluidity with increasing cell age. Factors which had been thought to be responsible for changes in membrane fluidity include: (i) cholesterol/phospholipid ratio, (ii) degree of unsaturation and length of fatty acid chains, (iii) lecithin/sphingolipid ratio, (iv) lipid/protein ratio, and (v) peroxidation of membrane phospholipids (107). The first three factors show little or no change (98-105) during erythrocyte aging. Normally, the lipid to protein ratio varies very little (slight decrease) during erythrocyte aging (109), although under conditions of severe anemia where stress reticulocytes are produced there is quite extensive remodelling with a

concomitant decline in the lipid to protein ratio (109). Bartosz (107) also found a decrease in lipid-to-protein ratio in bovine erythrocytes from a non-anemic animal; he suggests this may be due to the loss of protein poor vesicles during aging (101,110).

As cells age, their antioxidant capacity decreases (84,85), which leads to peroxidation of the lipids in the membrane. This peroxidation of the membrane lipids may lead to polymerization of membrane components, and thus a change in membrane fluidity is observed (111, 112).

Membrane Proteins and Glycoproteins

Protein accounts for about one-half of the dry weight of the membrane (113). During the past decade, several excellent reviews have dealt with the cytoskeleton and functional proteins of the red cell membrane (113-118). Briefly, the red blood cell membrane contains at least eight major Coomassie blue-stained polypeptides (see references 113-118) as evidenced from studies of ghost membrane proteins separated on sodium dodecyl sulfate (SDS) polyacrylamide gels using the procedure and nomenclature of Fairbanks and co-workers (119). Bands 1 and 2, which have molecular weights of about 240 kilodaltons, are known as spectrin and are part of the membrane skeleton (113). Band 3, the anion channel, is the most abundant protein in the membrane and has a molecular weight of 95 kilodaltons. Band 4.1 has a molecule weight of 80 kilodaltons and is also part of the membrane skeleton, while Band 4.2 (72 kilodaltons) function is unknown. Band 5, which is also

part of the membrane skeleton, has a molecular weight of 43 kilodaltons and is known as actin. Band 6 (molecular weight, 35 kilodaltons) is the enzyme glyceraldehyde-3-phosphate-dehydrogenase, and the eighth major polypeptide chain is Band 7, which has a molecular weight of 29 kilodaltons and whose function is unknown. In addition to these major polypeptides, there are several major carbohydrate-containing proteins which stain poorly with Coomassie blue. These polypeptides, the glycophorins, are visualized using a periodic acid-Schiff reagent (115).

A central question concerns the mechanism(s) underlying membrane protein changes which occur during the process of cell maturation and aging. A change in membrane protein may refer not only to peptide anabolism and catabolism, but also to any changes which occur in prosthetic groups e.g. carbohydrate side chains. In general changes in membrane protein composition can occur in several ways, namely: (i) membrane remodelling via endocytosis/exocytosis, (ii) protein synthesis, (iii) degradation of existing protein, and (iv) post-translational modification of existing protein.

The vast majority of studies (120-127, except reference 120) concerning membrane protein changes during maturation and aging has provided evidence for some alterations in membrane composition. In a series of five papers, the Singer group has investigated changes in membrane composition, in particular, changes in protein concentration through remodelling (121-125). Their conclusions suggest that remodelling is accomplished through endocytosis, with the endocytotic

vesicle being deficient in spectrin. Wraith and Chesterton (126) labelled the membrane proteins with radioiodine and compared them at different erythrocyte stages. They found that the more mature reticulocyte (i.e. normal marrow reticulocyte or circulating anemic reticulocyte) which resembled the mature erythrocyte had lower levels of erythroblast-specific proteins (molecular weights 25, 29, 36 and 40 kilodaltons) than the anemic marrow reticulocyte.

Although hemoglobin is the major protein synthesized by reticulocytes (30), both Lodish (127,128) and Koch et al (129) demonstrated that rabbit reticulocytes are capable of synthesizing proteins other than hemoglobin. The two major membrane proteins which were synthesized had molecular weights of 33 and 53 to 60 kilodaltons.

Many groups of investigators have studied the chronological order of appearance of membrane proteins during red cell differentiation and maturation. The concensus is that membrane proteins are synthesized asynchronously and that high molecular weight proteins are predominated synthesized earlier (129). Thus, in 1976 Chang et al (130) found that when a six week old mouse was given a single injection of $^{35}S$-methionine, the label appeared in spectrin and actin before Band 3, and Band 3 was synthesized before Bands 4.1 and 4.2. Tong and Goldwasser (131), using SDS-polyacrylamide gel electrophoresis (PAGE) showed that when bone marrow cells from a polycythemic rat were induced to differentiate with erythropoietin, the time at which synthesis commenced for glycophorin, Band 3 and hemoglobin

occurred after 0, 18, and 24 hours, respectively. The rate of maximal synthesis for these three proteins commenced after 30, 66, and 96 hours respectively. The sequence of appearance of these proteins was partially corroborated by Fukuda (132). Using an antibody to the anion transport protein and the staphylococcus aureus 'rosette' technique (133), Foxwell and Tanner (134) showed that most of the anion transporter of the rabbit bone marrow cell is inserted into the erythrocyte membrane between the polychromatic normoblast and reticulocyte stages. Koch et al (135) showed that 8 hours after injecting phenylhydrazine-treated rabbits with $^{3}H$-leucine, Bands 4, 5, and 9 were labelled, while Band A, a high molecular weight protein and Bands 1, 2, and 3 had very little incorporated radioactivity. However, 20 hours after the injection all protein bands on a SDS-PAGE were labelled. The results of the preceding experiments suggest that Band 3 is synthesized early in erythrocyte development (130-132,135) and is inserted gradually into the membrane (134).

Keyhani and Maigne (136) followed the synthesis of the membrane protein, acetylcholinesterase, by a cytochemical localization method. They showed that synthesis started at the basophilic normoblast stage, and continued in the polychromic normoblast stage, where it was present in the endoplasmic reticuluum, golgi apparatus and nuclear membrane. Synthesis of the protein ceased at the orthochromic normoblast stage, as deduced by the fact that the enzyme was only located in the golgi apparatus.

During the course of maturation from reticulocyte to mature red blood cell several membrane proteins are lost, or dramatically reduced, as seen by SDS-PAGE (137). The course of events which leads to the loss of these proteins is unclear; however, this loss may be due to remodelling or proteolysis (138).

Many groups (103,127,129,135,139-143) have investigated membrane protein composition changes during red blood cell maturation using polyacrylamide gel electrophoresis. Most of these studies were concerned with the reticulocyte-to-mature red blood cell maturation stage. Although numerous alterations in the membrane protein profile have been elucidated, two particular protein bands (molecular weights of 33 and 53 to 60 kilodaltons) have been observed by several groups of investigators (103,127,135). Three groups of researchers (111,112,143,144) have examined the effect of inducing peroxidation of membrane proteins and thereby trying to mimic the in vivo peroxide exposure of the cells. The results of their work reveal that there is an increase in high molecular weight protein polymers and a loss in Bands 1 and 2 of spectrin.

Danon and Marikovsky (145) reported a reduced surface charge which is related to the sialic acid content of the membrane in old red blood cells compared to young cells. Although this was corroborated by Yaari (146), the issue has remained controversial. Recently, a collaborative study carried out by Luner, Szklarek, Knox, Seaman, Josefowicz, and Ware (147), using different techniques, failed to show a difference in electrophoretic mobility of red blood cells of varying ages

(for review see 148). The reason for these discrepancies is that the earlier groups failed to remove all the contaminating leucocytes which contain large amounts of sialidases (120). Other groups of investigators (149-151) have shown decreases in surface carbohydrates, other than sialic acid, with increasing red blood cell age.

### 1.2.C Changes in Some Functional Components of the Red Blood Cell Membrane

Apart from the structural changes described in the previous sections, which appear to confer certain qualitative changes in viscosity, shape, and deformability to the red blood cell membrane, a number of membrane functions is either reduced or disappears during maturation of the reticulocyte. The mechanism(s) underlying these changes is (are) unknown.

In the following sections changes in membrane function will be discussed in terms of changes associated with cell maturation as well as with ontogeny. The functional components to be discussed are divided into two categories: (i) plasma membrane receptors and (ii) membrane transport systems.

#### 1.2.C.1. Plasma Membrane Receptors

The loss of several membrane receptors, including insulin, transferrin, concanavalin A, and beta-adrenergic receptors has been reported by several groups of investigators.

## Insulin Receptors

Thomopoulos et al (152) first demonstrated that the number of specific insulin binding sites, which represented 85% of the total binding, is about ten-fold higher in phenylhydrazine induced rabbit reticulocytes (1800 sites/cell) compared to mature red blood cells. Other investigators have corroborated the fact that the number of specific insulin sites (153-157) increases with increasing reticulocyte count. Also relevant is the finding of Ginsberg and co-workers (158), who showed that when the Friend erythroleukemic cell, a model for the basophilic normoblast, was induced to differentiate into a cell resembling the orthochromic normoblast, there was a decrease in the number of insulin receptors on erythrocytes, from 17,200 to 4,300 sites per cell. Other groups of researchers (159-162) investigating the number of insulin receptors as a function of the age of the experimental animal (rat, sheep, human), have shown a net decrease during the first months of life.

## Transferrin Receptors

It has been known for some time that the uptake of transferrin bound iron requires the transferrin receptor and that this uptake occurs in immature but not mature red blood cells. Thus, Hemmaplardh and Morgan (163) showed that the uptake of transferrin occurs by endocytosis, and that this phenomenon takes place only in rabbit bone marrow cells and reticulocytes, but not in mature red blood cells. Similarly, several investigators (164-172) have observed a loss of the transferrin receptor during reticulocyte maturation. For example,

Frazier, Caskey, Yoffe, and Seligman (171) have shown by an immunoassay technique that the number of transferrin receptors per cell decreases from approximately 400,000 to 46,000 in dextran gradient separated erythrocytes from a patient with autoimmune hemolytic anemia.

## Concanavalin A Receptors

Even though receptors for Concanavalin A have no known function, they have been used by the Singer group (121-123) to investigate membrane changes which occur during reticulocyte maturation. These researchers showed that the number of receptors for Concanavalin A and their mobility decreases during reticulocyte maturation (123). This phenomenon of reduced Concanavalin A receptors is also seen when neonatal and adult human mature red blood cells are compared (125).

## Beta-adrenergic Receptors

The beta-adrenergic receptor/adenylate cyclase system, is comprised of at least three proteins, the beta-adrenergic receptor, the adenylate cyclase catalytic protein, and a guanine nucleotide binding regulatory protein (173). This system decreases during reticulocyte maturation (173). The relative extent of the maturation-associated decrease differs, however, for the different components. For example, during maturation adenylate cyclase activity may decrease by 25-fold (174), but there is only a 2- to 3-fold decrease in the number of beta-adrenergic receptors (174-176). Bilezikan (174) suggests that the beta-adrenergic receptor becomes uncoupled from the adenylate cyclase protein.

### 1.2.C.2 Plasma Membrane Transport Systems

The following is a brief review of maturation-associated and ontogenic-associated transport changes, with special emphasis on the two systems investigated in the present study, namely, $Na^+$-dependent glycine transport and the $Na^+,K^+$-pump. Other transport systems which have been investigated include anion transport, nucleoside transport, sugar transport, transport of a variety of amino acids, and cation transport.

#### Anion Transport

Since Band 3, the anion transport protein, is the major red blood cell membrane protein (31), some researchers have attempted to elucidate possible changes in this protein during differentiation and maturation. Law, Steinfeld, and Knauf (177) attempted to use the human K562 erythroleukemic cell line as a model for studying maturation-associated changes in the anion transport system. Their results suggest, however, that the K562 cells have a different anion transport system from that present in red blood cells and that there are few, if any, functioning Band 3 monomers on the K562 cell membrane.

Weise and Hoffman (178) studied the anion transport system in chicken red blood cells. They showed that the activity is higher in embryonic compared to adult cells, but only when the anion studied was sulfate. However, in most cases, when comparing one species to another, the change in monovalent and divalent anion transport occur at the same rate (179-

181). According to Weise and Hoffman the difference in chloride and sulfate transport may be due to differences in their carbonic anhydrase activity, since this enzyme, under certain conditions, appears to influence anion movements (182).

Nucleoside Transport

Maturation-associated changes in red blood cell nucleoside transport have been studied primarily to ascertain the role nucleoside transport plays in determining the intracellular ATP level. Jarvis and Young (183) showed that in sheep there is a genetic dimorphism with respect to nucleoside transport. It appears that there are basically two mechanisms of nucleoside permeation in sheep. A high affinity ($K_m$ = 0.2 mM) facilitated diffusion system, which transports both purine and pyrimidine nucleosides, is present in only 5% of all sheep (184). A second transport system is a non-saturable selective route for adenosine uptake (184). Thus, all sheep red blood cells are permeable to 5 mM adenosine, while only 5% of sheep have erythrocytes permeable to 5 mM inosine (184). However, reticulocytes of both types of sheep are permeable to both inosine and adenosine. Jarvis and Young (185), and Tucker and Young (186) have shown a loss in the high affinity nucleoside transport system ($^{14}$C-uridine) during in vitro culture of sheep reticulocytes. This loss in activity is not due to a change in kinetics of the transporter but to a decrease in the amount of the nucleoside transport protein (185). Thus, the number of sites per cell decreases from 2,500 in sheep reticulocytes to 20 in mature cells. In these studies the transport protein

was quantitated by measuring the number of specific nitrobenzylthioinosine (NBTI) binding sites, NBTI being a nucleoside analogue which specifically inhibits the uptake of the high affinity nucleoside transport system by binding to the transport protein. It is particularly interesting to note, however, that a kinetic change in nucleoside transport has been observed following cold storage of human blood. In this case, the nucleoside carrier changes from directional symmetry in freshly drawn blood to directonal asymmetry in outdated blood (187). Maturation-associated changes in nucleoside transport have also been observed by Gordon and Rubin (188), who showed the loss in uridine transport activity after Friend erythroleukemic cells were induced to differentiate.

Jarvis and Young (185) have also observed the loss of red cell nucleoside transport activity during ontogeny. This loss is attributed to cell replacement, while in the adult the loss in activity is due to maturation of the individual cells.

Sugar Transport

Using the non-metabolizable substrate, 3-O-methylglucose, Kim et al (189-191) showed that glucose permeability is several orders of magnitude greater in fetal erythrocytes of the pig compared to either reticulocytes or mature red blood cells of the adult animal. Reticulocytes, in turn, have greater glucose permeability than mature erythrocytes which are glucose impermeable. Kondo and Beulter (192), and Lee et al (193) corroborated Kim's finding of a maturation-

associated loss in glucose transport in guinea pig and dog erythrocytes, respectively. In addition, Kondo and Beutler (192), using SDS polyacrylamide gel electrophoresis, showed that the loss of glucose transport activity closely paralleled the loss of Band 4.5. They concluded the glucose transport protein was probably Band 4.5, a finding which other investigators (194,195) have recently substantiated. In the foregoing studies the glucose transporter was identified as Band 4.5 using an antibody (195) or radiolabelled cytochalasin B (194,195), which binds specifically to the glucose transporter.

Amino Acid Transport

Amino acid transport has been studied in mammalian red blood cells for approximately thirty years. At present at least six different amino acid transport systems have been described in the human erythrocyte: (i) the L system, which transports L-valine, L-leucine, L-phenylalanine, and L-methionine (196, 197), (ii) the T transport system for the aromatic amino acids tyrosine, tryptophan, and phenylalanine (198), (iii) the Ly system for the dibasic amino acids lysine, arginine, and ornithine (199), (iv) the ASC system for small neutral amino acids such as alanine, serine, and cysteine (200), (v) the glycine transport system (201), and (vi) the anion transport protein which is capable of transporting some small neutral amino acids (202), such as glycine, serine, and cysteine. This activity is inhibited by the anion transport inhibitor, SITS (4-acetamido-4'-isothiocyanostilbene-2, 2'-disulphonate) which shows half-maximal inhibition at a concentration of 3.5 nM (202).

Some (203) but not all (204,205) amino acids are concentrated intracellularly. In addition, some amino acids which are concentrated intracellularly do not require sodium (206,208). Instead, they are concentrated by exchange diffusion (208). This ability to concentrate amino acids intracellularly occurs in the red blood cells of many species (204,206,209).

## $Na^+$-Dependent Glycine Transport

Ellory, Jones and Young (201) have further delineated five different transport systems for glycine in human erythrocytes. According to their studies the major component is the $Na^+$- and chloride-dependent glycine system. The other $Na^+$-dependent glycine transport system is the ASC system which is chloride independent. The three sodium independent glycine transport systems are: (i) the L amino acid transport system, (ii) the SITS-sensitive Band 3 amino acid transport system, and (iii) an uncharacterized component of sodium independent glycine uptake. At 0.2 mM glycine, 42% of glycine is transported by the $Na^+$- plus chloride-dependent glycine transport system, 11% by the ASC system, 15% by the L system, 16% by Band 3, and the remainder by the other sodium independent route (201).

$Na^+$-dependent glycine transport system has been extensively studied in red blood cells since 1952, when Christensen and co-workers (203) illustrated the sodium-dependency of glycine uptake in avian (duck) erythrocytes. They observed that glycine was not accumulated against its concentration gradient if the sodium in the medium was replaced by potassium. In 1964,

Vidaver, Romain, and Haurowitz (210) elucidated the specificity of the $Na^+$-dependent glycine transport system. Using avian (pigeon) erythrocytes, they observed that the only other amino acids capable of inhibiting $Na^+$-dependent glycine uptake were N-methyl and N-ethyl glycine. The kinetic effects of sodium were examined by Wheeler and Christensen (211) using rabbit reticulocytes. They showed that extracellular sodium affected (decreased) the $K_m$ for glycine uptake.

In 1964, Vidaver (212, 213) proposed that an electrical potential may also be important in allowing the avian red blood cell to accumulate glycine. He showed that a Donnan potential (inside positive), effected by replacing the chloride anion in the medium with mucate (a large impermeant anion), abolished any glycine accumulation. Gibb and Eddy (214), and Heinz, Geck and Pietrzyk (215) have since corroborated the importance of the electrical gradient in glycine accumulation in Ehrlich ascites tumor cells. Terry and Vidaver reported that although $Na^+$ stimulates the rate of glycine transport, an inward sodium <u>electrochemical</u> gradient is required for glycine accumulation (216, 217). This conclusion was based on an experiment in which gramicidin was used to equilibrate sodium and potassium across the cell membrane (218) and thus abolish any electrochemical cation gradients. This work by Terry and Vidaver confirmed that the energy source for glycine accumulation was the cation gradient, a finding which had been proposed by other investigators (219,220).

In addition to its requirement for a sodium electrochemical gradient, $Na^+$-dependent glycine transport may require an anion such as chloride, as observed by Ellory, Jones, and Young (221), using human red blood cells. Previously, Imler and Vidaver (222) demonstrated that pigeon erythrocytes require chloride for glycine transport. However, the chloride anion could be replaced by several other small anions ($F^-$, $NO_3^-$, $HCO_3^-$, $SCN^-$ or $I^-$) but not by large ones (214).

According to Vidaver (223, 224), each glycine molecule is transported across the red blood cell membrane with two sodium ions. This stoichiometric relationship was determined directly by measuring the co-transport of glycine and sodium as well as being inferred from kinetic studies of the $Na^+$-dependency of glycine transport. Thus, the activity with respect to the sodium concentration obeyed Michaelis-Menten kinetics when the data were analyzed as a function of the binding of two $Na^+$ to the Michaelis complex. According to other researchers, the coupling between sodium and solute can be either 1:1 (225-227) or variable (228, 229), depending on conditions and the amino acid tested. Paterson, Sepulveda, and Smith (229) suggest a fixed stoichiometry of 1:1 under all conditions; however, under some conditions the amino acid may be able to cross on the $Na^+$-dependent carrier in the absence of sodium.

Vidaver and Shepherd (230), and Johnstone (231) showed that the $Na^+$-dependent glycine transport system is asymmetric with respect to influx and efflux.

Using gramicidin to abolish any effects due to an electrochemical $Na^+$-gradient, Johnstone (231) found that for Ehrlich ascites cells the $K_m$ for glycine efflux is an order of magnitude larger than influx. Similarly, using pigeon erythrocytes, Vidaver and Shepherd (230) derived equations from their model for $Na^+$-dependent glycine transport, which showed that the $K_m$ for efflux is approximately five fold greater (sodium concentration = 126 mM) than for influx. Whether this apparent asymmetry is real or secondary to other factors related to differences in the medium composition at the two surfaces of the membrane, remains to be determined.

The amino acid transport system is one of the several transport systems whose activity either disappears or decreases drastically during maturation. In 1952, Riggs, Christensen, and Palatine (232) first demonstrated that phenylhydrazine induced rabbit reticulocytes and not mature red blood cells were able to concentrate glycine against its concentration gradient; this finding suggested that the loss is due to maturation-associated changes. Later it was shown that during the process of differentiation of erythroleukemic cells (188,233,234) and maturation of sheep reticulocytes (186,208,235) in <u>in vitro</u> culture, the cells lose their ability to concentrate amino acids. In some instances, amino acids, which are dependent upon sodium for transport, appear to lose their sodium dependency but are still transported (233). Some other amino acid transport systems are lost at different rates (207) during maturation.

## Cation Transport

Cation transport systems and their enzymic counterparts (cation-activated ATPases) have been studied in cells fractionated according to density and presumably age. Using density fractionation to separate cells, Kim and co-workers (236) found that the calcium activated ATPase activity in pig erythrocytes decreases with increasing density and thus, presumably, decreases with cell age. In contrast, Cameron and Green (237) found no significant difference between the magnesium-dependent calcium-stimulated ATPase activities in phenylhydrazine induced rabbit reticulocytes and mature red blood cells.

Cardiac glycosides, such as ouabain, bind specifically to the $Na^+,K^+$-pump enzyme (238,239), so that the number of $Na^+,K^+$-pump sites can be easily estimated from measurements of specific binding (240,241). In human red blood cells, it was shown that young cells have more specific $^3H$-ouabain binding sites per cell than older cells (242).

Lee, Woo, and Tosteson (243) were the first to demonstrate that reticulocytes from sheep of the low potassium (LK) genotype have 4 or 5 times more $Na^+,K^+$-pumps than mature LK red blood cells. Subsequently, other investigators have reported a loss in active cation ($Na^+$ or $K^+$) transport (191,244,245) and sodium-activated ATP hydrolysis ($Na^+$-ATPase) (246,247) in sheep red blood cells during maturation. In addition to the loss in activity observed in sheep cells, a difference between immature and mature cells with respect to the

kinetic response to potassium has also been observed (246-249). The changes are complex, however, since they reflect the genetic dimorphism of the $Na^+,K^+$-pump of ruminant red cells. Thus, two-thirds of all sheep have low potassium levels (10-20 milliequivalents $K^+$/litre), while the other third has high potassium levels (70-90 milliequivalents $K^+$/litre) (250-252). The reticulocytes of both types of sheep contain even higher intracellular potassium levels (101-128 milliequivalent $K^+$/litre). Table 1 and Figure 3 summarize and illustrate, respectively the differences in pump (intact cells) and $Na^+$-ATPase (broken membranes) activities between high potassiumm (HK) and low potassium (LK) mature sheep cells and between mature and immature cells of both types.

Figure 3 illustrates the kinetic differences which exist between the immature and mature LK erythrocytes with respect to the sodium, potassium pump ($Na^+,K^+$-pump). In addition, this figure shows the kinetic difference in the $Na^+$-ATPase activity of broken membranes derived from immature and mature HK erythrocytes. As shown, the $Na^+,K^+$-pump in LK cells is much more sensitive to inhibition by intracellular $K^+$ than either the pumps of HK cells or immature LK or HK cells. The $Na^+$-ATPase activity of membranes derived from LK cells is also more sensitive to inhibition by added $K^+$ compared to HK $Na^+$-ATPase; in this case, however, immature LK or HK cells, while also similar to each other, are different from mature HK cells and resemble LK mature cells. Thus, immature LK and HK cells have pumps which resemble each other, but <u>differ from both mature LK and HK cells</u> with respect to pump and $Na^+$-ATPase kinetics, respectively.

**Table 1**
**Characteristics of the Sodium Pump System of HK and LK Mature and Immature Red Cells**

1. The $K^+$ response profile of HK refers to the relative insensitivity of the pump to increasing $K^+$ intracellular and activation of HK $Na^+$-ATPase by addition of 5 mM KCl or less.

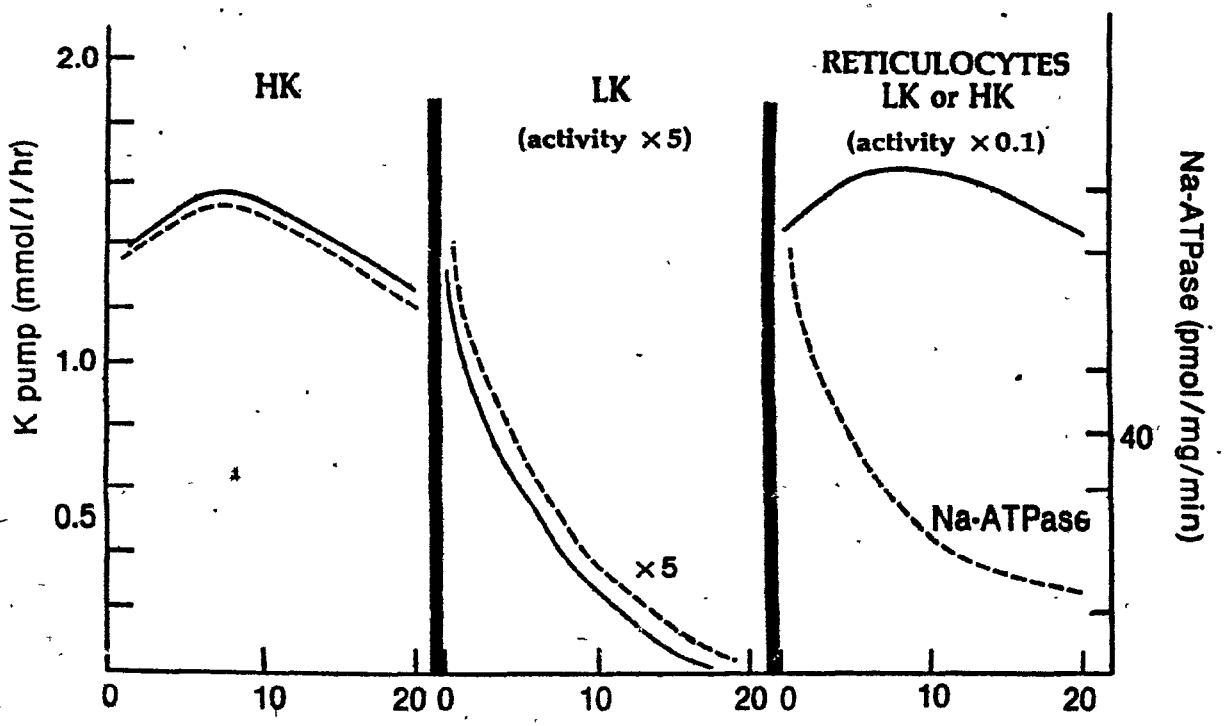
2. The $K^+$ response profile of LK refers to the marked inhibition of the pump by increasing $K^+$ intracellular and marked inhibition of LK $Na^+$-ATPase by addition of KCl. From Blostein, Drapeau, Benderoff and Weigensberg (250).

| Characteristic | Cell Type | | | |
|---|---|---|---|---|
| | HK | | LK | |
| | Mature | Reticulocyte | Mature | Reticulocyte |
| Intracellular $K^+$ concentration (250,251) | High | High | Low | High |
| Na pump (physiological [ATP]) | | | | |
| (i) Total activity (243,245) | High | High | Low | High |
| (ii) Intracellular $K^+$ response profile (248,249) | $HK^1$ | HK-Like | $LK^2$ | HK-like |
| $Na^+$-ATPase (200 nM ATP) | | | | |
| (i) Total activity (247, 254) | High | High | Low | High |
| (ii) $K^+$ response profile (247,254) | $HK^1$ | LK-Like | $LK^2$ | LK-Like |

## Figure 3

### Potassium Response Profiles of the $Na^+,K^+$-pump and the $Na^+$-ATPase Activities

The dotted lines represent $Na^+$-ATPase activity in pmol/mg protein/minute while the solid lines represent the $K^+$ pump activity in mmol/litre of cells/hour. The x-axis is the amount of KCl (mM) added ($Na^+$-ATPase) or the intracellular potassium (mM) concentration ($K^+$ pump).


HK
LK
(activity ×5)
RETICULOCYTES
LK or HK
(activity ×0.1)
K pump (mmol/l/hr)
2.0
1.0
0.5
×5
Na-ATPase
40
Na-ATPase (pmol/mg/min)
0
10
20
0
10
20
0
10
20

During ontogeny the change in the overall activity and kinetics of the $Na^+,K^+$-ATPase appears to occur by cell replacement (256-258). However, until now, it has not been clear if, or to what extent, changes in $Na^+,K^+$-ATPase associated with maturation of reticulocytes from mature sheep reflect cell maturation (259) versus cell replacement. Previous experiments were done by comparing characteristics of stress reticulocytes with those of mature cells, without following the time-dependent changes occurring in the same population of reticulocytes (259). Thus, the possibility remained that immature cells released into circulation following severe anemic stress may be different from 'normal cells' with respect to their pump characteristics.

Decreases in the activity of the $Na^+,K^+$-ATPase enzyme have also been observed in other species (242, 260,261) and in the Friend erythroleukemic cell line (234).

# 2 METHODS AND MATERIALS

## 2.1 Sources and Preparation of Red Blood Cells

### 2.1.A Care and Classification of Sheep

Male or female sheep were obtained from a local farm or from MacDonald College, McGill University and were housed in the Animal Centre of the McIntyre Medical Sciences Building. They were fed hay, water, and purina chow (Ralston Purina) ad libitum. The sheep were classified according to their intracellular red blood cell potassium concentrations. The high potassium (HK) sheep had intracellular potassium levels between 80 and 90 milliequivalents per litre cell water and the low potassium (LK) sheep had intracellular potassium levels between 20 and 30 milliequivalents per litre.

### 2.1.B Sheep Reticulocyte Production

Reticulocytosis was induced by repeated phlebotomy as follows: One litre of blood was initially withdrawn by jugular venipuncture (16 gauge needle) into heparinized (10,000 units sodium heparin/ml, 0.1 ml sodium heparin/100 ml blood) vacuum bottles on three days within a five day period. This was followed by removal of 0.5 - 1.0 L every 3 to 4 days depending on the animal's hematocrit, i.e. for a hematocrit greater than 17%, 1.0 L was removed, for hematocrit values between 15% and 17%, 0.8 L was removed and for a hematocrit value between 13% and 15%, 0.5 L was removed. The animals were maintained in this anemic state for 6 to 10 weeks during which time they received weekly intramuscular iron injections (250 mg iron/5 ml dextran, Fisons).

## 2.1.C Reticulocyte Isolation

The fresh sheep blood was centrifuged and the cells washed two or three times by repeated centrifugation at 3,000 x g and suspended in 0.9% NaCl at 4°C. The packed washed erythrocytes (hematocrit between 70% and 85%) were then centrifuged at 3,000 x g for 35 to 60 minutes at 4°C in a swinging bucket rotor. The top 20% to 25% of the cells (including the white blood cells) was removed and recentrifuged a second time as described above (262). After the second centrifugation, the cells were carefully separated, from top to bottom, into 3 to 5 approximately equal portions. Each fraction, which was usually pooled from several tubes and had a total volume of approximately 1 ml, was washed 3 to 5 times in order to remove the buffy coat. The erythrocyte washing with isotonic NaCl was done at room temperature in an IEC table-top clinical centrifuge at maximal speed for 4 minutes.

## 2.1.D Separation of Erythrocytes According to Density Using Phthalate Esters

Red blood cells were washed three times with isotonic saline and then separated according to their specific gravity using different ratios of two phthalate esters (dibutylphthalate, specific gravity = 1.048, diethylphthalate, specific gravity = 1.118) (146). Washed red blood cells were layered on top of a 5 ml phthalate ester cushion (specific gravity as indicated in the text) and centrifuged at room temperature (21°C) for 7 minutes at 25,000 x g. The red blood cells which passed through the phthalate ester cushion were recentrifuged on a phthalate ester cushion of greater specific gravity.

## 2.2. Preparation of Membrane Vesicles, Fragments and Cell Lysates

### 2.2.A Hemoglobin-Free Membrane Fragments

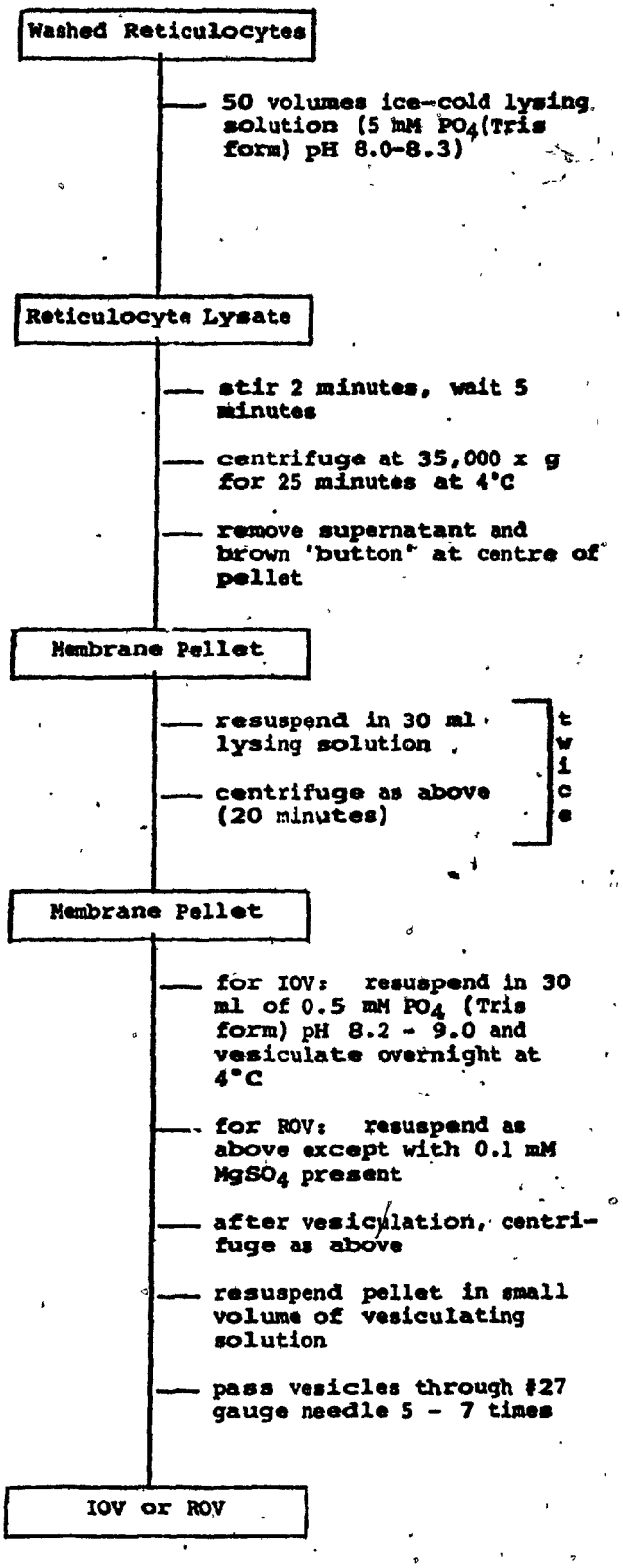
Hemoglobin-free membranes used to assay $Na^+$-ATPase activity were prepared from washed erythrocytes by modifying the method of Blostein (263). Washed erythrocytes were lysed with 10 volumes of ice-cold water. After stirring vigorously for 2 minutes and waiting an additional 5 minutes, the lysate was centrifuged for 20 minutes at 35,000 x g. The pellet was washed 4 times as above with: (i) 1 mM Tris-HCl in 1 mM EDTA, pH 7.4 (twice), (ii) 10 mM Tris-EDTA, pH 7.4 (once), (iii) 2 mM Tris-HCl, pH 7.4 (once). The membranes were resuspended in a small volume of the final wash solution at a protein concentration between 4 and 6 mg/ml and stored at 4°C if they were used within 4 days; otherwise they were frozen at -20°C.

### 2.2.B Inside-Out and Right Side-Out Membrane Vesicles

Inside-out (IOV) and right side-out (ROV) membrane vesicles were prepared from sheep reticulocytes by a modification of the method of Steck and co-workers (ref. 264; see also Figure 4). Washed reticulocytes were lysed with 50 volumes of ice-cold 5 mM $PO_4$ (Tris form) pH 8.0-8.3 (lysing solution). The lysate was stirred vigorously for 2 minutes and after an additional 5 minutes it was centrifuged at 35,000 x g for 25 minutes. The supernatant was removed by aspiration and any residual brown 'button' at the centre of the pellet

**Figure 4**

**Schematic Representation of IOV or ROV Preparation**


Washed Reticulocytes
50 volumes ice-cold lysing solution (5 mM $PO_4$(Tris form) pH 8.0-8.3)
Reticulocyte Lysate
stir 2 minutes, wait 5 minutes
centrifuge at 35,000 x g for 25 minutes at 4°C
remove supernatant and brown "button" at centre of pellet
Membrane Pellet
resuspend in 30 ml lysing solution
centrifuge as above (20 minutes)
twice
Membrane Pellet
for IOV: resuspend in 30 ml of 0.5 mM $PO_4$ (Tris form) pH 8.2 - 9.0 and vesiculate overnight at 4°C
for ROV: resuspend as above except with 0.1 mM $MgSO_4$ present
after vesiculation, centrifuge as above
resuspend pellet in small volume of vesiculating solution
pass vesicles through #27 gauge needle 5 - 7 times
IOV or ROV

was discarded. The pellet was washed twice by resuspension in approximately 30 ml of lysing solution and after 5 minutes on ice was centrifuged for 20 minutes. Following the second wash, pellets from two centrifuge tubes were combined and to obtain IOV, resuspended in approximately 30 ml of 0.5 mM $PO_4$ (Tris form) pH 8.2-9.0 (vesiculation solution). The best results were obtained when the pHs of the lysing and vesiculating solutions were 8.1 and 8.2, respectively. If ROV were desired, 0.1 mM $MgSO_4$ was added to the vesiculation solution. The membranes were incubated overnight (approximately sixteen hours) at 0°C and then centrifuged at 35,000 x g for 20 minutes. The membranes were resuspended in a small volume (usually between 1 and 3 ml) of vesiculation solution and the vesicles were released by passing the membranes through a stainless steel 27 gauge needle five to seven times. The vesicles were suspended in 15 volumes of 0.2 mM $MgCl_2$, 10 mM MOPS (Tris form) pH 7.4 to pH 7.7, as desired. After 10 minutes on ice they were centrifuged for 20 minutes at 35,000 x g, resuspended in the same solution and used within 4 days.

## 2.2.C. Preparation of Red Blood Cell Lysate

Reticulocytes or mature erythrocytes which were washed thoroughly with 0.9% NaCl were lysed with an equal volume (or 1.6 volumes) of ice-cold water. In some experiments as indicated, the cells were first passed through a column consisting of a 1:3(w/w) mixture of Sigmacell (Type 50): alpha-cellulose in 0.9% NaCl to remove leucocytes according to the method of Beutler and West (265). The lysate was centrifuged at 17,000 x g for 25 - 60 minutes (266). The clear supernatant was removed and used immediately or stored in liquid nitrogen.

## 2.3 In Vitro Incubation of Sheep Reticulocytes

### 2.3.A Long-Term In Vitro Culture

Reticulocytes were isolated as previously described using sterile technique and incubated (0.4 to 0.8 ml red blood cells/100 ml incubation medium) in a sterile medium in either Erlenmeyer flasks or 1 L Roller bottles (267). The vessels were gassed before sealing for five minutes with 95% $O_2$, 5% $CO_2$. The cells were kept in suspension by gentle continuous agitation at 37°C, with separate flasks being removed for analysis at intervals up to 14 days. The incubation medium consisted of 5% fetal calf serum, essential and non-essential amino acids, vitamins, 0.075% $NaHCO_3$, penicillin - streptomycin (0.125 mg/ml, 125 units/ml, respectively), Hank's balanced salt solution (268), 2 mM adenosine, and 5 mM glucose. The pH was adjusted to 7.4 with Tris base; the solution was then filtered through a 0.45 micron filter. At specified intervals during incubation, sterile 5 ml aliquots of 40 mM adenosine, 100 mM glucose and 100 mM Tris-HCl pH 8 were injected into the culture flasks containing 100 ml cell suspensions to maintain the cellular ATP levels.

### 2.3.B Short-Term In Vitro Incubation

The reticulocytes were isolated as previously described (not using sterile technique) and incubated at a hematocrit of 0.4 to 0.8 percent. The medium consisted of 130 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 20 mM MOPS (Tris form) pH 7.4, 2% fetal calf serum, 2 mM glutamine, vitamins, essential and non-

essential amino acids, 125 units/ml penicillin, and 0.125 mg/ml streptomycin. The final pH was adjusted with HCl or KOH (pH 7.0 - 7.7, as indicated) and the solutions were filtered through a 0.22 micron filter. Incubation at 37°C was carried out for periods up to 42 hours. Samples were removed at various intervals and stored at 4°C until they were assayed at the end of the incubation period.

## 2.4 Preparation of [$\gamma$- $^{32}$P] ATP

The method used to prepare [$\gamma$- $^{32}$P] ATP was similar to the method of Glynn and Chappell (269) as modified by Post and Sen (270), using the coupled reactions.

$$\text{GAP} + {}^{32}P_i + \text{NAD} \xrightarrow{\text{GAPDH}} 1,3D\ {}^{32}\text{PG} + \text{NADH}$$

$$1,3D^{32}\ \text{PG} + \text{ADP} \xrightarrow{\text{PGK}} [\gamma\text{-}{}^{32}\text{P}]\ \text{ATP} + 3\text{-PGA}$$

Accordingly, one mC of inorganic $^{32}P_i$ in 0.2 ml of 0.02 M HCl was transferred to a heavy-walled conical centrifuge tube and the vial containing the $^{32}P_i$ was rinsed twice with three drops of water. Ten microlitres of 0.6M Tris base were added to neutralize the HCl (pH was between 6 and 8 as tested on pH paper). Twenty microlitres of a cofactor solution (23 mM Tris-ATP, 4.5 mM Tris-ADP, 23 mM 3-phosphoglycerate, 18 mM EDTA, and 0.9 mM NAD), 0.01 ml 0.5 M Tris-HCl, pH 8.1, 0.001 ml beta-mercaptoethanol (10-fold dilution of stock), 0.001 ml phosphoglycerate kinase (0.5 mg/ml diluted from

stock with 0.2 M Tris-HCl, pH 7.4, 1806 units/mg), and 0.005 ml glyceraldehyde-3-phosphate dehydrogenase (0.4 mg/ml diluted from stock with 0.1 M HCl, pH 7.4, 80 units/mg) were added separately. One hundred microlitres of 1 mM $MgCl_2$ were then added slowly, in small aliquots, with gentle vortexing to prevent precipitation of magnesium ammonium phosphate. After one hour at room temperature, the solution was immersed in boiling water for two minutes, chilled, diluted to 6 ml with water and then applied to an ion exchange column (Dowex-1-Cl, 200-400 mesh, 2% crosslinked, 0.1 ml bed volume, contained in a pasteur pipette plugged with glass wool and washed consecutively with acetone, water, 1 N NaOH, water, 1 N HCl and then thoroughly with water). After the radioactive mixture was applied, the column was washed with 5 ml of ice-cold 0.02 N HCl in 0.02 N $NH_4Cl$ and then 6 ml of water. The [$\gamma$ - $^{32}$P] ATP was eluted with 3 ml of ice cold 0.25 N HCl. Three 1 ml fractions were collected and neutralized with 40 mg of Tris (final pH, 7.3-7.4). The ATP concentration of each fraction was determined spectrophotometrically at 259 nm with a correction for non-specific ultraviolet absorption by correcting for the fractional (0.15) absorption at 280 nm as follows (271):

$$\frac{(A_{259} - A_{280})}{15.9 \times 0.85} = \text{ATP concentration } (\mu M)$$

The percent incorporation of $^{32}P_i$ into [$\gamma$-$^{32}$P] ATP was measured by the charcoal adsorption method described below (Section 2.4.A). The specific radioactivity was 5 to 10 Ci/mmole.

## 2.4.A Separation of [$\gamma$-$^{32}$P] ATP from Inorganic $^{32}P_i$

A small aliquot, e.g. 0.001 ml, of a reaction mixture containing $^{32}P_i$ and [$\gamma$-$^{32}$P] ATP was added to 1 ml of a TCA solution (TCA 1 comprising 5% TCA, 2.5 mM $Na_2ATP$, 5 mM $NaH_2PO_4$) followed by addition of 0.5 ml of charcoal suspension (1.5 g Norit A-activated charcoal/10 ml 5% TCA). After 20 - 60 minutes on ice, with occasional vortexing, the suspension was either centrifuged or filtered. Aliquots were removed before and after charcoal treatment to determine the percentage of $^{32}P_i$.

## 2.5 Electrophoretic Separation of Sheep Hemoglobins

Washed packed red blood cells were lysed with equal volumes of water. Fifty microlitres of carbon tetrachloride were added to approximately 0.5 ml of lysate and the stroma was removed by centrifugation for 5 minutes in an Eppendorf (Model 3200) microcentrifuge. Sheep hemoglobins A, B, and C were separated by electrophoresis (272) using a Beckman (Model R-100) Microzone Electrophoresis System, with a boric acid-EDTA buffer, pH 8.6 (3.15 g boric acid, 1 g EDTA, and 15 g Tris/litre, adjusted to pH 8.6 with Tris). Before applying the hemoglobin lysates to the cellulose acetate strips, the strips were soaked for 20 minutes in buffer and blotted. Following electrophoresis at 4°C for 90 minutes at an applied voltage of 425 volts, the strips were removed, dried and the areas corresponding to hemoglobins A and C were cut out and eluted with an aliquot, usually 0.5 ml, of buffer. The relative amounts of the

hemoglobins were determined by measuring the optical density at 540 nm using an extinction coefficient of 0.885 l/kg cm (273). All spectrophotometric determinations were performed using a Zeiss Model PMQII spectrophotometer.

## 2.6 Assays of Membrane Transport Systems

### 2.6.A Transport Assays in Intact Cells

#### 2.6.A.1 $Na^+$-Dependent Glycine Transport

Cells were washed three times with 150 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 20 mM sucrose, 5 mM potassium phosphate pH 7.4 and 10 mM MOPS (Tris form), pH 7.4 and then diluted (hematocrit 20% - 50%) five-fold with the above wash solution containing 0.125 mM ouabain; in some instances potassium was replaced by sodium as indicated. The red blood cell suspension was then pre-incubated 5 minutes at 37°C at which time gramicidin D (0.01 volumes of an ethanolic solution containing 0.7 mg/ml) was added and the cells were chilled. The cells were then aliquotted (0.095 ml) and pre-warmed (2 minutes) at 37°C. The reaction was started by adding 0.005 ml of 20 mM $^{14}C$-glycine (final specific activity 4.8 x $10^3$ dpm/nmole). After 5 minutes the reaction was terminated by removing 0.08 ml of the suspension and mixing it thoroughly with 1 ml of ice-cold wash solution layered above 0.15 ml dibutyl phthalate. The cells were separated from the medium by centrifuging for 10 to 15 seconds in an Eppendorf microfuge. An alternate method used to terminate the reaction involved adding 1 ml of ice-cold wash solution to the erythrocyte suspension and

then layering 1 ml of this suspension on 0.15 ml of the dibutylphthalate. Following centrifugation, the aqueous layer was removed and replaced with water which was also aspirated. After an additional centrifugation (10 seconds) the oil layer was removed. The pellet was lysed with 0.2 ml water. The stroma was removed by centrifugation (2 minutes) before aliquots of the lysate were removed for determinations of hemoglobin (optical density at 527 nm, extinction coefficient 0.532 l/kg) (273) and radioactivity using a LKB-Wallac 1215 Rack Beta II liquid scintillation counter with application of the appropriate quench curve correction for $^{14}C$.

2.6.A.2 Ouabain-Sensitive $^{86}Rb$ Uptake

The red blood cells were washed three times as previously described in Section 2.6.A.1, except that potassium was replaced by sodium. The cells were loaded with sodium, potassium, and choline using the nystatin method of Cass and Dalmark (274) modified as follows: the cells were diluted with at least ten volumes of ice-cold 135 mM NaCl, 5 mM glucose, 70 mM sucrose, 10 mM MOPS (Tris form), pH 7.4, a 50 mM mixture of varying ratios of KCl and choline chloride as desired, and 0.05 mg/ml nystatin (Squibb, sterile powder, diluted from a stock solution of 25 mg/ml in methanol). The cells were incubated at 0-4°C for 20 minutes and after centrifugation, this 'loading procedure' was repeated. The cells were then washed six times with the above loading solution without nystatin, and then three times with 150 mM NaCl, 10 mM MOPS (Tris form), pH 7.4 using rapid (40 seconds) centrifugation in a Clay Adams Serofuge II.

The $^{86}Rb$ uptake assay was started by diluting 37°C prewarmed cells 5-fold with a prewarmed medium consisting of 150 mM NaCl, 10 mM MOPS (Tris form), pH 7.4 and 3-5 mM $^{86}RbCl$ (specific activity approximately $5.0 \times 10^9$ cpm/mmole) in the presence or absence of 0.1 mM ouabain. After a period ranging from 10 to 60 minutes at 37°C, the reaction was stopped as described in Section 2.6.A.1, except that the solution above the phthalate ester consisted of 150 mM NaCl and 10 mM MOPS (Tris form), pH 7.4. After the cells were lysed, an aliquot was used for hemoglobin determination (O.D. 540 nm). Another aliquot was precipitated with an equal volume of 10% TCA before the amount of radioactivity was determined in the clear supernatant. The radioactivity was determined in either a Searle Delta 300 or Packard Tri-Carb liquid scintillation counter.

## 2.6.B Transport Assays in Vesicles

### 2.6.B.1 The Vesicle Transport Assay Technique

Vesicle transport assays were carried out by Millipore filtration as described by Blostein (275). Briefly, 15-30 seconds prior to termination of the uptake assay, a pre-moistened cellulose nitrate filter (Sartorius or Millipore 1.2 micron pore size, 25 mm diameter) was placed on the lower section of a Millipore funnel (catalogue number XX10 025 02) and 10 ml of ice-cold stop solution (ionic composition identical to final flux medium) was added to the upper section (catalogue number XX10 025 14). To terminate the reaction, an aliquot of the reaction mixture (0.03 - 0.15 ml) was added,

rapidly mixed with this solution and the vesicles were then separated from the medium by vacuum filtration. The vesicles were then washed on the filter with an additional 10 ml of ice-cold 'stop' solution. The upper section was then removed and the rim of the filter was washed with an additional 2 - 4 ml of ice-cold 'stop' solution. The filter was then dried and suspended in 5 - 10 ml of liquid scintillation counting cocktail (1:1 dilution of Aquasol with toluene or Hydrocount ). The radioactivity was determined as described in Section 2.6.A.1.

2.6.B.2 $Na^+$-Dependent Glycine Uptake

IOV and ROV which were equilibrated in 0.2 mM $MgCl_2$ and 10 mM MOPS (Tris form), pH 7.4, were diluted with an equal volume of 10 mM MOPS (Tris form), pH 7.4, 2 mM $MgCl_2$ and KCl (80-200 mM). After overnight equilibration at 0°C followed by 15 to 30 minutes at 37°C the vesicles were concentrated 2 to 5-fold by centrifugation at 12,000 x g for 10 minutes.

The transport assay was then started by adding 0.01 ml of prewarmed (37°C) vesicles to 0.09 ml of a prewarmed (37°C) equiosmolar medium or vice versa. The flux media consisted of 1 mM $MgCl_2$, 10 mM MOPS (Tris form), pH 7.4, varying amounts of NaCl or KCl as indicated and 0.1 to 2.0 mM glycine (specific activity 5 x $10^3$ - 2 x $10^4$ cpm/nmole). The assay was terminated at the indicated periods by adding 0.08 to 0.09 ml aliquots to 10 ml of ice-cold 'stop' solution and $^{14}C$-glycine retained by the vesicles was measured as described in Section 2.6.B.1.

2.6.B.3 ATP-Dependent Sodium Uptake

IOV which were equilibrated as described above (Section 2.6.B.2) in 0.2 mM $MgCl_2$ and 10 mM MOPS (Tris form), pH 7.4, were diluted with an equal volume of 2 mM $MgCl_2$, 10 mM MOPS (Tris form), pH 7.4 and 80 mM KCl and were then concentrated to an appropriate volume. The transport assay was carried out by the method described for glycine transport, except that the medium consisted of 1 mM $MgCl_2$, 10 mM MOPS (Tris form), pH 7.4, 36 mM $^{22}NaCl$ (specific activity approximately 100 cpm/nmole) and 4 mM KCl. The reaction was terminated and the intravesicular $^{22}Na$ was measured as previously described.

2.6.C $^{3}H$-Ouabain Binding to Intact Cells

The red blood cells were loaded with sodium by the nystatin method described in Section 2.6.A.2. The concentrations of NaCl, KCl and choline chloride in the external medium were 135mM, 5 mM, and 45 mM, respectively.

The binding assay was started by diluting the erythrocyte suspension (20 - 50% hematocrit) 5-fold in a medium consisting of 150 mM NaCl, 10 mM MOPS (Tris form), pH 7.4, 5 mM glucose (occasionally) and $4 \times 10^{-7}$ M $^{3}H$-ouabain (specific activity $1.4 \times 10^{13}$ dpm/mmole). Two sets of tubes were incubated, one with and one without $4 \times 10^{-4}$ M unlabelled ouabain, to measure nonspecific and specific binding, respectively. The suspension (0.15 ml) was incubated from 2 to 2½ hours at 37°C and then diluted with 3 ml of ice-cold wash solution (150 mM

NaCl, 10 mM MOPS (Tris form), pH 7.4) and centrifuged. The pellet was washed twice and then lysed with 0.5 ml of ice-cold 10 mM MOPS (Tris form), pH 7.4. The lysate was further centrifuged for 3 minutes in the Eppendorf centrifuge to remove the stroma. Hemoglobin concentrations in the clear supernatant were determined by measuring the optical density at 527 nm. The stroma was transferred quantitatively to a liquid scintillation counting vial and the radioactivity was determined using a liquid scintillation counter with application of the appropriate quench curve correction for $^{3}H$.

An alternate method which was used for terminating the ouabain binding assay was described in Section 2.6.A.1. The ouabain binding was terminated by centrifuging the cell suspension through oil. The radioactivities in both the stroma (total) and supernatant (0.1 ml aliquot of a 0.2 ml lysate) were determined following lysis and centrifugation.

## 2.6.D $Na^+$-ATPase Assay

Prior to the $Na^+$-ATPase assay (276), hemoglobin-free membranes were frozen and thawed twice in dry ice and acetone to ensure that the membranes were not sealed. Prewarmed (37°C) membranes were diluted five-fold with prewarmed medium, in a total volume 0.1 ml consisting of 0.012 mM $MgCl_2$, 200 nM [$\gamma$-$^{32}P$] ATP (specific activity approximately 1 x $10^6$ cpm/nmole), 20 mM Tris-glycylglycine, pH 7.4 and 60 mM chloride salts (sodium, potassium and choline, as indicated); following 1 - 15 minutes at 37°C, 0.9 ml of an ice-cold TCA I solution was added and the percentage of $^{32}P_i$

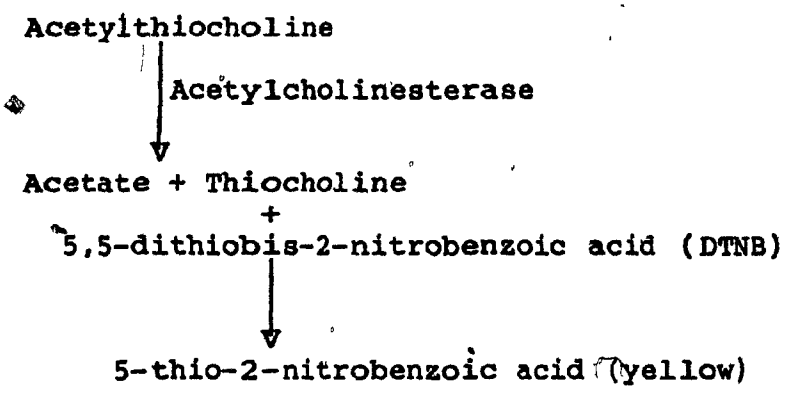
released from [$\gamma$-$^{32}$P] ATP was measured as described in Section 2.4.A. The baseline activity was measured with NaCl omitted and 50 mM KCl added.

## 2.7 Enzyme Assays Used to Determine Vesicle Sidedness

### 2.7.A Acetylcholinesterase

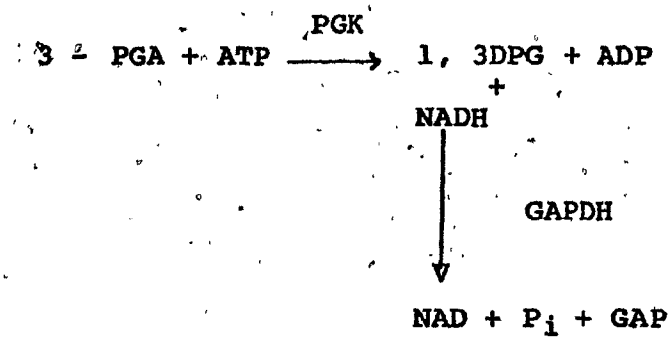
The enzyme acetylcholinesterase is normally active at the extracellular surface of the erythrocyte membrane. Its accessibility in vesicles was measured by modifying the method of Steck and Kant (36). To 0.01 ml of vesicles at room temperature, 0.9 ml of 0.2 mM $MgCl_2$, 10 mM MOPS (Tris form), pH 7.4 and 0.04 ml of 10 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were added. After mixing, the solution was transferred to a cuvette and the optical density at 420 nm was set at 0.1. Fifty microlitres of 12.5 mM acetylthiocholine were then added and the rate of increase in optical density was measured. In order to determine the enzyme's inaccessibility to the substrate and thus the percentage of vesicles which were inside-out, the assay was repeated with Triton X-100 added to disrupt the vesicular structure, i.e. 0.01 ml of 2% Triton X-100 was added to 0.01 ml of vesicles 3 minutes prior to the addition of the assay reagents.

The percentage of IOV was calculated as the difference in activities in the presence and absence of the detergent divided by the total activity observed with detergent added. In this assay the thiocholine released reacts with DTNB to form the yellow chromogen 5-thio-2-nitrobenzoic acid.




2.7.B Glyceraldehyde-3-phosphate Dehydrogenase

The enzyme glyceraldehyde-3-phosphate dehydrogenase, accessible at the internal side of the erythrocyte membrane, was assayed by a modification of the method by Beutler (277). Ten microlitres of vesicles at room temperature were mixed with 0.85 ml of a reagent consisting of 10 mM ATP, 12.5 mM $MgCl_2$, 0.7 mM EDTA, 0.25 mM NADH, 138 mM Tris-HCl, pH 8.0, and 25 - 50 units/ml PGK (Sigma Type IV from yeast). The optical density of the mixture was adjusted to 0.4 - 0.5 at 340 nM. One hundred microlitres of 100 mM 3-PGA were added and the decrease in optical density due to NADH oxidation was recorded at ten second intervals for a period of two minutes. In order to determine the percentage of vesicles which were right side-out, the enzyme assay was repeated with Triton X-100 added to disrupt the vesicular structure; i.e. 0.01 ml of 0.2 % Triton X-100 was added just prior to the addition of 0.85 ml of the previously described reagent. In this assay NAD production from NADH via reversal of the GAPDH reaction is measured.




## 2.8 Measurement of ATP Concentration

ATP was measured by the luciferin-luciferase method (278). Briefly, an erythrocyte suspension was lysed with two volumes of 4 mM $MgSO_4$, 10 mM potassium phosphate buffer, pH 7.4 (lysing solution), precipitated with an equal volume of 10% TCA and then centrifuged. The supernatant was used directly or neutralized (pH 6 - 7) with Tris-base or MOPS (Tris form), pH 7.4, and stored at -20°C.

To each assay vial (liquid scintillation counting type) 1 ml water, 1 ml lysing solution containing either 0.025 ml of 0 - 5 x $10^{-4}$ M ATP (standard curve) or 0.05 - 0.10 ml of ATP sample and 0.5 ml of a filtered arsenate buffer (40 mM $MgSO_4$, 100 mM $Na_2HA_sO_4$ adjusted to pH 7.4 with $H_2SO_4$) containing a luciferase extract (Sigma FLE-250, 6 - 10 mg/10 ml buffer) was added. The light emissions during the first 5 or 6 seconds after the addition of the enzyme extract solution were determined by liquid scintillation spectrometry using the tritium channel of a Searle Delta 300 spectrometer. The standard curve consists of a double logarithm plot of total light emissions versus moles of ATP.

## 2.9 Measurement of Free Amino Groups

The method used was essentially the method of Moore and Stein (279). Ice-cold 50% TCA was added to a specific volume of hemolysate, so that the final TCA concentration was 10%. Following centrifugation the supernatant was removed and stored at -20°C.

Prior to the assay, the TCA was extracted with anhydrous ether (4 or 5 times), or the sample was neutralized by adding 0.5 M Tris base. The samples (0.025 ml - 0.05 ml) and the standard curve (0 - 0.02 ml of 10 mM glycine, diluted from 50 mM glycine with 0.8 M NaCl) were diluted with 0.8 M NaCl to 0.25 ml before 0.75 ml of a ninhydrin solution was added (279). The test tubes were placed in boiling water for 20 minutes, cooled and 4 ml of a 50% n-propanol solution was added. The colour was allowed to develop for ten minutes before the optical density at 570 nm was measured.

## 2.10 Measurement of Protein

Protein was determined by the method of Lowry et al (280).

## 2.11 Determination of Sodium and Potassium Levels

Potassium and sodium concentrations were determined by flame photometry, using an internal lithium standard solution with an Instrumentation Laboratories Inc. flame photometer, Model 143.

## 2.12 Hemoglobin and Hematocrit Determinations

The hemoglobin concentration of stroma-free hemolysate was measured spectrophotometrically at either 540 or 527 nm. The amount of hemoglobin was determined by using the appropriate extinction coefficient (0.885 l/kg cm at 540 nm or 0.532 l/kg cm at 527 nm (273). Microhematocrit measurements were carried out in a hematocrit centrifuge (Clay Adams or IEC) or in an IEC refrigerated centrifuge (Model PK-B).

## 2.13 Estimation of Reticulocyte Number

The percentage of cells with visible reticulum were counted after staining with new methylene blue (281). In some experiments the presence of contaminating leucocytes was evaluated by counter staining with Wright's stain (282) as follows: After the initial staining (with new methylene blue) the slides were immersed for five minutes in Wright's stain and then transferred to a mixture of Wright's stain and Wright's buffer (0.15 M $KPO_4$ buffer pH 6.8). In order to obtain a green metallic sheen on the surface of this solution, the approximate ratio of Wright's stain to the buffer was 1:5. After four minutes the slides were washed several times with water, air-dried and visualized using an oil immersion objective lens.

## 2.14 Expression of Results

All results are expressed as the mean ±S.E.M. of 3 - 5 replicate samples. For duplicate measurement, only mean values are shown. The results are representa-

tive of two or more similar experiments. For the vesicle experiments, the results are expressed per mg of membrane protein or per microlitre of vesicle space.

## 2.15 Source of Reagents

All radioactive material was purchased from New England Nuclear. All enzyme preparations were obtained either from Sigma Chemical Company or Boehringer Mannheim unless otherwise indicated. Other materials were purchased from Fisher Scientific or Canlab.

# 3 CHANGES IN $Na^+$-ATPase AND $Na^+$, $K^+$-PUMP DURING MATURATION OF SHEEP RETICULOCYTES

A number of transport functions which are readily apparent in immature mammalian red blood cells (reticulocytes) are either absent or weakly active in mature cells (see Chapter 1). This holds true for systems which accumulate amino acids (205,208,235) and for the $Na^+$, $K^+$-pump (243,249). In the latter case, there are also maturation-associated changes in the properties of the pumps in red blood cells of sheep which are dimorphic with respect to their ability to pump sodium and potassium (248,249). This dimorphism is apparent only in mature cells; the reticulocytes of all sheep have typical high potassium and low sodium (251, 252) concentrations, whereas the mature cells of one type (HK) have high pump fluxes and those of the other (LK) have low fluxes. The differences in pump and ATPase activities between HK and LK mature cells and between mature and immature cells of both types have been summarized in the Introduction (Table 1 and Figure 3).

Reticulocytes of both HK and LK sheep have high pump activity with pump kinetics resembling HK mature cells (249). However, there remains a distinct difference between reticulocytes of either type and HK mature cells, namely the response of their $Na^+$-ATPase to $K^+$ (247). Figure 3 illustrated the difference in $K^+$ response properties of HK immature and mature cells. This $K^+$ response pattern is characterized by a marked inhibition of $Na^+$-ATPase in LK but stimulation in HK at a potassium concentration of 5 mM or greater when activ-

ity is measured at very low levels of ATP ($\leq$ 200 nM). Thus, both the HK and LK reticulocyte $Na^+$-ATPase resemble mature LK $Na^+$-ATPase with respect to the inhibitory $K^+$ response profile, despite the fact that their absolute levels of activity are high, even greater than mature HK cells. In addition, there is a kinetic difference between reticulocytes of either type and LK mature red blood cells as shown by the response of the $Na^+,K^+$-pump to varying intracellular potassium levels. This $K^+$-response pattern shows marked inhibition of the $Na^+,K^+$-pump in LK mature cells but not in LK or HK reticulocytes or mature HK cells. Thus both HK and LK reticulocyte pumps resemble the mature HK pumps with respect to the $K^+$ response profile, even though their absolute levels of activity are higher than mature HK cells.

In the past, studies with reticulocytes have involved cells which were produced following severe anemic stress. As described in the Introduction, it is plausible that stress reticulocytes released into circulation following severe anemic stress differ from 'normal' reticulocytes with respect to their pump characteristics. Thus the questions which remain are:

(i) whether these differences in pump properties are due to alterations in genetic expression elicited by the anemic stress,

(ii) to what extent, if any, the properties of the stress reticulocytes revert to those of the normal mature cell by the process of cell maturation, or

(iii) whether the apparent changes in characteristics during recovery from anemia reflect replacement of the stress reticulocytes by 'normal' cells.

## 3.1 In Vitro Studies

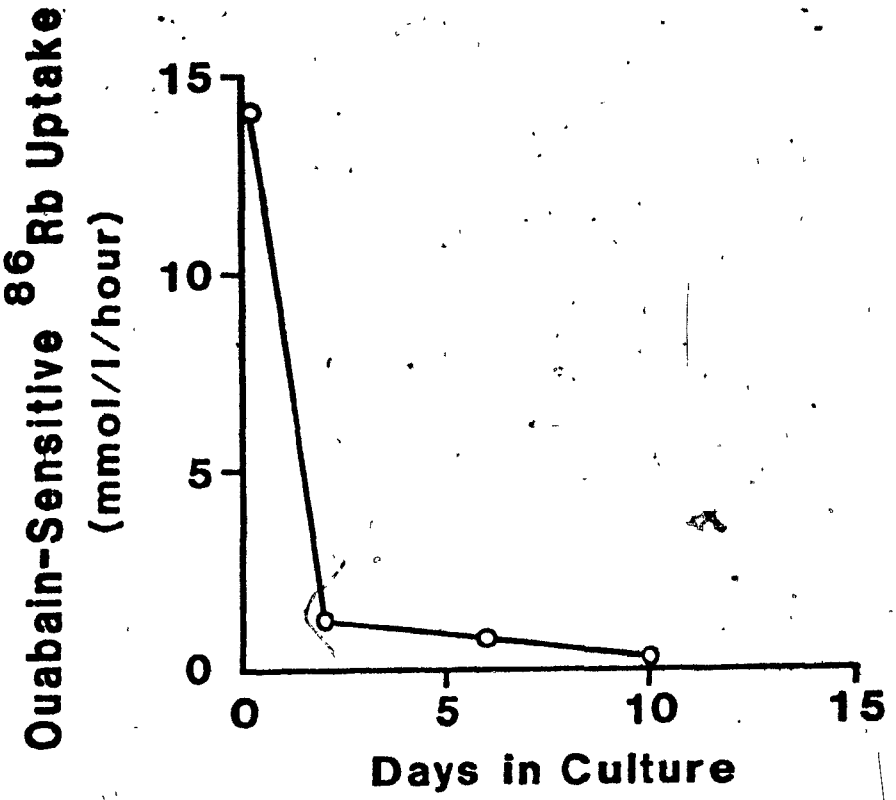
In an effort to answer the foregoing questions, long term in vitro incubations of sheep reticulocytes were carried out according to the method of Kepner and Tosteson (267). Previously Benderoff and co-workers (208,235) demonstrated that reticulocytes from sheep of the HK genotype can be maintained in culture for several days with neither loss of intracellular potassium nor a substantial decrease in ATP.

Pump Activity: In vitro culture was used to follow the maturation-associated changes in $Na^+,K^+$-pump activity in LK sheep red blood cells in, which differences between immature and mature cells are apparent with respect to both total activity and pump kinetics (intracellular $K^+$-response profile). Figure 5 shows the results of an experiment in which changes in pump activity of LK reticulocytes were followed for up to ten days. In this experiment, the cells remained intact (7% lysis or less) throughout the period of incubation. In cells with approximately 20 mM intracellular $K^+$, pump activity (ouabain-sensitive $^{86}Rb$ uptake) in the lightest fraction of LK cells showed a marked decrease (approximately 90%) in activity within the first two days of incubation. A marked decrease in $K^+$ pump and leak fluxes has been similarly observed by Kim et al (259) within six hours of in vitro incubation. The effects of long-term culture on ouabain-sensitive $^{86}Rb$ influx as a

**Figure 5**

**Changes in $^{86}Rb$ Uptake of Immature LK Cells During Long-Term In Vitro Incubation**

Changes in ouabain-sensitive $^{86}Rb$ uptake during in vitro maturaton of LK (#133) reticulocytes were followed using reticulocytes isolated by centrifugation as described in Methods and Materials (Section 2.1.C). Long-term incubation was carried out as described in Section 2.3.A at 0.5% hematocrit. $^{86}Rb$ uptakes were done in duplicate sets of $K^+$ loaded cells ($[K^+]$=20 mM, see Section 2.6.A.2), one without and one with $10^{-3}$ M ouabain, and the values shown are the ouabain-sensitive uptakes. Uptake measurements were carried out for 20 minutes for cells at days 0 and 2, 33 minutes at day 6 and 60 minutes at day 10. Media pH values on days 0, 2, 6, and 10 days were 7.6, 7.5, 7.5, and 6.9 respectively. (Data taken from '20 mM KCl values' in Figure 6)


Ouabain-Sensitive $^{86}Rb$ Uptake
(mmol/l/hour)
15
10
5
0
0
5
10
15
Days in Culture

function of varying $K^+$ are depicted in Figure 6. As shown, the $K^+$-response profile shows little change, if any, despite the large decrease in the total activity (See Figure 5). Thus, the marked inhibition of pump activity by intracellular $K^+$ observed in mature LK cells could not be observed during long-term culture.

$Na^+$-ATPase Activity: In a recent study done in collaboration with Blostein, Drapeau, and Benderoff (253) we showed that the $Na^+$-ATPase activity of LK reticulocytes incubated for five days in vitro decreases exponentially from 248 pmol/mg per minute to 70 pmol/mg per minute ($T_{\frac{1}{2}}$= 2.3 days), although the final level of activity observed in that experiment is still about one order of magnitude higher than that observed in mature LK red blood cells. Figure 7 shows a similar loss in $Na^+$-ATPase activity during in vitro culture of unfractioned red cells obtained from an anemic HK sheep. After six days of in vitro incubation the $Na^+$-ATPase activity decreased markedly, in this case to the level usually observed in mature HK erythrocytes (243-245).

In a similar experiment, I followed membrane $Na^+$-ATPase as a function of varying amounts of added $K^+$ using membranes from HK immature cells cultured in vitro. The typical (LK-like) HK reticulocyte $K^+$-response profile observed at the begining of culture did not usually change in the direction expected, i.e. to mature HK-like, except in the experiment shown in Figure 8.

## Figure 6

**Changes in $^{86}Rb$ Uptake and Kinetic Response to Varying Concentrations of Intracellular Potassium During Long-Term In Vitro Incubation of Immature LK Cells**

Changes in ouabain-sensitive $^{86}Rb$ uptake during *in vitro* maturation (Section 2.3.A) of LK reticulocytes were followed using reticulocytes isolated by centrifugation as described in Methods and Materials (Section 2.1.C.). Fractions rich in reticulocytes (80%) were combined. Long-term incubations at 37°C were carried out with cells suspended at a 0.5% hematocrit. $^{86}Rb$ uptakes were done in duplicate sets of $K^+$-loaded cells (Nystatin treated, see Section 2.6.A.2), one without and one with $10^{-3}$ M ouabain as described in Figure 5. The values shown are the ouabain-sensitive uptakes. Values in parentheses represent the number of days of *in vitro* incubation of LK cells. From Blostein, Drapeau, Benderoff and Weigensberg (253).

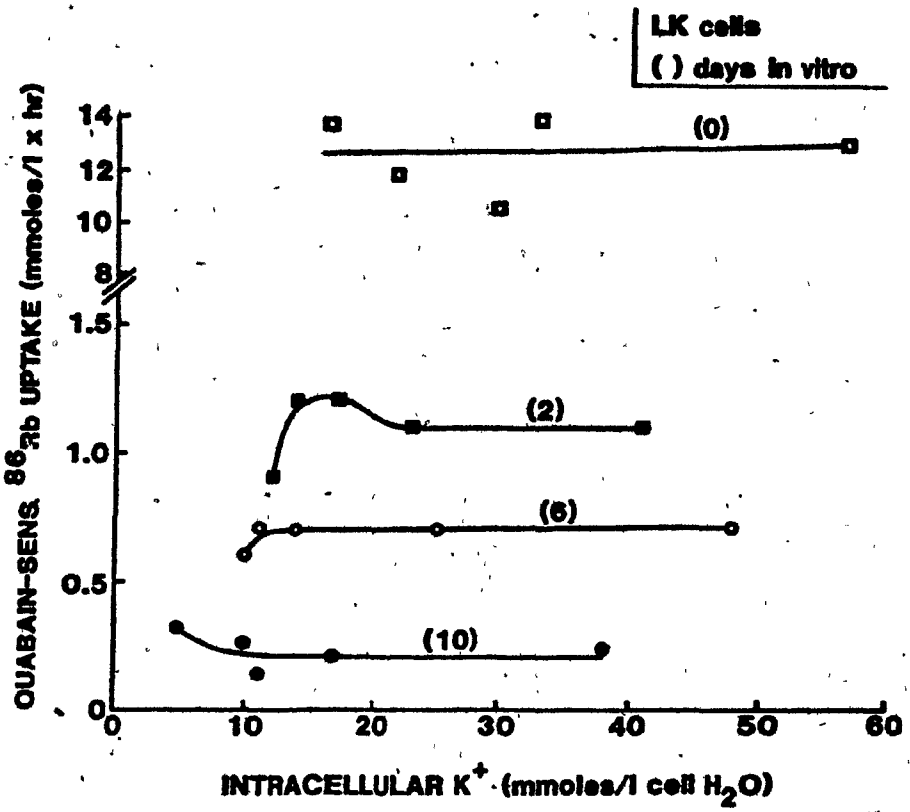
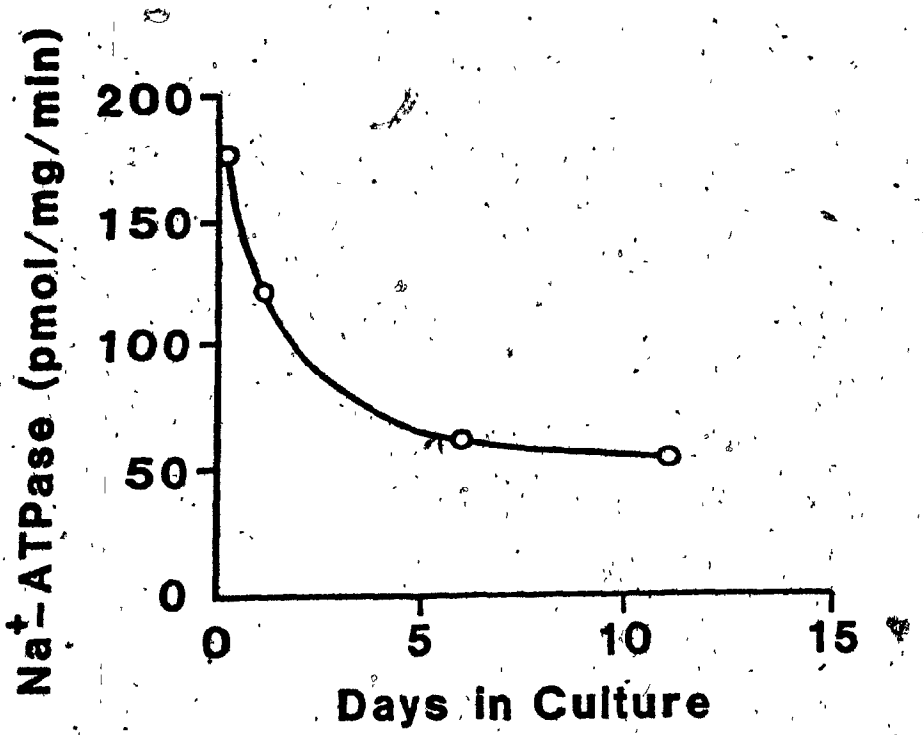

LK cells
( ) days in vitro
(0)
(2)
(6)
(10)
OUABAIN-SENS. $^{86}Rb$ UPTAKE (mmoles/l x hr)
14
12
10
8
1.5
1.0
0.5
0
0
10
20
30
40
50
60
INTRACELLULAR $K^+$ (mmoles/l cell $H_2O$)

**Figure 7**

**Changes in $Na^+$-ATPase Activity During In Vitro Maturation of HK Erythrocytes**

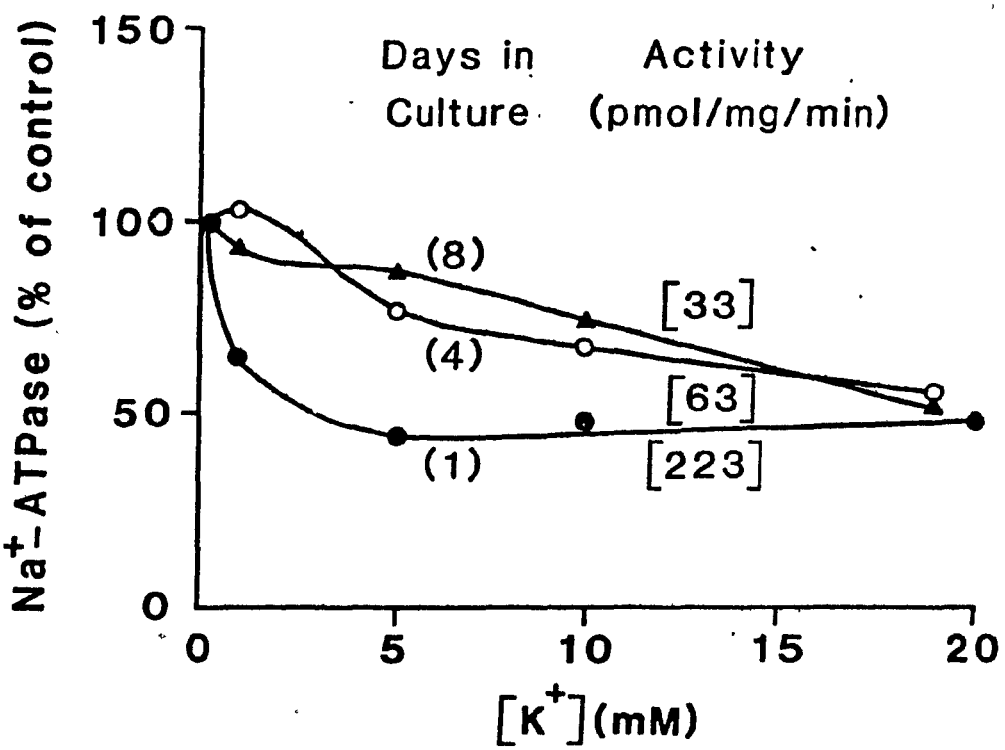
Long term incubation was carried out using unfractionated sheep blood (HK sheep #9) as described in section 2.3.A at 0.5% hematocrit. $Na^+$-ATPase activity was determined (Section 2.6.D) in quadruplicate sets of membranes derived from the erythrocytes which had been removed from the incubation medium at specified intervals. The values are the differences in activity between samples assayed with NaCl and KCl (baseline).


$Na^+$-ATPase (pmol/mg/min)
200
150
100
50
0
0
5
10
15
Days in Culture

## Figure 8

**Changes in the $Na^+$-ATPase Activity and Kinetic Response to Added $K^+$ During In Vitro Culture of HK Cells**

Changes in the $Na^+$-ATPase activity and kinetic response to added $K^+$, were followed during long-term in vitro culture. HK (#9) reticulocytes were isolated by centrifugation (Section 2.1.C) and long-term incubation was carried out as described in Section 2.3.A. $Na^+$-ATPase activity was measured (Section 2.6.D) at 200 nM ATP in quadruplicate sets of membranes derived from the reticulocytes removed at specified intervals. The values shown are the difference in activity between samples assayed with NaCl (and varying KCl) and KCl (baseline, no NaCl). The percentages of reticulocytes for days 1, 4 and 8 are 60, 30 and 1 respectively. The assay time for the $Na^+$-ATPase activity on days 1, 4 and 8 was 5, 10 and 15 minutes respectively. Values in parentheses represent the number of days in in vitro incubation of HK cells, while the square brackets represent 100% $Na^+$-ATPase activity (no KCl added).


Days in Culture
Activity (pmol/mg/min)
(8)
[33]
(4)
[63]
(1)
[223]
$Na^+$-ATPase (% of control)
150
100
50
0
0
5
10
15
20
$[K^+]$(mM)

## 3.2 In Vivo Studies

From the foregoing results it remains possible that kinetic changes in pump activity (LK cells) and $Na^+$-ATPase activity (membranes from HK cells), if they do occur, may do so at a much slower rate than the overall loss in activity. This possibility is, in fact, suggested by an in vivo maturation study of kinetic changes in HK cells. Thus, Blostein, Drapeau, Benderoff, and I examined the effects of added $K^+$ (253) on the kinetics of HK $Na^+$-ATPase. These experiments were carried out using membranes prepared from density-fractionated cells obtained at periods during recovery from anaemic stress. $Na^+$-ATPase kinetics of the membranes prepared from the top 10% and the bottom 10% were followed as a function of increasing amounts of added KCl.

The results (Figure 6 of reference 253, experiment carried out by P. Drapeau) indicated that the LK-like $K^+$ inhibition profile of the least dense cells (top fraction) persist, but to diminishing extents during the total six week recovery period. The $K^+$-response profile in the $Na^+$-ATPase of the bottom 10% of the fractionated cells was sensitive to $K^+$ inhibition during the first three weeks of recovery, suggesting that repeated massive bleeding results in a high proportion of young cells, even in the heaviest fraction. However, in contrast to the lightest fraction, a dramatic change in $K^+$ response from inhibition to activation was observed in the bottom fraction after four weeks, although a similar change was not observed in the top fraction $K^+$ inhibition diminished with time after bleeding.

Although the foregoing result suggested that cell maturation rather than cell replacement is the basis for the change in kinetics seen during recovery from anemic stress, the possibility remains that during in vivo maturation new cells released into circulation at later times increase in density faster than those produced in the earlier phase of recovery from anemic stress. To rule out this possibility, an experiment was carried out using blood from an animal with hemoglobins A and B, bled to induce the well-documented switch from HbA to HbC (283-286) and then allowed to recover; recovery from anemia is associated with a reversion to HbA production.

An experiment verifying that the younger cells, identified by their HbA, are less dense than the older cells, identified by HbC, is shown in Table 2. Similarly, the results depicted in Figure 9 show that on the 27th day of recovery, the percent HbC was greatest in the heaviest and least in the lightest fraction, consistent with the conclusion that under these conditions of recovery from anemic stress, the younger cells do appear in the lighter fractions. Concomitant measurements of total $Na^+$-ATPase activity show decreases in activity with increasing density (Figure 9). Moreover, the change in the $K^+$-response profile, which is similar to the change observed previously (Figure 6 of reference 253) is consistent with the notion that the apparent maturation-associated changes in the $Na^+,K^+$-pump are due to cell maturation and not cell replacement.

**Table 2**
**Relationship Between Cell Density and Cell Age as Measured by the Ratio of Hb A:Hb C in Cells Obtained 34 Days After Recovery From Anemic Stress**

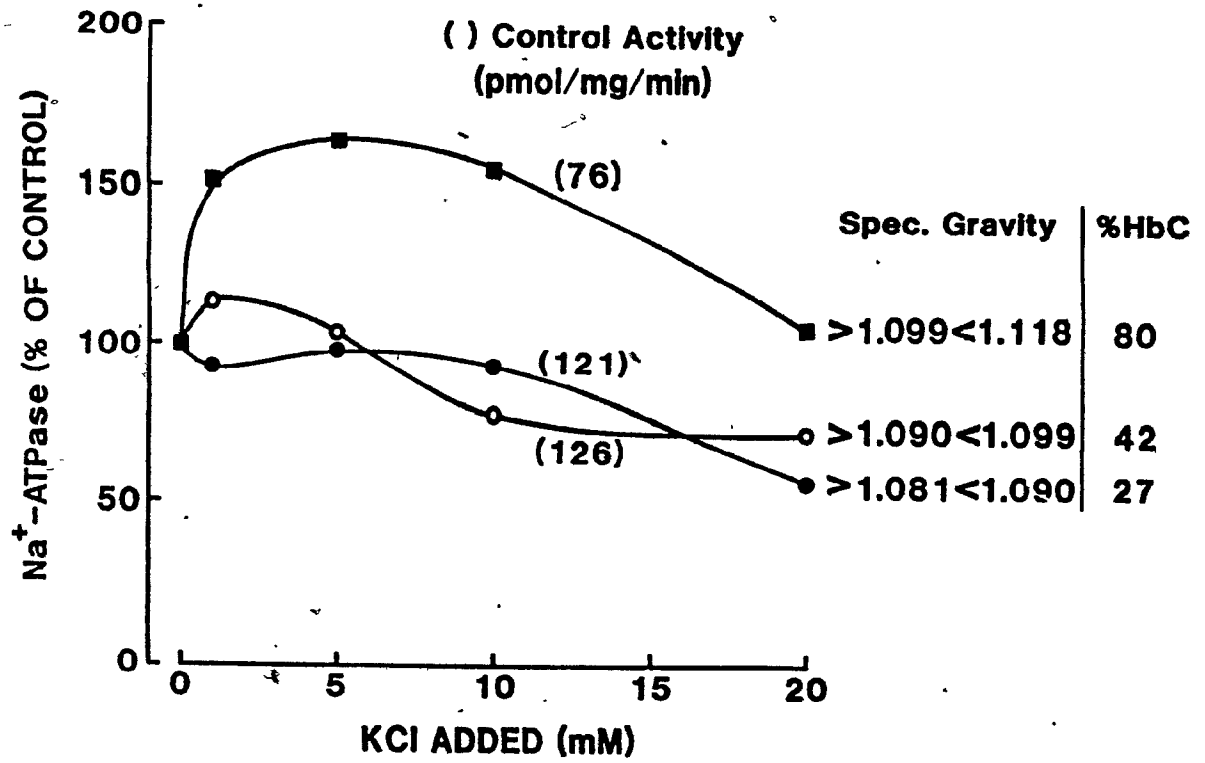
In this experiment an HK (#89) animal (heterozygote Hb A and Hb B) was bled repeatedly so that most of the Hb A was replaced by Hb C. Thirty-four days after the last bleeding a sample (100 ml) of blood was removed and fractionated as described in Methods and Materials (Section 2.1.D.). The cells were lysed and the hemoglobins separated and quantitated as described in Section 2.5. The Hb C and Hb A are expressed as a percentage of the total Hb A plus Hb C.

| Range of Specific Activity | % Hb A | %Hb C |
|---|---|---|
| 1. >1.078 <1.098 | 73 | 27 |
| 2. >1.09 <1.098 | 64 | 36 |
| 3. >1.098 <1.106 | 35 | 65 |
| 4. >1.106 <1.114 | 0 | 100 |

## Figure 9

### Changes in Percent Hemoglobin C and $Na^+$-ATPase Activity and Kinetics

For this experiment an HK animal (heterozygote, Hb A and B) was bled repeatedly so that most of the Hb A was replaced by Hb C. Twenty-seven days after the last bleeding the red cells were fractionated according to their density using mixtures of dibutyl- and diethylphthalate, as described in Section 2.1.D. The cell fractions were identified according to the densities of the two oil mixtures of which they were heavier and lighter, respectively. The red cells thus separated were used for the isolation of membranes for $Na^+$-ATPase measurements and lysates for hemoglobin electrophoresis. $Na^+$-ATPase was assayed at varying KCl concentrations as described in Section 2.6.D. The hemoglobins (Hb A, Hb B, and Hb C) were quantitated as described in Methods and Materials. Hb C is expressed as a percentage of total Hb A plus Hb C. Values in parentheses represent the activity obtained in the absence KCl (control) in picomoles per milligram per minute. From Blostein, Drapeau, Benderoff, and Weigensberg (253).


( ) Control Activity
(pmol/mg/min)
200
150
100
50
0
Na+-ATPase (% OF CONTROL)
(76)
(121)
(126)
0
5
10
15
20
KCl ADDED (mM)
Spec. Gravity
%HbC
>1.099<1.118
80
>1.090<1.099
42
>1.081<1.090
27

# 4 THE ROLE OF METABOLIC STATUS IN MATURATION-ASSOCIATED LOSSES IN MEMBRANE TRANSPORT

As mentioned earlier, the activities of many enzymes, membrane transport sytems, and other membrane components (see Introduction) are dramatically reduced during maturation and aging of the mammalian red blood cell. These changes have been shown indirectly by comparing activities in young and old populations of red blood cells and more directly, by following the activity in newly formed cells as they mature and increase in density during *in vivo* maturation. Loss in transport activities has also been seen during *in vitro* maturation of reticulocytes. Thus, amino acid transport carried out by neutral exchange (208) and electrogenic $Na^+$-dependent glycine uptake (235), as well as nucleoside transport (185), are rapidly lost after only a few days in culture; sodium pump activity is also reduced, particularly during maturation of sheep erythrocytes of the low potassium genotype as descried in the Introduction and the previous chapter. The mechanism(s) underlying these changes is (are), however, unknown. As a first step toward gaining insight to this process, the possible role of metabolic energy in maturation- and aging-associated functional losses was examined. The markers used for this study were the $Na^+,K^+$-pump and the $Na^+$-dependent glycine transport system.

## 4.1 Effects of Energy Depletion on the Loss of $Na^+$-Dependent Glycine Transport

As described in the Introduction, five glycine transport systems have been described in the human red blood cell (201). Two are sodium dependent, and the

difference between them appears to be their anion dependency. One transport system is chloride dependent, while the other is chloride independent. In an experiment illustrated in Figure 10, reticulocytes from an anemic sheep were equilibrated with either sulfate or chloride at an hematocrit of 5% after which $Na^+$-dependent $^{14}C$-glycine influx was measured. As shown, the substitution of most of the chloride for sulfate caused a decrease in $Na^+$-dependent glycine transport by 70% - 80%; this is consistent with the conclusion that the chloride-dependent system is the main $Na^+$-dependent glycine transport system. The residual activity in the sulfate medium may, in fact, be due to small amounts of chloride still present.

The effect of metabolic energy on the loss of $Na^+$-dependent glycine transport was studied during in vitro culture for periods of up to 2 days at 37°C. In the experiment shown in Figure 11, $Na^+$-dependent glycine transport was measured in the presence of ouabain to inhibit sodium pump activity and gramicidin D in order to abolish any electrochemical cation gradients. This was done so that any transport differences between fed and starved cells after culture could not be attributed to an electrochemical cation gradient difference. It was observed that after 17 hours incubation at 37°C, glycine transport was reduced markedly (60%) in the fed, ATP replete cells (sample B) or partially depleted cells (sample C) compared to the 0°C contol (sample A). In contrast, transport was reduced 6-13% in the starved, ATP depleted cells (samples D and E).

**Figure 10**

**Effect of Anions on $Na^+$-Dependent Glycine Uptake in Sheep Reticulocytes.**

Reticulocytes (90%) were isolated (Section 2.1.C) from an anemic LK sheep (#91) and incubated at approximately 5% hematocrit overnight at 4°C in a balanced salt solution (154 mM NaCl, 30 mM sucrose, 6 mM glucose and 5 mM Tris-HCl pH 7.4 or 103 mM $Na_2SO_4$, 30 mM sucrose, 6 mM glucose and 5 mM Tris-$H_2SO_4$ pH 7.4) containing either the chloride or sulfate anion. After the cells were washed twice with isotonic $MgCl_2$ or $MgSO_4$, the glycine uptake was carried out as follows. Reticulocytes (0.08 ml) in isotonic $MgCl_2$ or $MgSO_4$ were added to 0.74 ml of a balanced salt solution (5.5 mM potassium phosphate pH 7.4, 6.6 mM glucose, 1.1 mM $MgCl_2$ or 1.65 mM $MgSO_4$, 5.5 mM KCl or 3.7 mM $K_2SO_4$, 11 mM Tris-HCl, pH 7.4, or 11 mM Tris-$SO_4$, pH 7.4, 165 mM NaCl or KCl or 110 mM $Na_2SO_4$ or $K_2SO_4$, with 1.1 mM ouabain) containing either the chloride or sulfate anions. The samples were preincubated 5 minutes at 37°C at which time 0.09 ml of 1 mM $^{14}C$-glycine was added (specific activity 550 cpm/nmole). At specified time points, 0.2 ml of the red blood cell suspension was removed and the $^{14}C$-glycine uptake was determined as described in Section 2.6.A.1.

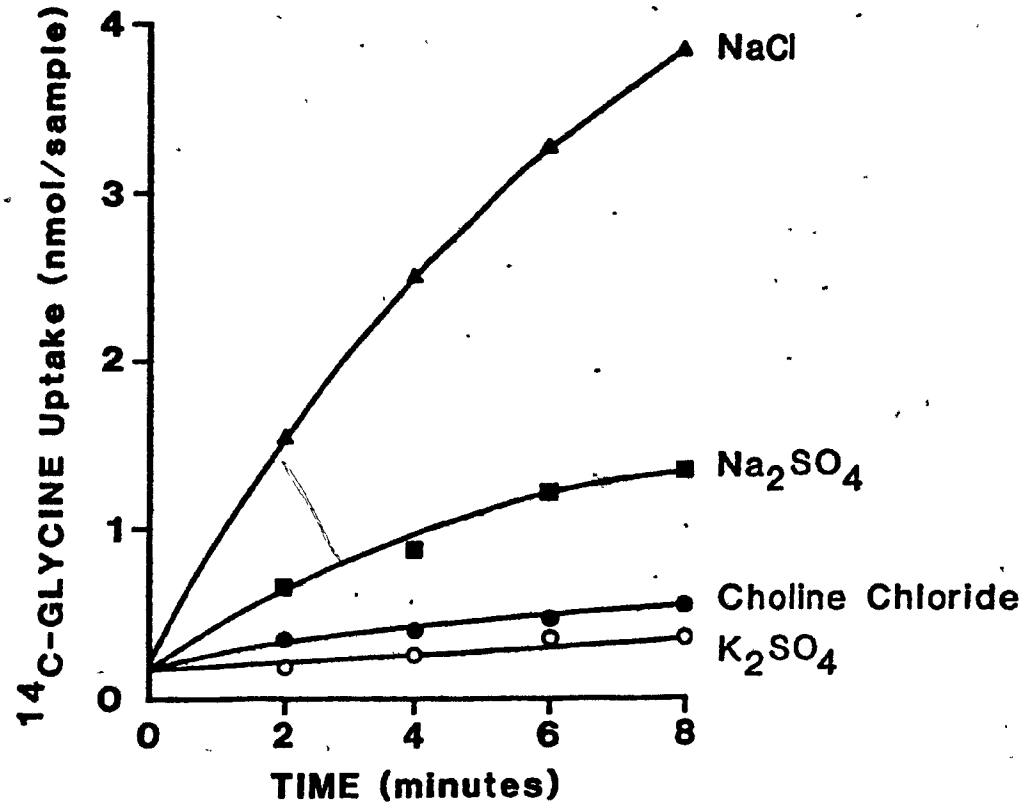
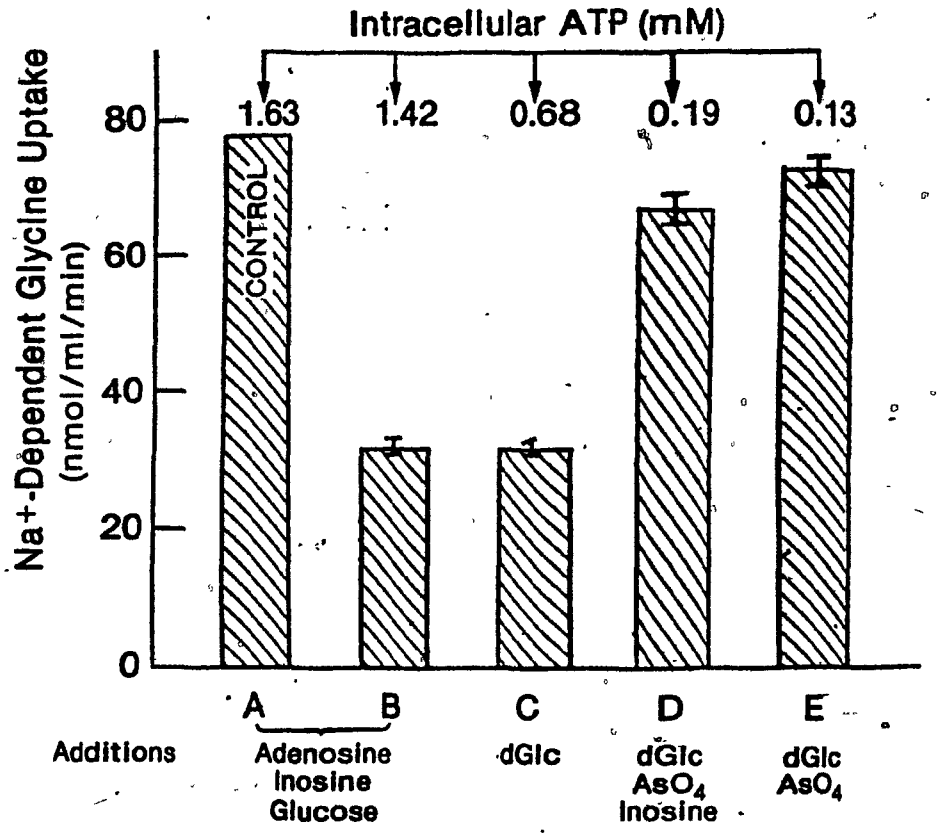

$^{14}$C-GLYCINE Uptake (nmol/sample)
0
1
2
3
4
0
2
4
6
8
TIME (minutes)
NaCl
$Na_2SO_4$
Choline Chloride
$K_2SO_4$

**Figure 11**

**The Effect of the ATP Level on $Na^+$-Dependent Glycine Transport In Vitro**

Relationship between ATP level and $Na^+$-dependent glycine transport activity in cultured reticulocytes was studied. Immature sheep erythrocytes (50% retulocytes) from a high $K^+$ sheep were incubated in tissue culture medium for 17 hours as described in Section 2.3.B. When arsenate (10 mM) was included, phosphate (1 mM) was omitted. Two samples (A and B) were supplemented with 10 mM inosine and 5 mM adenosine. One was incubated at 0°C (control, sample A) and the other, at 37°C (sample B). In the third sample (C) 20 mM 2-deoxyglucose (dGlc) was the only added substrate. Sample D had 20 mM dGlc with 20 mM sodium arsenate and 20 mM inosine, and sample E had dGlc and arsenate. The final pH was 7.6. At the end of the incubation, the cells were assayed as described in Section 2.6.A.1. Intracellular ATP levels (mM) (Section 2.8) at the end of 1 hour of culture were 1.5, 0.59, 0.86 and 0.49 in samples B, C. D. and E respectively. Mean cell hemoglobin concentrations (g/ml of cells) were 0.303, 0.336, 0.333, 0.331 and 0.318 in samples A, B, C, D, and E, respectively. Glycine uptake values (nmol/ml of cells per min) in $K^+$ medium were 39.0 ± 0.1, 15.7 ± 0.3, 15.1 ± 0.1, 24.0 ± 0.4 and 24.0 ± 0.5 in samples A, B, C, D and E, respectively. Values shown are uptakes in $Na^+$ medium after subtraction of the $K^+$ baseline values (15.7 ± 0.3 nmol/ml of cells per min) measured in the 37°C ATP-replete sample (B). Each value is the mean ± S.E.M. From Weigensberg and Blostein (287).


Intracellular ATP (mM)
1.63
1.42
0.68
0.19
0.13
Na+-Dependent Glycine Uptake
(nmol/ml/min)
80
60
40
20
0
CONTROL
A
B
C
D
E
Additions
Adenosine
Inosine
Glucose
dGlc
dGlc
AsO4
Inosine
dGlc
AsO4

ATP depletion was accomplished by substituting 2-deoxyglucose for glucose and adding arsenate with or without inosine. In no case was hemolysis observed; however, some methemoglobin formation was found to occur in the starved cells. Other experiments showed that arsenate had no effect on activity of cells kept at 0°C; furthermore, activity in arsenate-treated cells after 37°C incubation was not due to an effect of arsenate, per se, since, as shown in Table 3, similar results were obtained using the proton conductor carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) to deplete ATP. Thus, after 16 hours incubation, $Na^+$-dependent glycine transport was reduced 35% in fed cells (4.24 mM ATP), but only 14% in depleted cells (0.01 mM ATP).

Although the extracellular pH remained constant (pH 7.6) throughout the incubation, it is likely that the intracellular pH of fed cells was shifted to more acidic values, relative to starved cells, as a consequence of their presumably higher concentration of organic phosphates (288). The question is whether a difference in intracellular pH is a primary factor underlying the process resulting in a greater loss of transport activity in the fed cells. The assumption that the intracellular pH varies directly with extracellular pH, albeit to different extents in fed and starved cells, led to the testing of the effect of long-term incubation of cells in media of various pHs. The results in Figure 12 indicate that loss in transport activity in ATP replete cells decreases as medium pH is decreased, but remains relatively constant in ATP-depleted cells. These results indicate that intracellular pH differences are not the basis for the differences

## Table 3
## The Effect of Different Methods of ATP Depletion on the Loss of $Na^+$-Dependent Glycine Transport

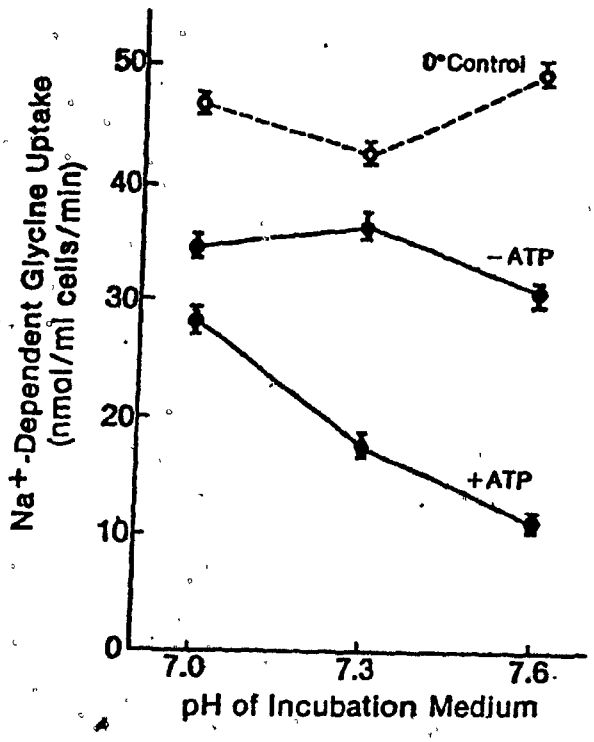
Immature sheep erythrocytes (60% reticulocytes) from an LK (#8279) sheep were incubated as described in Section 2.3.B for 16 hours. When arsenate (10 mM) was included, phosphate (1 mM) was omitted. Four samples (A,B,C,D) were supplemented with 10 mM inosine and 5 mM adenosine. One (control, A) was incubated at 0°C while the others (B, C and D) were incubated at 37°C. Sample C had 10 mM arsenate, sample D was supplemented with 20 mM 2-deoxyglucose and 0.02 mM CCCP. The final pH was 7.6. At the end of the incubation, $Na^+$-dependent glycine uptake was measured as described in Section 2.6.A.1. The mean cell hemoglobin concentrations (g/ml cell) were 0.279, 0.292, 0.294 and 0.282 in samples A, B, C and D, respectively. Glycine uptake values (nmol/ml cell/min) in $K^+$ medium were 10.2 ± 0.2, 8.4 ± 0.2, 9.4 ± 0.1 and 9.9 ± 0.9 in samples A, B, C and D, respectively. Values shown are uptakes in $Na^+$ medium after subtraction of the $K^+$ baseline value for B which was measured in the fed reticulocytes incubated at 37°. Each value is the mean ± S.E.M.

| Incubation Conditions | $Na^+$-dependent $^{14}C$-glycine uptake nmol/min/ml cells | ATP Concentration (mM) |
|---|---|---|
| A. 0°C, Control Fed | 18.1 ± .81 | 3.10 |
| B. 37°C, Fed | 11.8 ± .41 | 4.24 |
| C. 37°C Starved, arsenate | 17.9 ± .32 | 0.04 |
| D. 37°C, Starved, CCCP | 15.5 ± .57 | 0.01 |

## Figure 12

### The Effect of pH on $Na^+$-Dependent Glycine Transport In Vitro

Reticulocytes (55%) from an HK animal were incubated for 17 hours (Section 2.3.B) as described in Figure 11, except that the pH values of the media were adjusted to the pH value shown. The control (0°C), ATP-replete and ATP-depleted cells contained the same additives as described in the legend to Figure 11 for samples A, B and E respectively. The cells were assayed as described in Section 2.6.A.1. Each value is the mean ± S.E.M. From Weigensberg and Blostein (287).


0°Control
-ATP
+ATP
Na+-Dependent Glycine Uptake (nmol/ml cells/min)
50
40
30
20
10
0
7.0
7.3
7.6
pH of Incubation Medium

observed in fed and starved cells. In fact, the loss in energy replete cells is enhanced as pH is increased.

The question of whether this energy-requiring loss could be reactivated was addressed in another experiment described in Table 4. In this experiment, reticulocytes were incubated as described in Table 3. After 16.5 hours, an aliquot of starved cells was washed and incubated an additional 24 hours in a replete medium (i.e. a medium containing the same additions as the 'fed' medium). The results indicate that the rate of loss of $Na^+$-dependent glycine transport can be increased by refeeding starved cells. Thus, between 16.5 and 40.5 hours, transport activity decreased fromn 16.2 to 10.2 nmol/ml cell/min in starved cells and from 16.2 to 7.2 nmol/ml cell/min in refed cells.

## 4.2 Effects of Energy Depletion on the Loss of Sodium Pump Sites

In order to assess the effects of metabolic depletion on the loss of sodium pump activity, it is important that effects observed do not reflect changes in intracellular ATP, $Na^+$ and $K^+$ concentrations per se. Therefore, the number of sodium pump sites was measured indirectly by estimating the total number of specific $^3H$-ouabain binding sites in cells adjusted to the same initial high $Na^+$, low $K^+$ content and assayed in high $Na^+$, $K^+$ free media. Prior to assessing the effects of metabolic depletion on the loss of sodium pump sites, it was necessary to first investigate the effects of both ATP and of artificially altering the cation content of erythrocytes (using the nystatin method) on the rate and total number of specific ouabain binding sites.

**Table 4**
**The Effect of Refeeding Upon the Energy Modulated Loss of $Na^+$-Dependent Glycine Transport**

Immature erythrocytes (45% reticulocytes) from an HK (#474) sheep were incubated for the first 16.5 hours as described in Table 3 (samples A, B, C respectively). One aliquot of starved cells was washed free of depleting medium and then incubated an additional 24 hours in substrate replete medium. At the end of the incubation $Na^+$-dependent glycine uptake was measured as described in Section 2.6.A.1. Values shown are uptakes in $Na^+$ medium after subtraction of the $K^+$ baseline values which were 5.6 ± 0.2 nmol/ml cell/min for the top fraction (16.5 hour fed sample) and 3.7 ± 0.2 nmol/ml cells/min for the bottom fraction (16.5 hour fed cells). Each value is the mean ± S.E.M.

| Cell Fraction | Incubation Conditions | $Na^+$-Dependent $^{14}C$-Glycine Uptake nmol/ml cell/min | ATP Concentration (mM) |
|---|---|---|---|
| Top | 0°C, (control) 16.5 hours | 25.1 ± 0.7 | 1.19 |
| | 37°C, 16.5 hours | | |
| | fed | 5.0 ± 0.4 | 1.56 |
| | starved | 16.2 ± 0.5 | 0.03 |
| | 37°C, 40.5 hours | | |
| | starved | 10.2 ± 0.8 | 0.01 |
| | refed, after 16.5 hours | 7.2 ± 0.3 | 0.09 |
| Bottom | 37°C, 16.5 hours | | |
| | fed | 0.2 ± 0.3 | 0.05 |

Figure 13 indicates that intracellular ATP, per se, increases only slightly the rate of ouabain binding. The small increase in the number of specific $^3$H-ouabain binding sites due to the nystatin treatment is presumably due to alteration in the intracellular cation content, since it has been shown by Hobbs and Dunham (289) that increasing intracellular $Na^+$ and/or decreasing intracellular $K^+$ increases the rate of specific ouabain binding and the total number of sites. On the basis of these results all subsequent ouabain binding assays were carried out for 2 - 2½ hours at 37°C. The results of two experiments are shown in Figures 14 and 15. One experiment is typical of a series carried out with reticulocytes from sheep of the low $K^+$ genotype (Figure 14); the other (Figure 15) is from a series carried out with high $K^+$ sheep reticulocytes. As illustrated, the loss of ouabain binding sites was significantly less in starved cells, particularly during the 17 to 40 hour period of the long term incubation. Similar results (Table 5) were obtained when energy stores were depleted by addition of CCCP (0.02 mM). Thus, specific $^3$H-ouabain binding was reduced 82% in fed cells and 48% in depleted cells.

Table 6 shows $Na^+$-dependent glycine transport and the number of $^3$H-ouabain binding sites estimated concurrently in the same samples of cultured cells. As shown, both systems decreased to approximately similar extents in fed and in starved cells. At the end of incubation, $Na^+$-dependent glycine transport and the number of $^3$H-ouabain binding sites in the starved cells were 2.5 and 2.0 times, respectively, the levels observed in the fed cells.

## Figure 13

**The Effect of Nystatin Treatment and Energy Status (ATP Level) on the Rate of Ouabain Binding and the Total Number of Ouabain Binding Sites**

Reticulocytes from a LK animal (#8279) were isolated as described in Section 2.1.C and incubated overnight at 0°C as described in Section 2.3.B, except that one-half of the reticulocytes had additions of 20 mM 2-deoxyglucose and 10 mM sodium arsenate (ATP depleting media), while the remaining cells were incubated in 5 mM glucose, 5 mM adenosine and 10 mM inosine (ATP repleting media). One-half of each sample was then treated with nystatin (Section 2.6.A.2) after which the number of ouabain binding sites was determined (Section 2.6.C). Non-specific $^{3}H$-ouabain binding relative to specific binding was $\leq$ 8%.

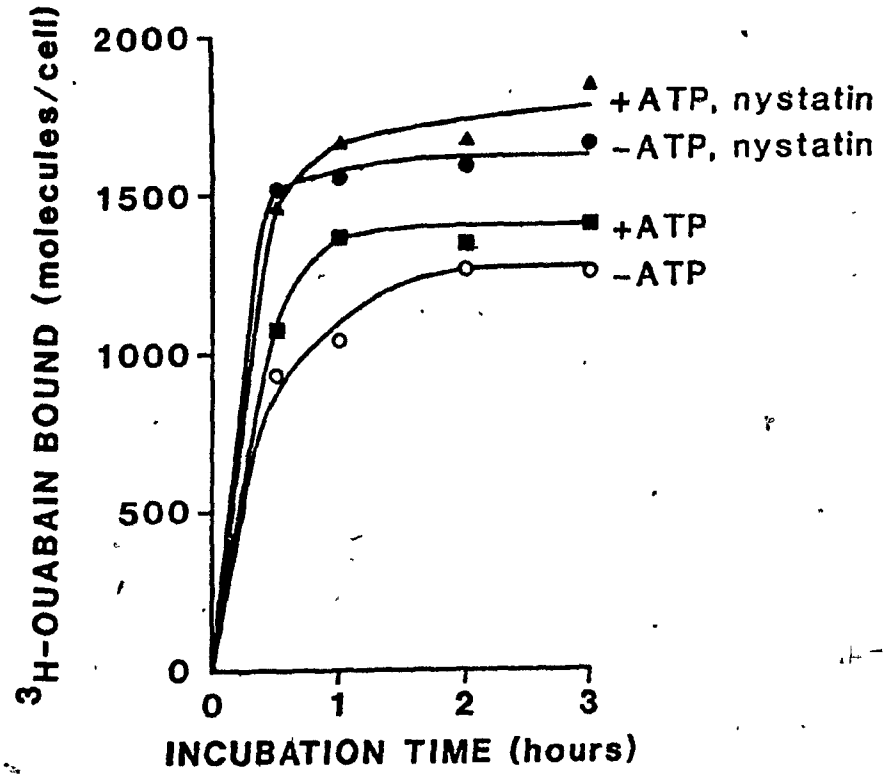
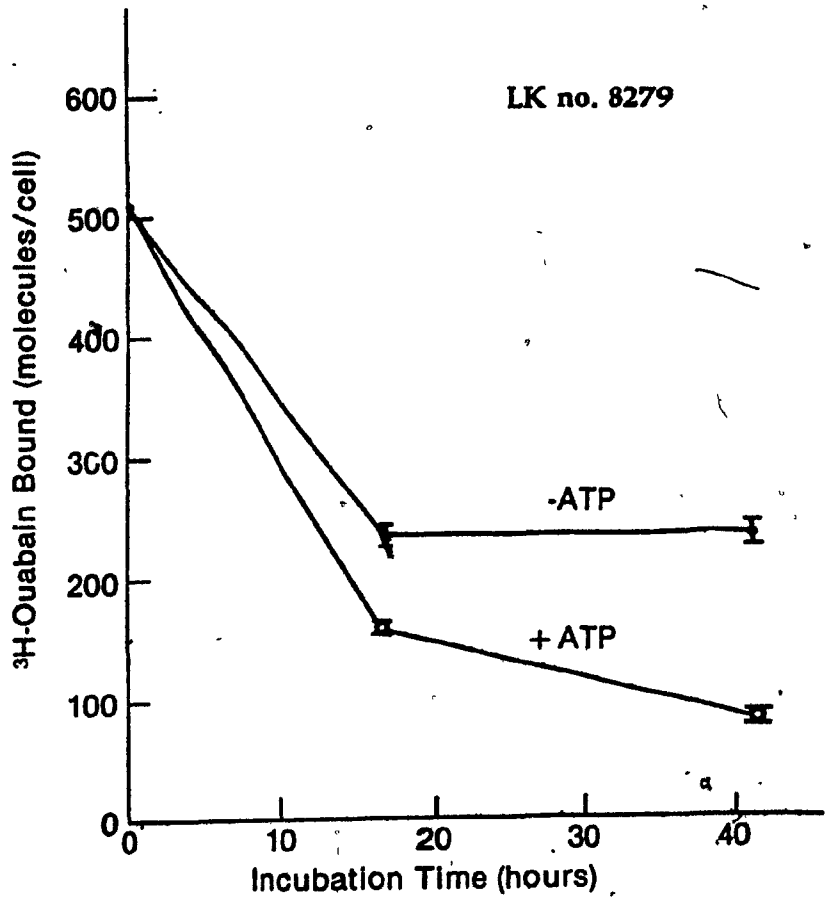

2000
1500
1000
500
0
0
1
2
3
$^3$H-OUABAIN BOUND (molecules/cell)
INCUBATION TIME (hours)
+ATP, nystatin
-ATP, nystatin
+ATP
-ATP

**Figure 14**

**The Effect of the ATP Level on Ouabain Binding in LK Sheep**

Reticulocytes (47%, from a low-$K^+$ animal) were incubated for 41½ hours, then $^3$H-ouabain binding sites were assayed (Section 2.6.C). The control (0°C), ATP-replete, and ATP-depleted cells contained the same additions as samples A, B, and E, respectively, described in the legend to Figure 11. Nonspecific $^3$H-ouabain binding relative to specific binding was ≤ to 2%. Each value is the mean ± S.E.M. From Weigensberg and Blostein (287).


LK no. 8279
600
500
400
300
200
100
0
0
10
20
30
40
$^3$H-Ouabain Bound (molecules/cell)
Incubation Time (hours)
-ATP
+ ATP

## Figure 15

### The Effect of the ATP Level on Ouabain Binding in HK Sheep

Reticulocytes (50%, from a high-$K^+$ animal) were incubated for 39½ hours. In this experiment $^3H$-ouabain binding sites were assayed after separation of the cells from the medium by centrifugation through dibutylphthalate (Section 2.6.C alternate method). Nonspecific $^3H$-ouabain binding was $\leq$ 10% relative to specific binding. Each value is the mean ± S.E.M. From Weigensberg and Blostein (287).

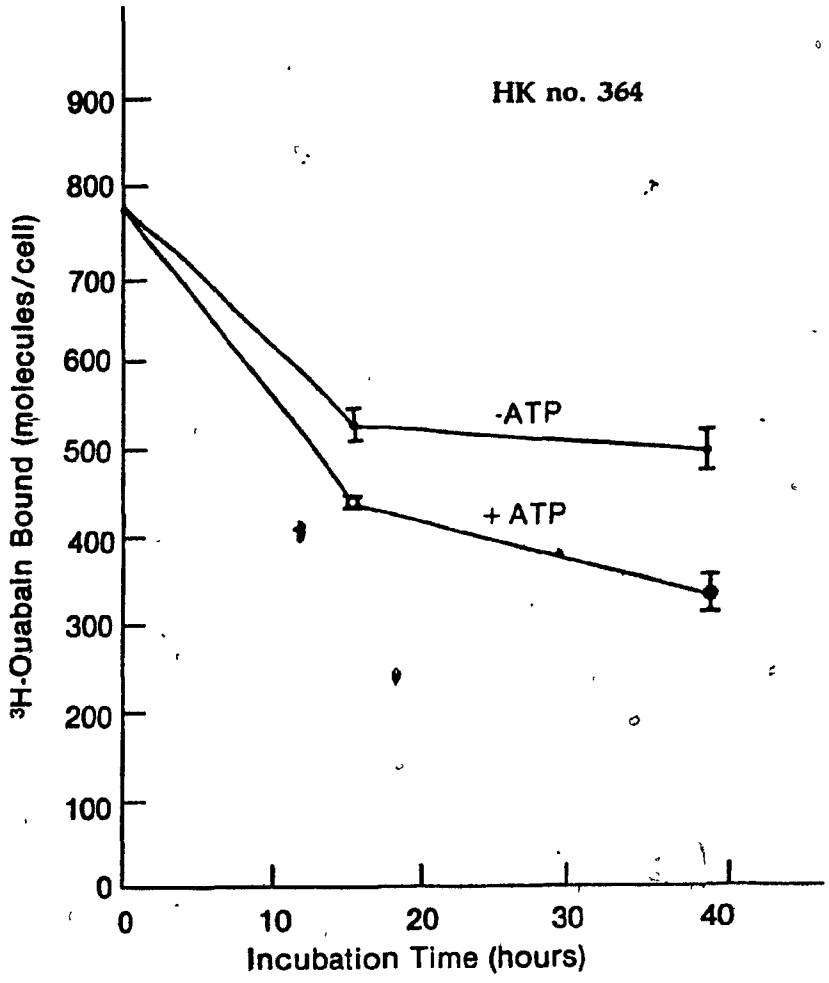

HK no. 364
900
800
700
600
500
400
300
200
100
0
-ATP
+ ATP
0
10
20
30
40
Incubation Time (hours)
³H-Ouabain Bound (molecules/cell)

**Table 5**
**The Effect of Different Methods of ATP Depletion on the Loss of Specific $^{3}H$-Ouabain Binding Sites**

Cells from the same incubation as described in Table 3 were used for measurements of $^{3}H$-ouabain binding. Ouabain binding was determined by the cell washing method described in Section 2.6.C. Non-specific $^{3}H$-ouabain binding ranged from 4 to 7 percent of specific binding observed in the control sample.

| Incubation Conditions | Specific $^{3}H$-Ouabain Binding (molecules/cell) | ATP Concentration (mM) |
|---|---|---|
| A. 0°C, Control Fed | 984 ± 20 | 3.10 |
| B. 37°C, Fed | 177 ± 8 | 4.24 |
| C. 37°C, Starved, Arsenate | 644 ± 28 | 0.04 |
| D. 37°C, Starved, CCCP | 509 ± 13 | 0.01 |

**Table 6**
**Concomitant Changes in Glycine Transport, Ouabain Binding Sites, MCHC and Reticulocyte Count During Culture of Fed and Starved Reticulocytes**

The top and bottom 5% of centrifuged cells from an anemic sheep (# 474, HK genotype) were incubated as described in the legends to Figures 13, 14 and 15. Control (100%) values for $Na^+$-dependent $^{14}C$-glycine influx and $^{3}H$-ouabain sites were 47.4 nmol/ml of cells per min and 338 molecules per cell, respectively.

[1]Most (70%) of the reticulocytes counted in the 40½ hour fed sample had only weakly detectable reticulum, whereas those in the other samples had clearly visible, well-stained reticulum. From Weigensberg and Blostein (287).

| Cell Fraction | Incubation Conditions | Activity, % of Control: $Na^+$-Dependent $^{14}C$-glycine Uptake | Molecules of Ouabain Bound Per Cell | MCHC, g/ml of cells | Reticulcoytes, % |
|---|---|---|---|---|---|
| Top | 0°C (control) | 100 | 100 | 0.244 | 78 |
| | 37°C, 17 hr | | | | |
| | Starved | 53 | 53 | 0.247 | 67 |
| | Fed | 38 | 35 | 0.261 | 72 |
| | 37°C, 40½ hr | | | | |
| | Starved | 53 | 39 | 0.262 | 61 |
| | Fed | 20 | 18 | 0.271 | 51[1] |
| Bottom | 0°C | 5 | 10 | 0.324 | 3 |

## 4.3 Changes in Hematological Parameters

Changes in cell morphology and cell volume were followed on portions of the same cells as those used to measure the transport systems (Table 6). As shown in Table 6, there was a slight (< 11%) increase in mean cell hemoglobin concentration (MCHC) during maturation. Differences in MCHC values or reticulocyte counts between fed and starved cells were either small as shown, or not observed (for example see Figure 11 legend). At the end of incubation, a striking decrease in the number of reticulocytes with clearly visible, well stained reticulum was apparent in the fed cells compared to the starved cells. This observation is reminiscent of the slowing of maturation under conditions of glucose deprivation reported almost forty years ago. (For review see reference 290.)

## 4.4 Changes in Other Cellular Functions

The foregoing results raise the issue of whether metabolic depletion has a general slowing effect on all maturation-associated changes. Therefore, the effect of metabolic depletion on changes in activity of several cytoplasmic enzymes, namely glucose-6-phosphate dehydrogenase, hexokinase and phosphoglucose isomerase, all of which decrease to varying extents during *in vivo* maturation (54) was investigated. The results (experiments carried out by R. Blostein and O. W. Kan) showed that ATP depletion did not affect the decrease in activity. In the bottom fraction (mature cells) of centrifuged cells from anemic sheep blood, glucose-6-phosphate dehydrogenase activity was 0.33 IU/g hemoglobin. How-

ever, the activity of the reticulocyte-enriched top fraction of density separated cells was 1.99 IU/g hemoglobin. During incubation at 37°C the activity of the reticulocyte-enriched fraction decreased as follows: 1) after 16 hours, the activity decreased 21% and 18% in fed and starved cells, respectively, 2) after 40 hours, the activity decreased 46% and 41% in starved and fed cells respectively. Significant differences in activities of hexokinase and phosphoglucose isomerase following in vitro maturation were similarly not detected in fed and starved cells.

# 5 TRANSPORT IN SHEEP RETICULOCYTE MEMBRANE VESICLES

In the studies described in Chapter 4, it was observed that the energy status and/or ATP level have a key role in the mechanism underlying the loss of membrane transport during maturation of sheep reticulocytes. In order to determine whether specific cytoplasmic factors are involved in this process, and if so, the mechanism of the effect, I have attempted to mimic in vivo conditions by using a reconstituted system. This system was comprised of inside-out vesicles (IOV), derived from reticulocytes and incubated with cytoplasm derived from either reticulocytes or mature erythrocytes. For this purpose, IOV and ROV from sheep reticulocytes were prepared by a modification of the procedure used to prepared IOV and ROV from mature human erythrocytes (264). This procedure was described in Methods and Materials and illustrated in Figure 4. The sealed membrane vesicles obtained by this procedure were first characterized with respect to their sidedness. This was accomplished by using the membrane enzyme markers, acetylcholinesterase which is exposed to the extracellular milieu (IOV) and glyceraldehyde-3-phosphate dehydrogenase which is exposed to the intracellular milieu (ROV). Thus, the percentage of IOV or ROV was determined by measuring the acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase activities, respectively, in the presence and absence of detergent. Table 7 shows that the percent of sealed vesicles with the desired orientation is usually greater than 80% of the total sealed vesicles.

## Table 7
## Sidedness of Vesicle Preparations

Reticulocytes were isolated as described in Section 2.1.C. Methods to obtain either sealed IOV or ROV are described in Section 2.2.B and Figure 4. The sidedness of each vesicle preparation was determined as desribed in Sections 2.7.A and 2.7.B. The percentage of IOV and ROV are expressed as the percentage of each type of vesicle compared to the percentage of total sealed membrane vesicles.

| Preparation No. | Method of Preparation | Sheep No. | Reticulocytes (%) | IOV (% of total vesicles) | ROV |
|---|---|---|---|---|---|
| 24-3-81 | IOV | 9 | 15 | 77 | 23 |
| 6-4-81 | IOV | 9 | 70 | 91 | 9 |
| 2-4-81 | IOV | 1023 | 68 | 100 | 0 |
| 24-2-82 | IOV | 91 | 51 | 97 | 3 |
| 24-3-82 | ROV | 1023 | 80 | 0 | 100 |
| 21-4-82 | ROV | 364 | 40 | 0 | 100 |

Before attempting to investigate the effects of cytoplasmic factors on the loss of membrane transport in a 'reconstituted' vesicle system, it was necessary to characterize the membrane transport systems ($Na^+,K^+$-pump and $Na^+$-dependent glycine transport) to ensure that the vesicle transport functions remain essentially similar to intact reticulocytes. In a series of experiments, the $Na^+,K^+$-pump was examined with respect to ATP-dependency and to inhibition by cardiac glycosides. The aspects of the glycine transport system examined using the reticulocyte-derived IOV amd ROV were: (i) the sodium and chloride dependency of the glycine transport system, (ii) the effect of the sodium electrochemical gradient on glycine transport, (iii) the extent of glycine accumulation inside the vesicles, (iv) sodium: glycine coupling ratio, and (v) the apparent $K_m$ values for both sodium and glycine.

## 5.1 Characterization of the Directional Symmetry of $Na^+$-dependent Glycine Transport and the $Na^+,K^+$-Pump in IOV and ROV

The question of symmetry or asymmetry of $Na^+$-dependent solute transport systems has been addressed in several studies, including those using lysed and restored pigeon red cells (230), intact Ehrlich ascites tumor cells (291-295), and rabbit ileum (296). In the former studies, Vidaver and Shepherd showed that although the system operates in a reversible mode whereby $Na^+$-driven net glycine flow appears to occur in either direction, the system is kinetically asymmetric.

One of the difficulties inherent in experiments with cells is that it cannot be ascertained if the behaviour, particularly efflux kinetics, is not complicated by the intracellular environment. Even in lysed and restored cells, the interior milieu is not completely replaced. Although membrane vesicles offer the advantage of eliminating this problem, until now preparations of distinct sidedness, particularly inside-out, which exhibit organic solute transport, have not been achieved (for a review see 297).

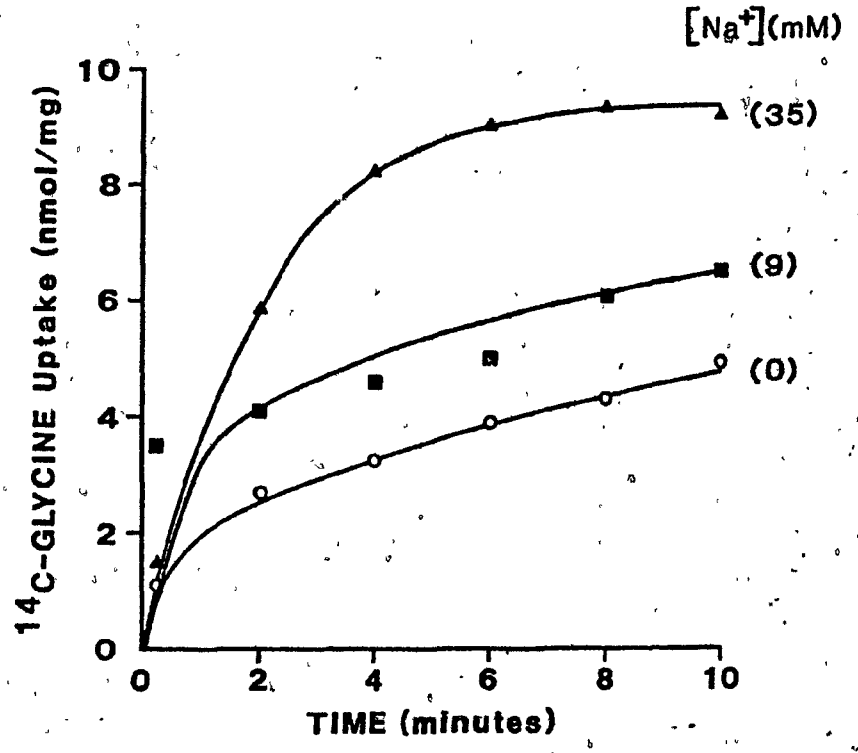
Since reticulocytes possess relatively active amino acid transport activities (208,235), these vesicles, particularly those which are inside-out, have unique advantages for examining the sidedness of amino acid transport in mammalian cells and the role of cytoplasmic factors in regulating the transport activity. A series of experiments were first carried out to test whether $Na^+$-dependent glycine transport in sheep reticulocyte vesicles can occur in the reverse mode where uptake is equivalent to normal efflux.

Figure 16 depicts a preliminary experiment to test the effect of extravesicular $Na^+$ on the time course of $^{14}C$-glycine uptake into $Na^+$ free (choline chloride-loaded) vesicles (IOV) incubated in media with and without 9 or 35 mM NaCl. As shown, glycine influx is stimulated by extravesicular $Na^+$ (normally cytoplasmic $Na^+$). A more detailed study of the kinetic effects of $Na^+$ are depicted in Figures 28 and 29. In those experiments initial rates of $^{14}C$-glycine influx were measured. In order to determine what percentage of the $Na^+$-dependent glycine transport system that is chloride dependent, IOV

**Figure 16**

**The Effect of Varying Sodium Concentrations on Glycine Uptakes**

IOV (approximately 0.5 mg protein/ml) derived from reticulocytes (70%) were equilibrated overnight at 0°C, then 23 minutes at 37°C with 38 mM choline chloride, 2 mM KCl, and 5 mM MOPS (Tris form) pH 7.4. They were then concentrated by centrifugation (12,000 x g for 10 minutes at 4°C) to 1.1 mg protein/ml. The reaction was started by adding 0.6 ml prewarmed (37°C) isoosmotic medium containing 1.1 mM $^{14}$C-glycine (1.93 x $10^3$ cpm/nmole) 38 mM choline chloride or NaCl as indicated, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM MOPS (Tris form) pH 7.4 to 0.06 ml prewarmed (37°C) vesicles. The reaction was terminated at the indicated times and the vesicles (0.01 ml aliquots) were filtered and washed as described in Section 2.6.B.1. Ninety percent of the total sealed membranes were IOV and 10% were ROV.


[Na+](mM)
(35)
(9)
(0)
14C-GLYCINE Uptake (nmol/mg)
TIME (minutes)
0
2
4
6
8
10

were equilibrated with either an all chloride or an all sulfate medium before $Na^+$-dependent glycine influx was measured. Figure 17 illustrates that more than 94% of the $Na^+$-dependent glycine influx is chloride-dependent.

In another experiment, ATP-stimulated $Na^+$-pump activity in reticulocyte vesicles was demonstrated under conditions in which concurrent measurements of ATP-dependent $^{22}Na$ uptake and unidirectional $^{14}C$-glycine efflux from $^{14}C$-glycine-loaded vesicles were carried out. As shown, ATP-stimulation of $^{22}Na^+$ uptake (Figure 18) and $^{14}C$-glycine efflux (Figure 19) were observed. These results support the notion that a sodium pump-mediated increase in intravesicular $Na^+$ (and/or change in membrane potential) stimulates $Na^+$-dependent amino acid efflux from these inverted membrane vesicles.

In order to further ascertain that the $Na^+,K^+$ pump mediated $Na^+$ influx affects glycine uptake, experiments were carried out measuring glycine influx with and without ATP and/or a sodium pump inhibitor (strophanthidin) present in the medium.

Figure 20 depicts a time-course of $^{14}C$-glycine uptake into $Na^+$-free (choline chloride-loaded) vesicles incubated in media with and without 35 mM NaCl. As expected, glycine influx is stimulated by extravesicular $Na^+$ (normally cytoplasmic $Na^+$), and the addition of ATP decreases $Na^+$-stimulated uptake. This decrease could be either a direct action of ATP on the $Na^+$-dependent glycine transport system, or indirect, whereby ATP, by virtue of its direct access to the cyto-

**Figure 17**

**The Effect of Chloride and Sulfate Anions on $Na^+$-Dependent Glycine Uptake**

Vesicles (100% IOV) derived from reticulocytes (30%) were equilibrated with either 50 mM choline chloride, 50 mM KCl, 1 mM $MgCl_2$ and 10 mM MOPS (Tris form) pH 7.4, or 25 mM $K_2SO_4$, 63.5 mM $MgSO_4$ and 10 mM MOPS (Tris form) pH 7.4 overnight at 4°C and then 30 minutes at 37°C before being concentrated to 2.9 mg protein/ml. The assay was started by adding 0.1 volume of prewarmed (37°C) IOV (either chloride or sulfate equilibrated) to 0.9 volumes of prewarmed medium (either 50 mM choline chloride, 1 mM $MgCl_2$, 50 mM NaCl, 0.11 mM $^{14}C$-glycine, 10 mM MOPS (Tris form) pH 7.4 or 63.5 mM $MgSO_4$, 25 mM $Na_2SO_4$, 0.11 mM $^{14}C$-glycine, 10 mM MOPS (Tris form) pH 7.4). The potassium salt was substituted for sodium as indicated. The reaction was terminated as described in Figure 16.

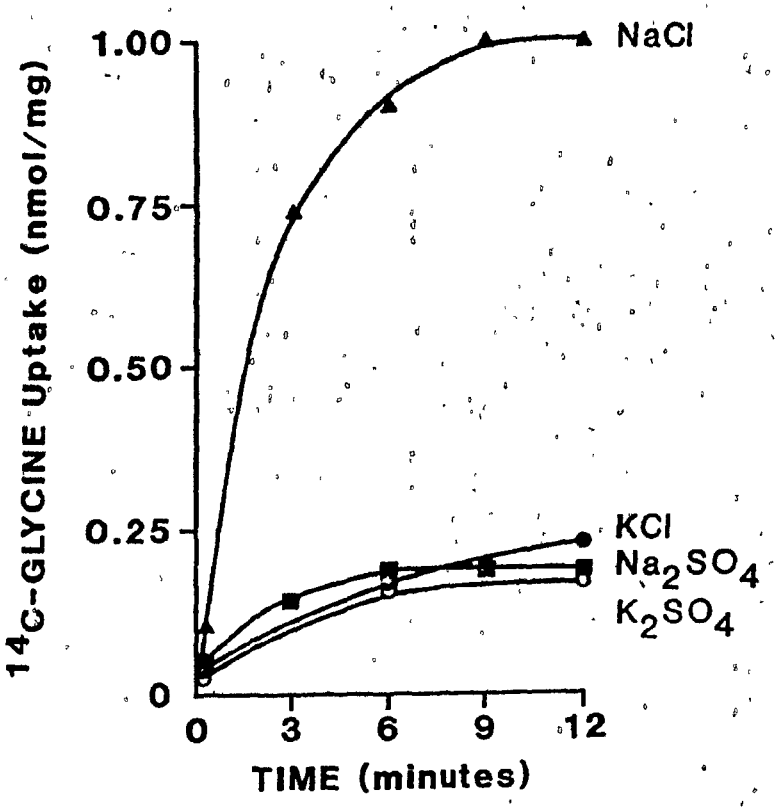

1.00
0.75
0.50
0.25
0
0
3
6
9
12
$^{14}$C-GLYCINE Uptake (nmol/mg)
TIME (minutes)
NaCl
KCl
$Na_2SO_4$
$K_2SO_4$

**Figure 18**

**ATP-Dependent $^{22}Na^{+}$ Uptake**

Vesicles derived from reticulocytes (55%) were equilibrated overnight at 0°C, then 2 hours at 37°C in 40 mM KCl, 5 mM MOPS (Tris form), pH 7.4, $^{14}C$-glycine ($1.3 \times 10^3$ cpm/nmole), and then concentrated to 3.85 mg protein/ml. The assays were initiated by adding 0.45 ml medium containing 5 mM MOPS (Tris form), pH 7.4, 1 mM $MgCl_2$, 0.1 mM nonradioactive glycine, and 40 mM $^{22}NaCl$ (63 cpm/nmole) with or without ATP to 0.05 ml vesicles. The reaction was terminated (0.05 ml aliquots) as indicated in Figure 16. Final concentrations were 36 mM NaCl, 1 mM ATP, and 0.1 mM glycine. Of the total sealed membranes, 100% were inside-out. From Weigensberg, Johnstone and Blostein (298).

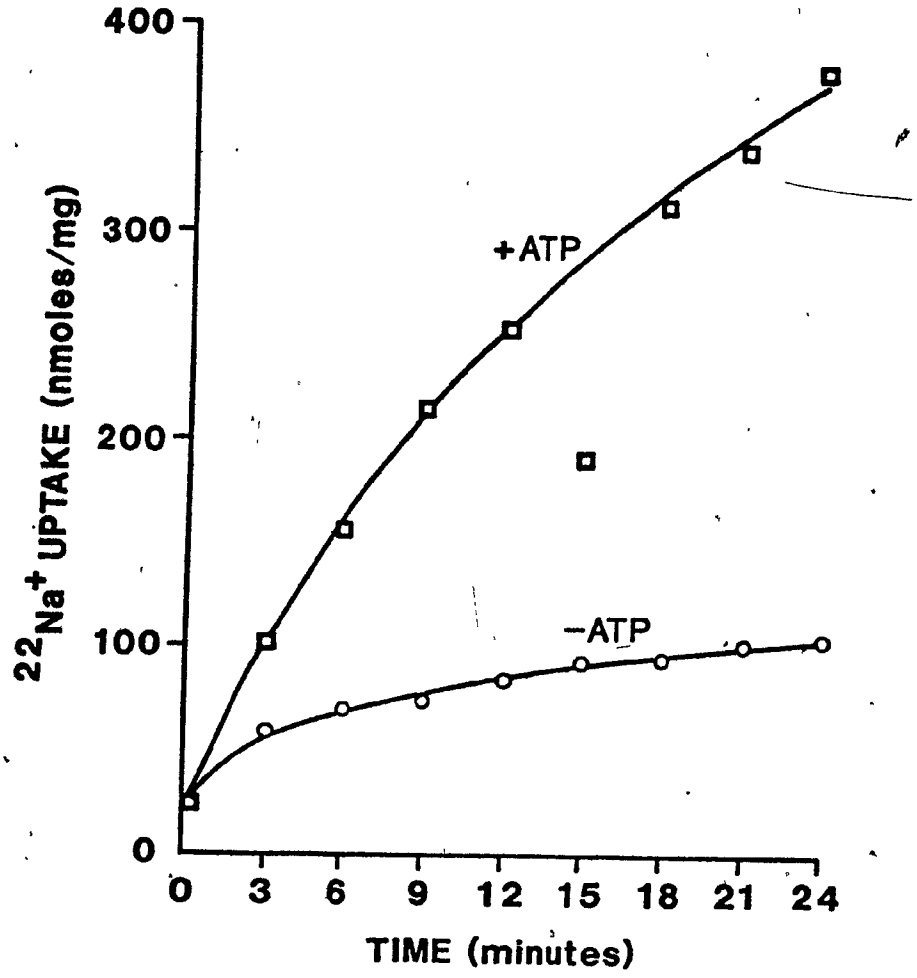
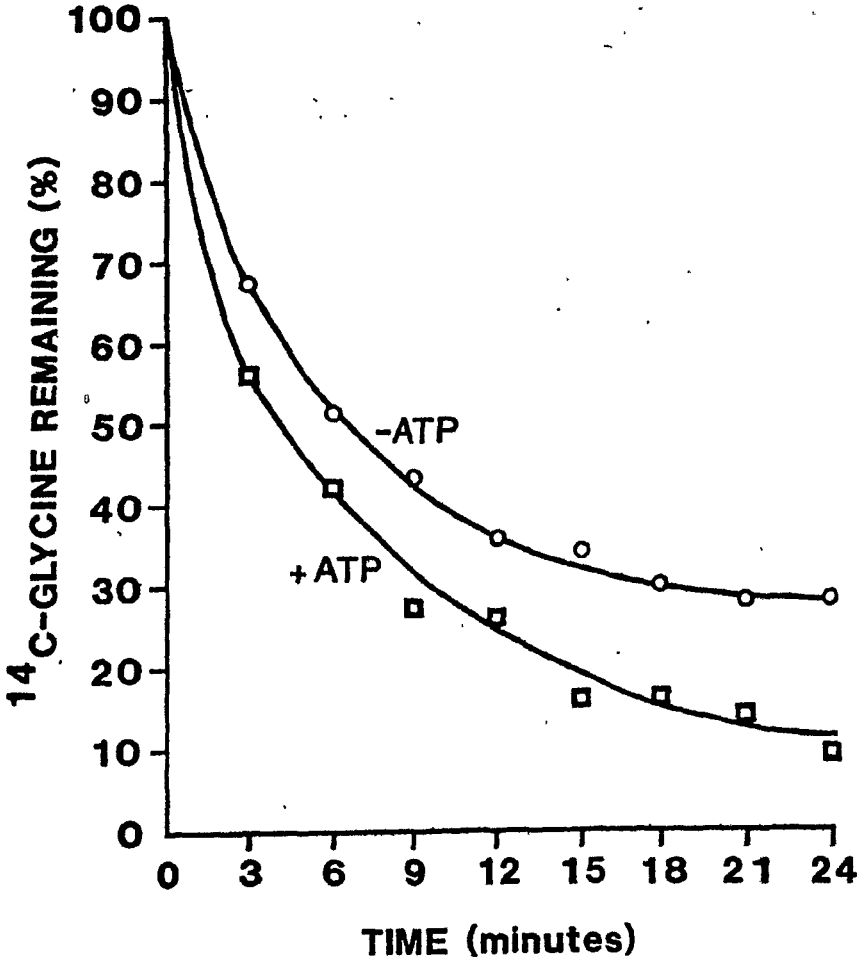

400
300
200
100
0
$^{22}Na^{+}$ UPTAKE (nmoles/mg)
+ATP
−ATP
0
3
6
9
12
15
18
21
24
TIME (minutes)

**Figure 19**

**The Effect of ATP on Glycine Efflux**

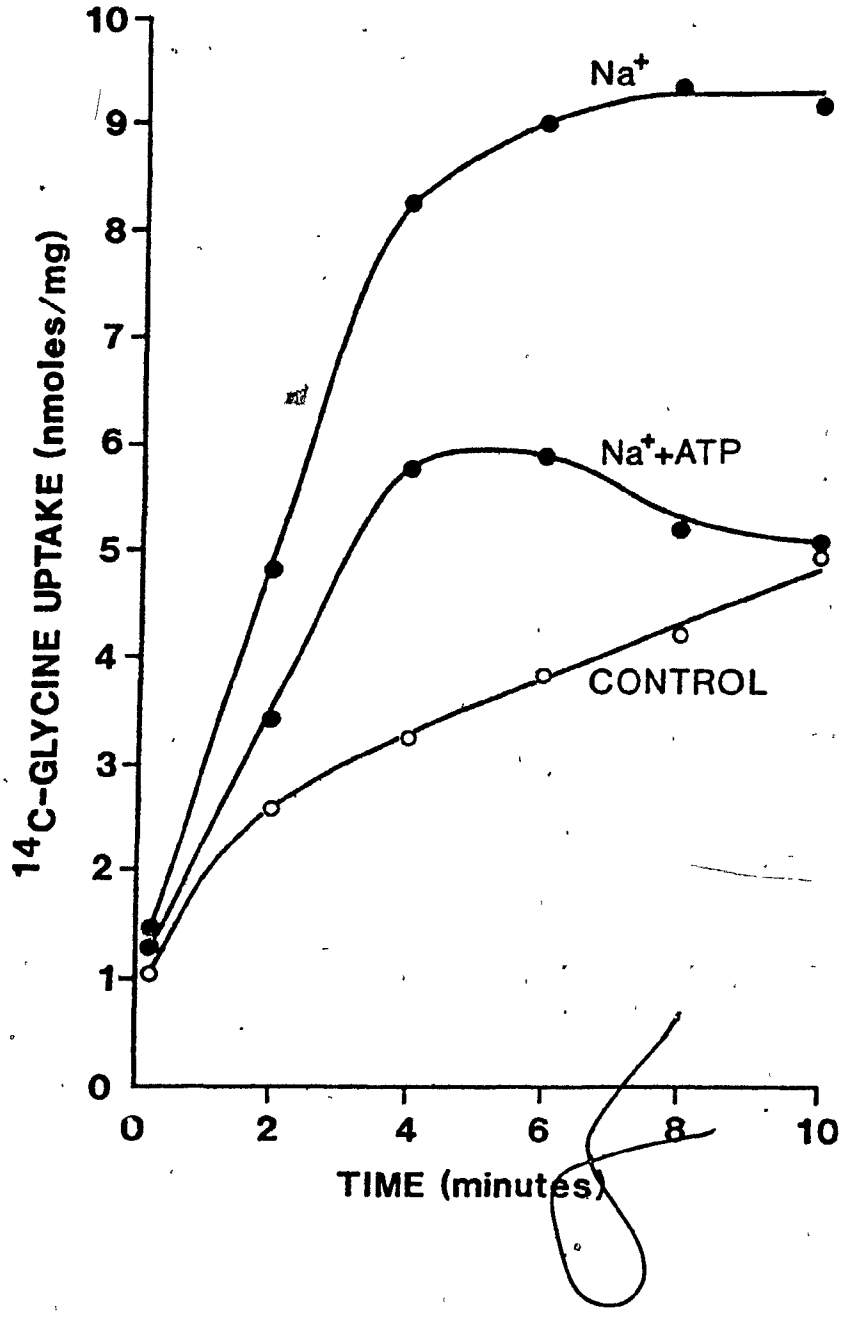
$^{14}C$-glycine efflux was measured in IOV. For details see the text and Figure 18. From Weigensberg, Johnstone and Blostein (298).


100
90
80
70
60
50
40
30
20
10
0
$^{14}$C-GLYCINE REMAINING (%)
-ATP
+ATP
0
3
6
9
12
15
18
21
24
TIME (minutes)

## Figure 20

### Effects of $Na^+$ and ATP on Glycine Uptake

The experiment was carried as described in Figure 16 except that only one concentration of $Na^+$ (35 mM) was used and, where indicated, the prewarmed media contained 0.55 mM ATP. From Weigensberg, Johnstone and Blostein (298).


10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
Na+
Na+ +ATP
CONTROL
14C-GLYCINE UPTAKE (nmoles/mg)
TIME (minutes)

plasmic surface of IOV, energizes the $Na^+,K^+$-pump. Therefore, the effect of pump inhibition on $Na^+$-dependent glycine uptake with ATP present was tested. Strophanthidin, rather than ouabain, was used since it is sufficiently lipophilic to permeate the vesicles and bind to the extracellular (intravesicular) cardiac glycoside binding site. As shown in Figure 21, $Na^+$-dependent glycine uptake was enhanced markedly with strophanthidin added to inhibit the $Na^+,K^+$-pump. This suggests that ATP inhibition is via activation of $Na^+$, $K^+$-pump activity which, in turn, dissipates the $Na^+$ chemical gradient (see Figures 18 and 19), and may generate a membrane potential (inside positive) (299).

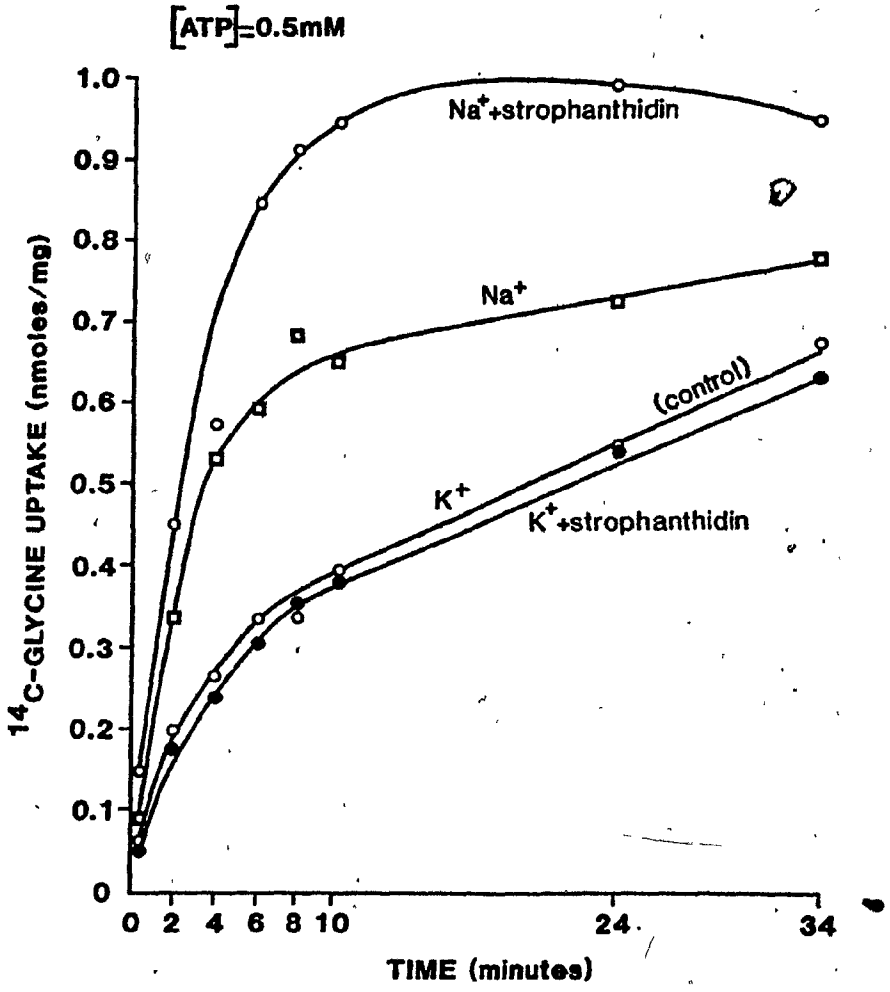
Although the foregoing kinetic experiments showed that $Na^+$-dependent glycine transport can operate in reverse, the question remained as to whether the system can perform net accumulation in reverse.

Reverse accumulation was investigated following equilibration of vesicle with KCl and 0.1 mM $^{14}C$-glycine, then diluting them with a medium containing $^{14}C$-glycine of the same concentration and specific activity and with $K^+$ or with $Na^+$ substituting for $K^+$. As shown in Figure 22, transfer to the $K^+$ medium did not result in further glycine uptake, whereas imposition of the $Na^+$ gradient effected a substantial (two-fold) accumulation of glycine. This was followed by a subsequent loss, to the level observed in the control. As shown, with 1.0 mM ATP present the peak accumulation, or "overshoot", is decreased and the final amount of glycine inside the vesicles is somewhat less than in the control. Although these preparations were 86% IOV and 14% ROV similar results were obtained with preparations lacking any detectable ROV.

**Figure 21**

**Effect of Strophanthidin on Glycine Uptake in the Presence of $Na^+$ and ATP**

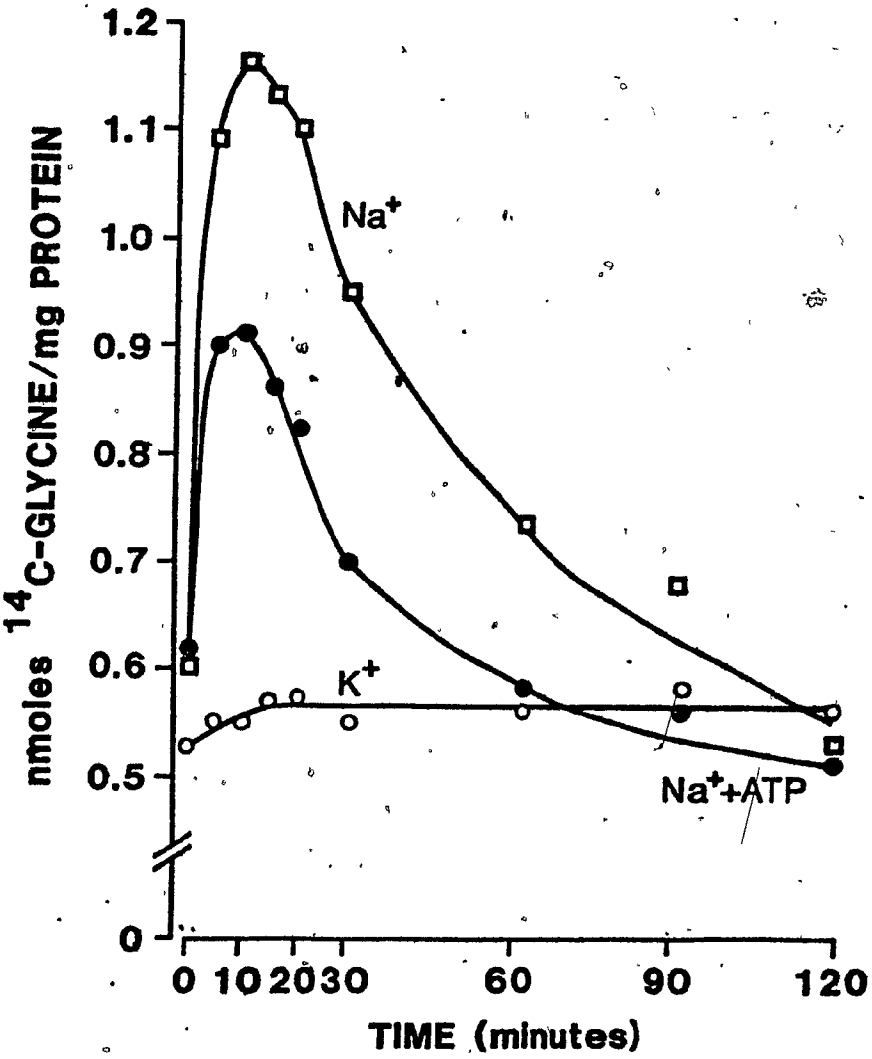
Vesicles derived from reticulocytes (71%) were equilibrated overnight at 0°C, then 15 minutes at 37°C with 40 mM KCl, 1 mM $MgCl_2$ and 5 mM MOPS (Tris form), pH 7.4, and then concentrated to 1.23 mg protein/ml as described in Figure 16. Assays were initiated by adding 0.6 ml isoosmotic $Na^+$ or $K^+$ medium to 0.15 ml vesicles and terminated by removing 0.08 ml aliquots at the indicated intervals as described in Figure 16. Final concentrations were 0.1 mM $^{14}C$-glycine ($1.42 \times 10^4$ cpm/nmole), 32 mM NaCl or KCl, and 0.5 mM ATP with either 0.02 mM strophanthidin added as a 0.5% ethanolic solution or 0.5% ethanol (control). Of the total sealed membranes, 77% were inside-out and 23% right side-out. From Weigensberg, Johnstone and Blostein (298).


[ATP]=0.5mM
Na+ +strophanthidin
Na+
(control)
K+
K+ +strophanthidin
14C-GLYCINE UPTAKE (nmoles/mg)
TIME (minutes)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0 2 4 6 8 10 24 34

## Figure 22

### $Na^+$ Gradient Stimulated Glycine Accumulation in IOV

Vesicles derived from reticulocytes (58%) were equilibrated with 0.1 mM $^{14}C$-glycine ($2.5 \times 10^4$ cpm/nmole) as described in Figure 18, concentrated to 6.56 mg protein/ml, and then assayed as described in Figure 16 except that 0.1 mM $^{14}C$-glycine of the same specific activity was included in the medium, and an additional sample with KCl replacing NaCl in the medium was included as indicated. ATP (1 mM) was present as indicated. Of the total sealed membranes, 86% were inside-out, and 14% right side-out. From Weigensberg, Johnstone and Blostein (298).


1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
nmoles $^{14}$C-GLYCINE/mg PROTEIN
Na+
K+
Na+ +ATP
0 10 20 30 60 90 120
TIME (minutes)

A similar experiment was carried out with ROV in an effort to compare the ability to accumulate glycine in the two types of vesicles. The experiment was done in exactly the same manner as the accumulation experiment in Figure 22, except that the glycine concentration was 1 mM. The results (Figure 23) indicate three important characteristics of glycine transport in ROV, namely: (i) the sodium dependency of glycine transport, (ii) the ability of these vesicles to accumulate glycine, and (iii) the lack of any ATP or sodium pump-mediated effect on either $Na^+$-dependent glycine uptake or total glycine accumulation which was 2.5 times the baseline level. This last point confirms that the vesicles are, indeed, right side-out, in which case ATP does not have access to the ATP binding site which is located intravesicularly. It appears that the IOV and ROV share a similar ability to accumulate glycine.

In another series of experiments the $Na^+$-dependent glycine transport system's stoichiometry or coupling ratio of sodium to glycine uptake in IOV and ROV was assessed. In these experiments, the glycine-enhanced uptake of $^{22}Na^+$ and sodium-enhanced uptake of $^{14}C$-glycine were followed concomitantly. The results of two representative experiments are shown in Table 8. The findings indicate that the stoichiometry of the $Na^+$-dependent glycine transport is the same for IOV and ROV, with two sodium ions being transported for each molecule of glycine transported.

## Figure 23

### $Na^+$ Gradient Stimulated Glycine Accumulation in ROV

Vesicles derived from reticulocytes (83%) were equilibrated with 1.0 mM $^{14}C$-glycine ($7.6 \times 10^3$ cpm/nmole) as described in Figure 18, concentrated to 2.19 mg protein/ml, and then assayed as described in Figure 16, except that 1.0 mM $^{14}C$-glycine of the same specific activity was included in the medium and an additional sample with KCl replacing NaCl in the medium was included as indicated. ATP (0.5 mM) was present as indicated. All of the sealed membranes were right side-out.

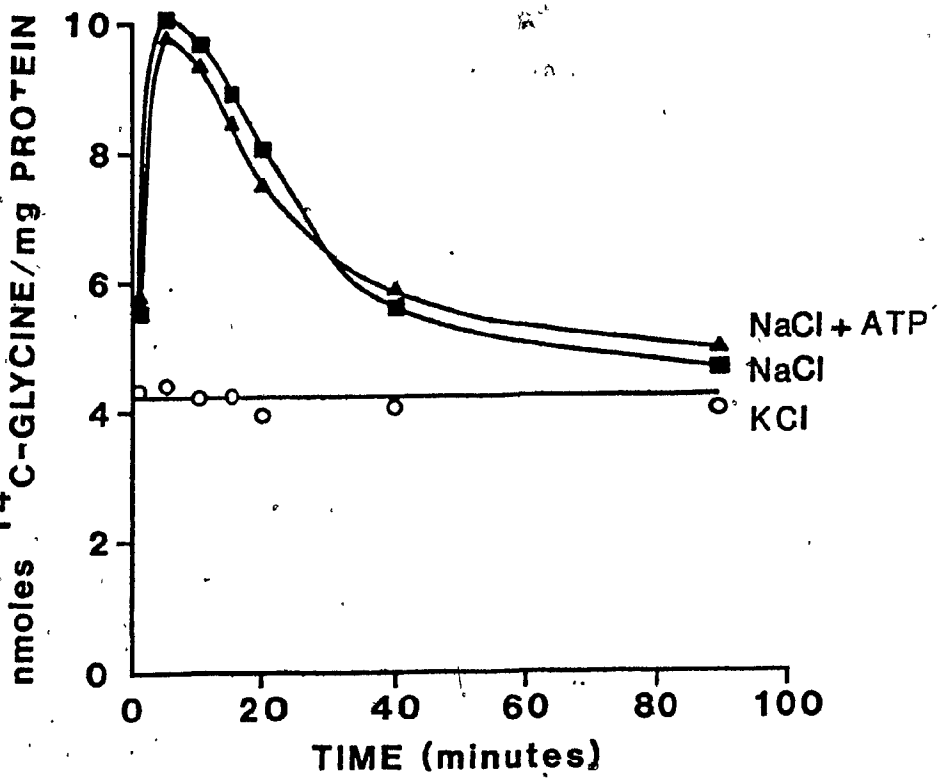

nmoles $^{14}$C-GLYCINE/mg PROTEIN
10
8
6
4
2
0
0
20
40
60
80
100
TIME (minutes)
NaCl + ATP
NaCl
KCl

**Table 8**
**Stoichiometry of $Na^+$-Dependent Glycine Uptake**

Vesicles (either IOV or ROV approximately 0.55 mg protein/ml) derived from reticulocytes (50%) were equilibrated overnight at 0°C, then for 30 minutes at 37°C with 40 mM KCl, 1 mM $MgCl_2$ and 10 mM MOPS (Tris form), pH 7.4. They were then concentrated (Section 2.6.B.2) to 3.55 and 3.98 mg protein/ml, respectively. The reaction was started by adding 0.09 ml prewarmed (37°C) isoosmotic medium to 0.01 ml prewarmed (37°C) vesicles. For glycine uptake the media contained 5.56 mM $^{14}C$-glycine (1550 cpm/nmole), 40 mM KCl or 22 mM NaCl plus 18 mM KCl, 1 mM $MgCl_2$ and 20 mM MOPS (Tris form) pH 7.4. For sodium uptake the media contained 22 mM $^{22}NaCl$ (1825 cpm/nmole), 18 mM KCl, 1 mM $MgCl_2$ and 10 mM MOPS (Tris form) pH 7.4 with and without 5.56 mM glycine the final $Na^+$ and glycine concentrations were 20 and 5 mM, respectively. The reaction was terminated after 5 minutes and the vesicles (0.08 ml aliquots) were filtered and washed as described in Section 2.6.B.1 using ice-cold solutions of identical ionic composition as the final reaction medium. Depending on the experiment 100% of the total sealed membranes were either IOV or ROV. All values are the mean ± S.E.M. of 3 or 4 determinations. The S.E.M. of the ratio was determined according to Colquhoun (300).

| Type of Vesicle | $^{14}$C-Glycine Uptake (nmole/mg protein) | | | $^{22}Na^{+}$-Uptake (nmole/mg protein) | | | |
|---|---|---|---|---|---|---|---|
| | $-Na^{+}$ a | $+Na^{+}$ b | b-a | -Glycine c | +Glycine d | d-c | Ratio $\frac{d-c}{b-a}$ |
| IOV | 7.03 ± .10 | 10.11 ± .06 | 3.08 ± .11 | 25.45 ± .31 | 31.44 ± .18 | 5.99 ± .36 | 1.94 ± .14 |
| ROV | 6.67 ± .19 | 11.86 ± .16 | 5.19 ± .25 | 32.11 ± .55 | 42.23 ± .81 | 10.12 ± .98 | 1.95 ± .21 |

In order to determine if there is directional asymmetry in the $Na^+$-dependent glycine transport system with respect to influx and efflux kinetics, the apparent affinities of the system for sodium and for glycine were measured at several glycine and sodium concentrations. The $K_m$ for glycine at two different sodium concentrations (36 and 90 mM) was determined by following the initial velocity of $^{14}C$-glycine influx estimated from samples taken at 3 time intervals within the initial 60 or 90 seconds. These short time intervals were used because of the relatively small intravesicular volume of these vesicles and thus short equilibration times. In Figures 24 and 25 the velocity (nmole/mg/min) versus glycine concentration curves for IOV and ROV, respectively, are illustrated. The velocity versus glycine concentration plots were analyzed by fitting the data to Eadie-Hofstee plots using linear regression analysis. As shown in Figures 26 and 27, $K_m$ (negative inverse of slope) and $V_{max}$ (x-intercept) values for IOV and ROV at various sodium concentrations are generally similar. However, it is difficult to directly compare these $V_{max}$ values, since IOV and ROV were derived from different reticulocyte preparations and the percentage of reticulocytes and of sealed vesicles are <u>not</u> usually the same for <u>each</u> IOV and ROV preparation.

Figures 28 and 29 illustrate velocity versus $Na^+$ concentration (10 to 80 mM) curves at four different glycine concentrations (0.1, 0.5, 1, 2 mM) for different preparations of IOV and ROV.

## Figure 24

### Velocity Versus Glycine Concentration Curves in IOV

Vesicles (IOV) derived from reticulocytes were equilibrated overnight at 0°C, then 15 minutes at 37°C in either 40 mM KCl, 10 mM MOPS (Tris form) pH 7.4 and 1 mM $MgCl_2$, or 100 mM KCl, 10 mM MOPS (Tris form) pH 7.4 and 1 mM $MgCl_2$, and then concentrated (Figure 16) to either 1.01 (open circles) or 3.9 (closed triangles) mg protein/ml. The assays were initiated by adding 0.1 volume of prewarmed (37°C) IOV to 0.9 volumes of prewarmed (37°C) medium containing 20 mM MOPS (Tris form) pH 7.4, 1 mM $MgCl_2$, 0.08 - 2.8 mM $^{14}C$-glycine (specific activity = $8.5 \times 10^3$ - $1.1 \times 10^5$ cpm/nmole) and either 40 mM or 100 mM NaCl respectively. The potassium salt was substituted for the sodium one in order to determine the glycine baseline uptake level. There was no ROV present in either preparation. The initial velocities were determined using linear regression analysis during the first minute of uptake.

| | Preparation # | % Reticulocytes | Sheep # | % Sealed Vesicles |
|---|---|---|---|---|
| ▲——▲ | 2-12-82 | 42 | 8279 | 32 |
| O——O | 21-04-82 | 40 | 364 | 28 |

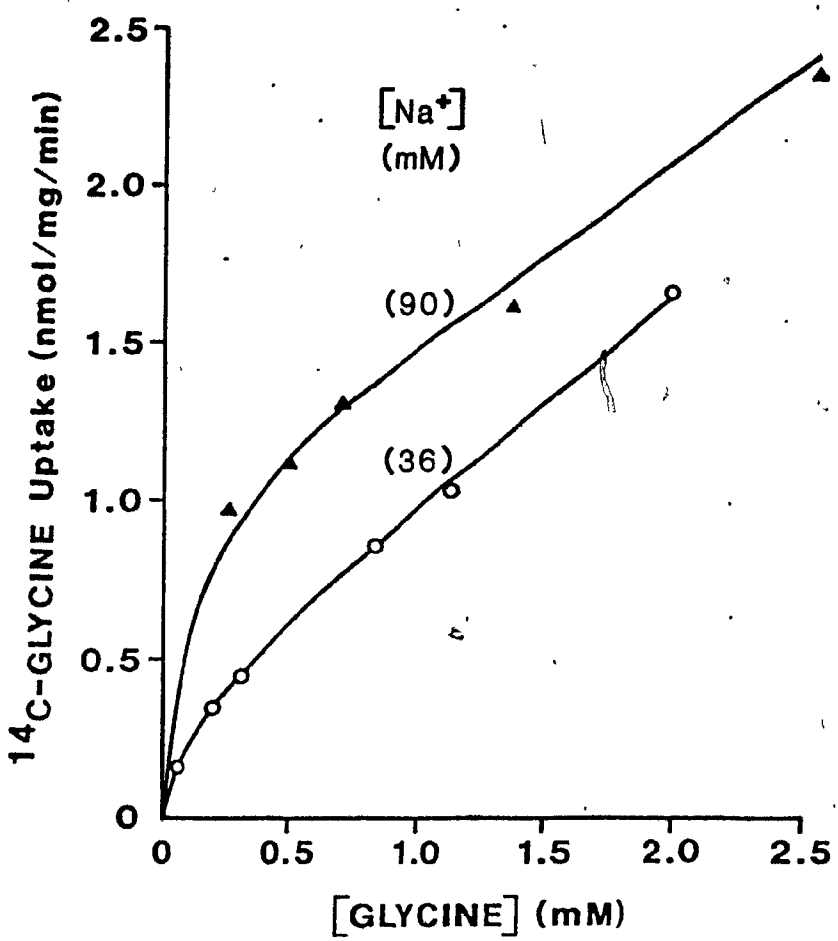

2.5
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
2.5
[Na+]
(mM)
(90)
(36)
14C-GLYCINE Uptake (nmol/mg/min)
[GLYCINE] (mM)

**Figure 25**

**Velocity Versus Glycine Concentration Curves in ROV**

Vesicles (ROV) derived from reticulocytes were equilibrated overnight at 0°C, then 15 minutes at 37°C in either 40 mM KCl, 10 mM MOPS (Tris form) pH 7.4 and 1 mM $MgCl_2$, or 100 mM KCl, 10 mM MOPS (Tris form) pH 7.4 and 1 mM $MgCl_2$, and then concentrated (Figure 16) to either 2.3 (open circles) or 1.8 (closed triangles) mg protein/ml. The assays were initiated by adding 0.1 volumes of prewarmed (37°C) IOV to 0.9 volumes of prewarmed (37°C) medium containing 20 mM MOPS (Tris form) pH 7.4, 1 mM $MgCl_2$, 0.08 - 2.8 mM $^{14}C$-glycine (specific activity = $8.5 \times 10^3$ - $1.1 \times 10^5$ cpm/nmole) and either 40 mM or 100 mM NaCl respectively. The potassium salt was substituted for the sodium one in order to determine the glycine baseline uptake level. There was no IOV present at the sodium concentration of 36 mM. However, at the 90 mM sodium concentration there was 3% IOV present. The initial velocities were determined using linear regression analysis during the first minute of uptake.

| | Preparation # | % Reticulocytes | Sheep # | % Sealed Vesicles |
|---|---|---|---|---|
| ▲——▲ | 12-05-82 | 55 | 1023 | 31 |
| ○——○ | 21-04-82 | 60 | 1023 | 13 |

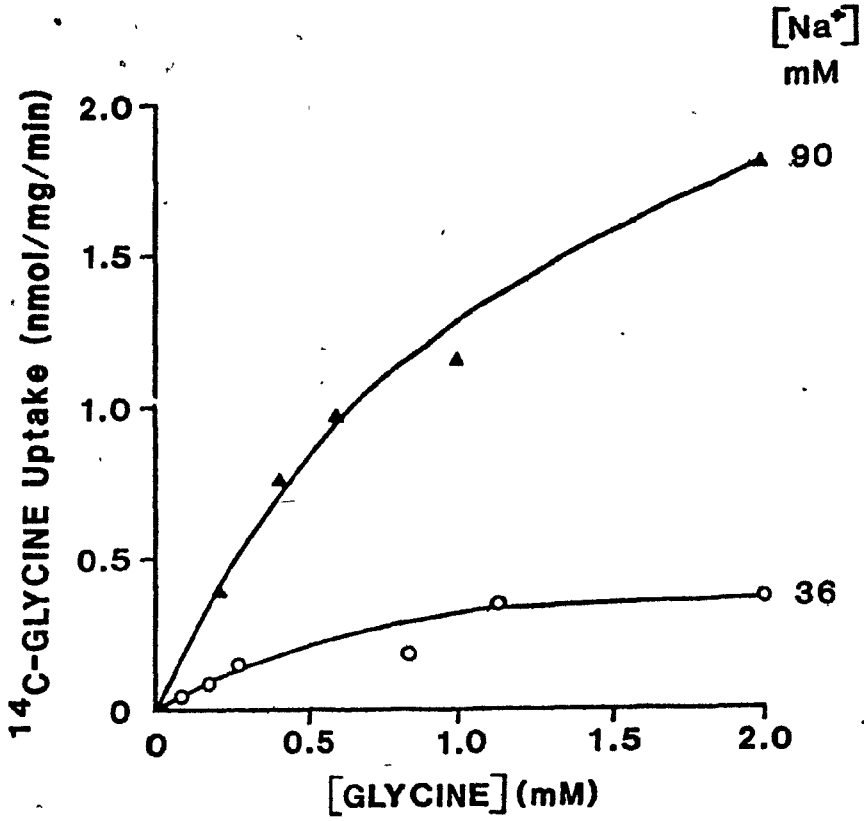

[Na+]
mM
90
36
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
14C-GLYCINE Uptake (nmol/mg/min)
[GLYCINE] (mM)

**Figure 26**

**Eadie Hofstee Plots to Determine the $K_{gly}$ in IOV**

Data shown are taken from Figure 24. Linear regression analysis of the plots were carried out and the apparent $K_m$ values obtained are shown in Table 9.

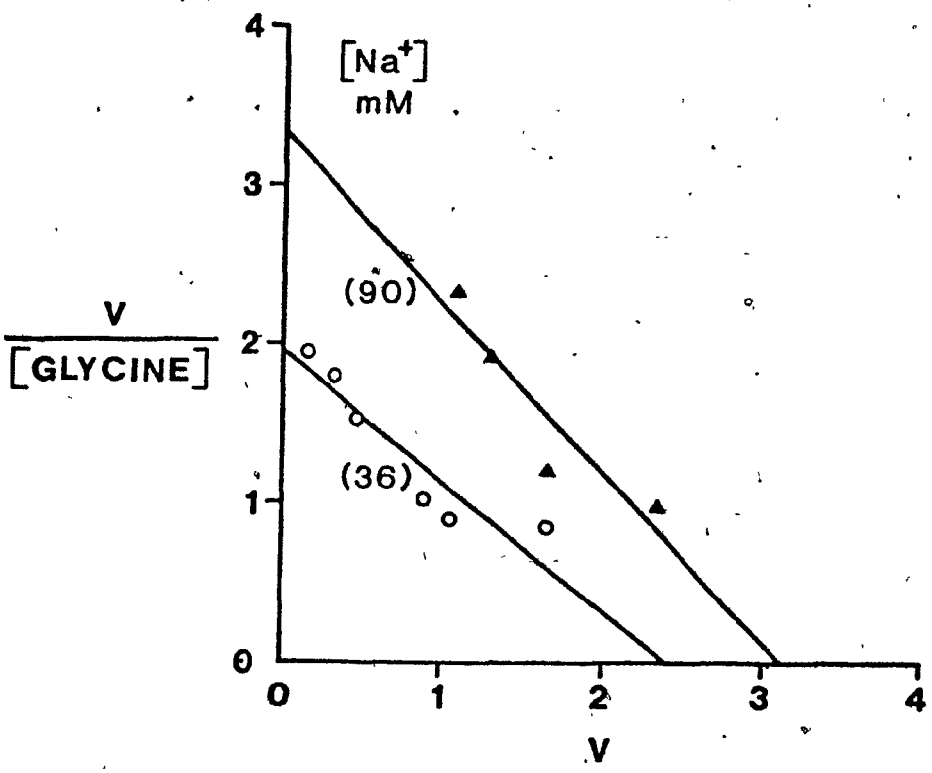
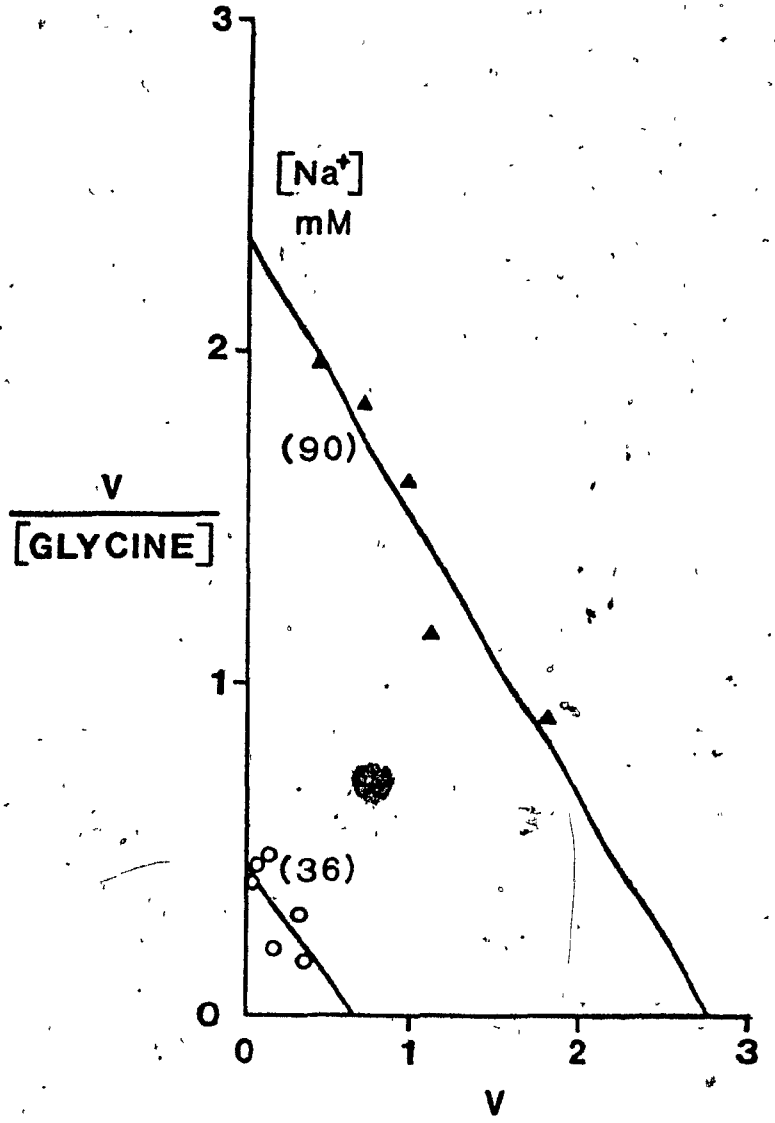

[Na+]
mM
(90)
(36)
V
[GLYCINE]
V
0
1
2
3
4

**Figure 27**

**Eadie-Hofstee Plots to Determine the $K_{gly}$ in ROV**

Data shown are taken from Figure 25. Linear regression analysis of the plots were carried out and the apparent $K_m$ values obtained are shown in Table 9.

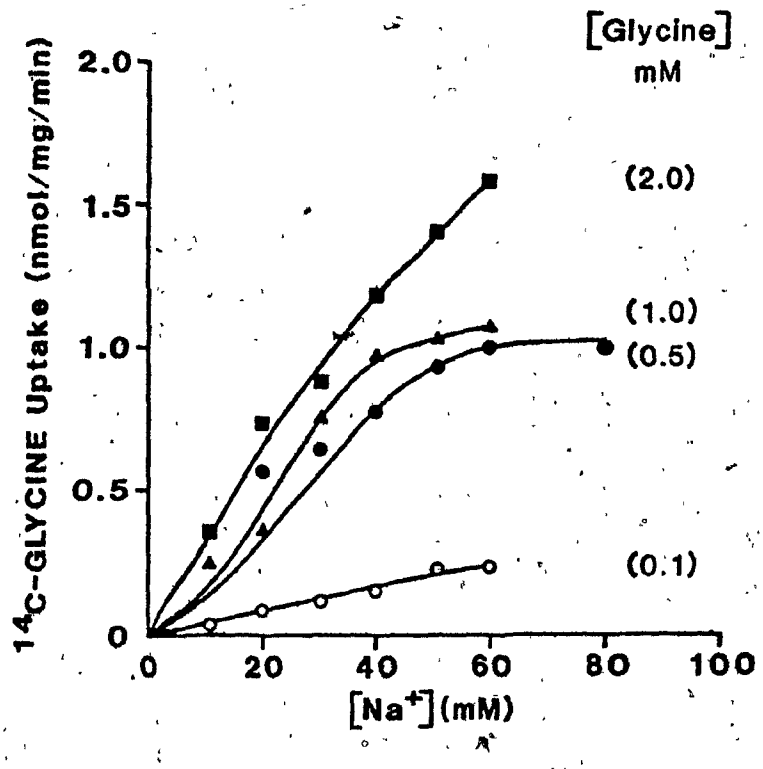

3
2
1
0
0
1
2
3
V
$\frac{V}{[GLYCINE]}$
[Na+]
mM
(90)
(36)

## Figure 28

### Velocity Versus Sodium Concentration Curves in IOV

Vesicles (IOV) derived from reticulocytes were equilibrated overnight at 0°C, then 15 minutes at 37°C in 80 or 100 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS (Tris form) pH 7.4 and then concentrated (Figure 16) to either 3.1 (open circles), 4.6 (closed circles), 2.3 (closed triangles) or 2.3 (closed squares) mg protein/ml, respectively. The assays were initiated by adding 0.1 volumes of prewarmed (37°C) IOV to 0.9 volumes of prewarmed (37°C) medium containing 10 mM MOPS (Tris form) pH 7.4, 1 mM $MgCl_2$, $^{14}C$-glycine (specific activity approximately 4 x $10^4$cpm/nmole) and 80 mM or 100 mM KCl. In this experiment, the sodium salt was substituted for the potassium one (10-80 mM). ROV were present (21%) in only one sample (glycine concentration equalled 1.0 mM). The initial velocities were determined using linear regression analysis during the first minute of uptake.

| | Preparation # | % Reticulocytes | Sheep # | % Sealed Vesicles |
|---|---|---|---|---|
| ■——■ | 16-03-82 | 37 | 364 | 34 |
| ▲——▲ | 27-10-82 | 53 | 474 | 67 |
| ●——● | 18-01-83 | 80 | 1023 | 53 |
| O——O | 01-12-82 | 42 | 8279 | 32 |

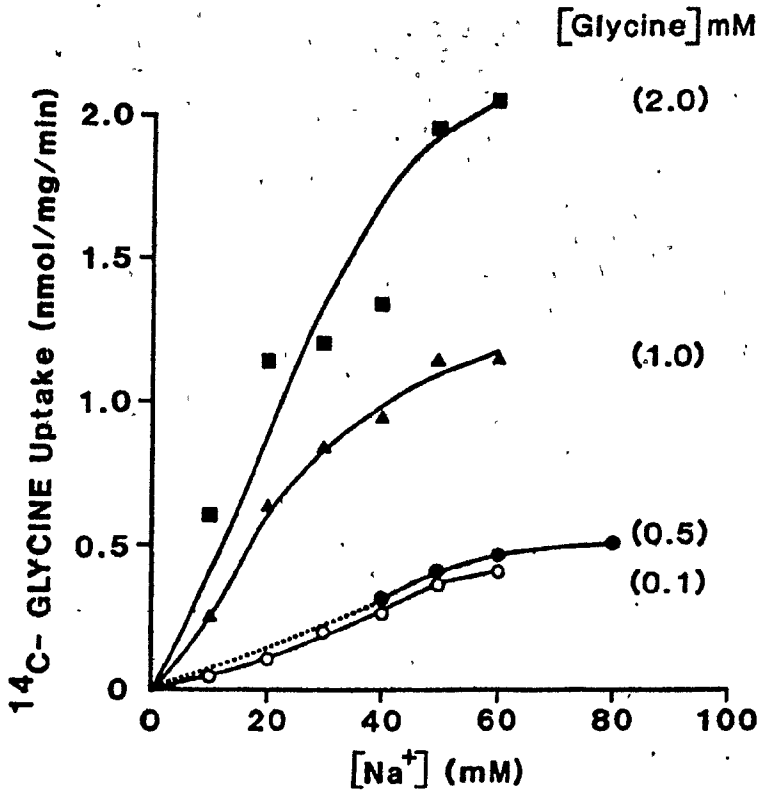

[Glycine]
mM
(2.0)
(1.0)
(0.5)
(0.1)
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
$^{14}$C-GLYCINE Uptake (nmol/mg/min)
[Na$^+$] (mM)

## Figure 29

### Velocity Versus Sodium Concentration Curves in ROV

Vesicles (ROV) derived from reticulocytes were equilibrated overnight at 0°C, then 15 minutes at 37°C in 80 or 100 mM KCl, 1 mM $MgCl_2$, 10 mM MOPS (Tris form) pH 7.4 and then concentrated (Figure 16) to either 2.7 (open circles), 4.1 (closed circles), 2.4 (closed triangles) and 2.7 (closed squares) mg protein/ml, respectively. The assays were initiated by adding 0.1 volumes of prewarmed (37°C) ROV to 0.9 volumes of prewarmed (37°C) medium containing 10 mM MOPS (Tris form) pH 7.4, 1 mM $MgCl_2$, $^{14}C$-glycine (specific activity approximately 4 x $10^4$ cpm/nmole) and 80 mM or 100 mM KCl. In this experiment the sodium salt was substituted for the potassium one (10-80 mM). There was no IOV present in the vesicle preparations. The initial velocities were determined using linear regression analysis during the first minute of uptake.

| | Preparation # | % Reticulocytes | Sheep # | % Sealed Vesicles |
|---|---|---|---|---|
| ■——■ | 16-03-82 | 45 | 364/1023 | 33 |
| ▲——▲ | 21-04-82 | 40 | 364 | 49 |
| ●——● | 26-01-83 | 65 | 1023 | 50 |
| ○——○ | 01-12-82 | 42 | 8279 | 63 |


[Glycine]mM
(2.0)
(1.0)
(0.5)
(0.1)
$^{14}$C- GLYCINE Uptake (nmol/mg/min)
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
[Na$^+$] (mM)

As shown, the curves are sigmoidal and since it has been shown that two sodium ions are transported per molecule of glycine (Table 8 and Ref. 223) the Michaelis-Menten equation was transformed to $V_{max}/v = (1 + K_m/[Na])^n$, according to the Garay and Garrahan model (301). Thus the Eadie-Hofstee plots of $v^{1/n}/Na^+$ versus $v^{1/n}$ for n = 2 approximated a straight line (Figures 30 and 31). The data are summarized in Table 9. As shown the apparent $K_m$ values for IOV and ROV at each different glycine concentration are generally similar. From these experiments it appears that the $Na^+$-dependent glycine transport system is symmetrical with respect to the apparent $K_m$ values for $Na^+$ and for glycine, and the ability to accumulate glycine.

## 5.2 The Effect of Cytoplasmic Factors on Membrane Transport

In an effort to elucidate the factor(s) that contribute to the loss of membrane transport a 'reconstituted' vesicle system comprised of IOV incubated with stroma-free lysate was utilized. In the experiments illustrated in Tables 10 and 11, IOV were incubated with reticulocyte lysate in the presence of an ATP regenerating or depleting system at 37°C for up to 18 hours. The control was incubated at 0°C in the presence of the ATP regenerating system. In Table 10 the $Na^+$-dependent glycine transport was reduced 75% following incubation in an ATP replete medium compared to the control, while in the absence of ATP a significant difference from the control was not detected. Table 11 depicts the results of an experiment in which $Na^+$-pump activity was

**Figure 30**

**Eadie-Hofstee Plots to Determine the $K_{Na}$ in IOV**

Data shown are taken from Figure 28. Linear regression analysis of the plots were carried out and the apparent $K_m$ values are shown in Table 9.

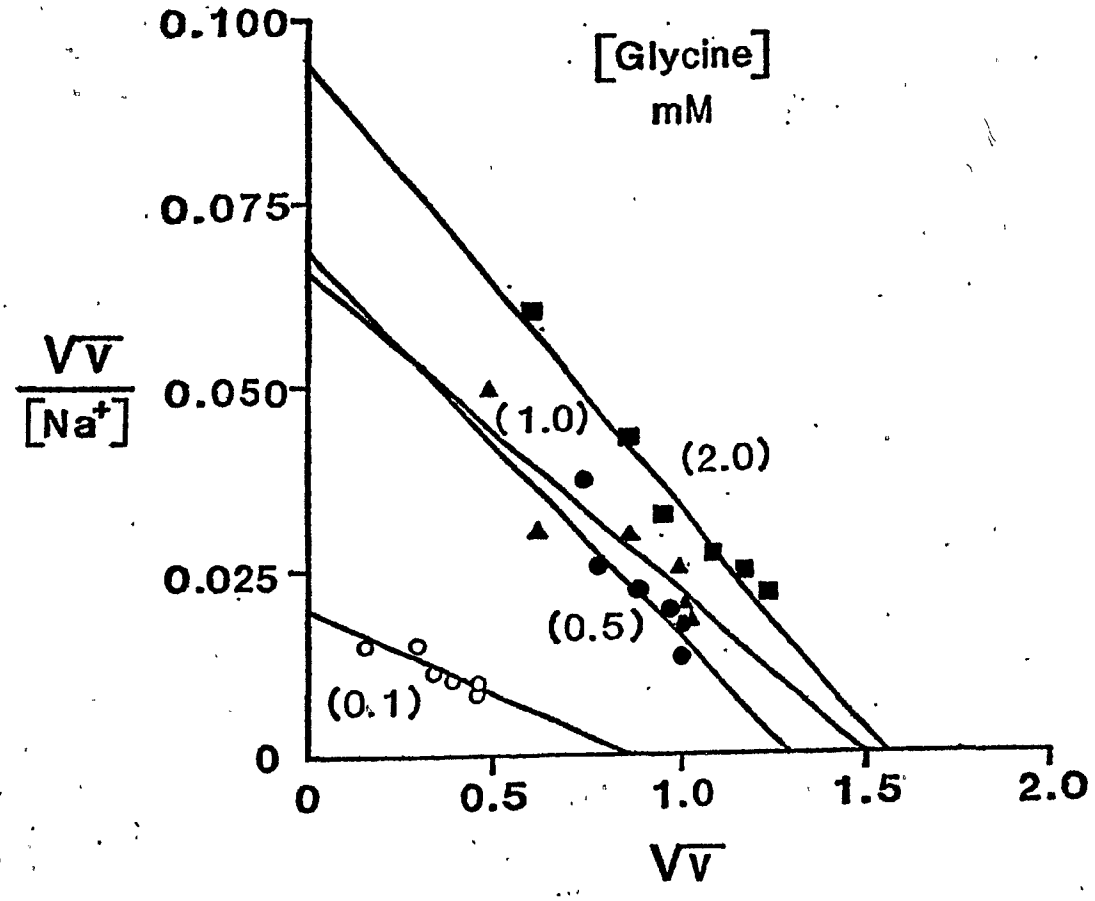
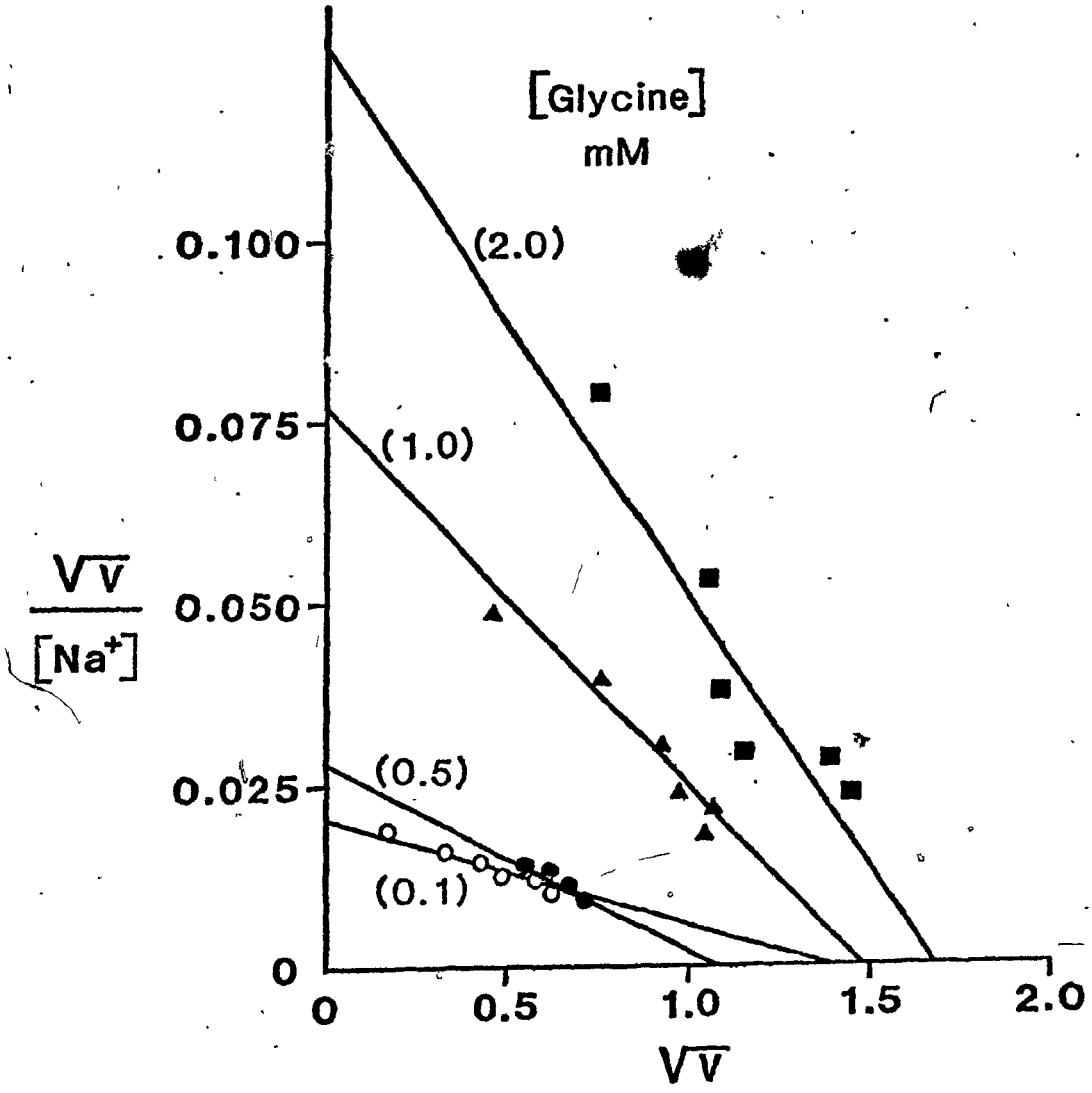

0.100
0.075
0.050
0.025
0
$\frac{V\sqrt{V}}{[Na^+]}$
[Glycine]
mM
(1.0)
(2.0)
(0.5)
(0.1)
0
0.5
1.0
1.5
2.0
$V\sqrt{V}$

**Figure 31**

**Eadie-Hofstee Plots to Determine the $K_{Na}$ in ROV**

Data shown are taken from Figure 31. Linear regression analysis of the plots were carried out and the apparent $K_m$ values obtained are shown in Table 9.


[Glycine]
mM
(2.0)
(1.0)
(0.5)
(0.1)
0.100
0.075
0.050
0.025
0
0
0.5
1.0
1.5
2.0
V/[Na+]
√V

## Table 9
## Comparison of Some Kinetic Parameters of the $Na^+$-Dependent Glycine Transport System in IOV and ROV

Table 9 summarizes the data presented in Figures 22[1], 23[2], 26[4], 27[5] 30[6], 31[7] and Table 8[3].

| Parameter Measured | | Type of Vesicle Preparation | |
|---|---|---|---|
| | | IOV | ROV |
| Accumulation of Glycine Intravesicularly | | 2.1-Fold[1] | 2.5-Fold[2] |
| Stoichiometry ($Na^+$:glycine coupling ratio) | | 1.94[3] | 1.95[3] |
| | Glycine Concentration (mM) | Apparent $K_m$s (mM) | |
| $K_{Na}$ | 0.1 | 43.0[6] | 66.0[7] |
| $K_{Na}$ | 0.5 | 18.9[6] | 38.9[7] |
| $K_{Na}$ | 1.0 | 23.0[6] | 19.1[7] |
| $K_{Na}$ | 2.0 | 16.5[6] | 12.9[7] |
| | Sodium Concentration (mM) | | |
| $K_{gly}$ | 36 | 1.43[4] | 1.24[5] |
| $K_{gly}$ | 90 | 1.2[4] | 0.93[5] |

**Table 10**
**Effects of ATP on the Loss of $Na^+$-Dependent Glycine Transport in a Reconstituted System Comprised of Vesicles Plus Cytoplasm**

IOV (100% IOV 0.09 ml) derived from sheep reticulocytes (65%) were equilibrated with 70 mM KCl, 10 mM $MgCl_2$ and 10 mM MOPS (Tris form) pH 7.7 (loading solution) for 40 minutes at 37°C, and then added to 0.136 ml of reticulocyte lysate (made from 65% reticulocytes as described in Section 2.2.C) and 0.074 ml of an ATP-depleting or regenerating system. The final composition of the ATP regenerating system consisted of 5 mM creatine phosphate, 8.8 mM ATP, 0.1% sodium azide and 5 units of creatine phosphokinase (CPK) in the loading solution . In the ATP-depleting system, 20 mM 2-deoxyglucose and 0.033 mM dinitrophenol (DNP) were substituted for the ATP and CPK, respectively. A control sample was incubated at 0°C with the ATP regenerating system, while the others were incubated at 37°C for 19 hours. The incubation of the reconstituted system was terminated by adding 0.6 ml of the loading solution pH 7.4 to it prior to centrifugation (12,000 x g for 10 minutes). The pellet was washed twice by resuspension and centrifugation. Prior to assay, the IOV were preincubated for 15 minutes at 37°C in 70 mM KCl, 5 mM $MgCl_2$ and 10 mM MOPS (Tris form) pH 7.4. $Na^+$-dependent glycine transport activity was then assayed for 2 minutes at 37°C as described in Section 2.6.B.2. The intravesicular spaces were measured by allowing the vesicles to equilibrate for 2 hours at 37°C with $^{14}C$-glycine in the absence of sodium. The intravesicular spaces for the control, +ATP and -ATP samples were 7.12 ± 0.45, 12.1 ± 0.45 and 3.23 ± 0.17 µl of vesicular space/ml vesicle, respectively.

| Incubation Conditions | $^{14}$C-Glycine Uptake pmole/µl vesicle space | | |
|---|---|---|---|
| | $-Na^+$(a) | $+Na^+$(b) | b-a |
| +ATP 0°C (control) | 25.6 ± 0.3 | 60.0 ± 2.5 | 34.4 ± 2.5 |
| +ATP 37°C | 29.0 ± 0.6 | 38.0 ± 1.3 | 9.0 ± 1.4 |
| -ATP 37°C | 101.6 ± 2.3 | 140.5 ± 1.9 | 38.9 ± 2.9 |

## Table 11
## Effect of ATP on the Loss of Sodium Pump Activity in Vesicles Following Incubation with Hemolysate

The incubation of the reconstituted system was carried out under conditions essentially the same as those described in Table 10, except that 0.65 ml IOV (100% IOV) derived from 55% reticulocytes (HK, sheep #474), 0.188 ml of reticulocyte lysate (made from 44% and 40% reticulocyte for 7 and 16 hour incubations respectively) and 0.047 ml of the ATP depleting or regenerating system were used. The incubation was terminated as described in Table 10. The ATP dependent sodium uptake (5 minute flux) was measured as described in Section 2.6.B.3. The intravesicular spaces were determined as described in Table 10. The 7 and 16 hour incubations were caried out on separate days.The intravesicular spaces for the control, +ATP (7 hr), -ATP (7 hr), +ATP (16 hr), -ATP (16 hr) samples were 29.2 ± 0.1, 27.6 ± 0.4, 24.8 ± 0.5, 17.2 ± 0.9 and 12.5 ± 0.3 µl of vesicle space/ml vesicle, respectively.

| Incubation Conditions | $^{22}Na$ Uptake (nmole/μl vesicle space) | | |
|---|---|---|---|
| | -ATP(a) | +ATP(b) | b-a |
| Control, 0°C (7 hr) | 0.99 ± 0.04 | 1.63 ± 0.03 | 0.64 ± 0.05 |
| +ATP ,37°C (7 hr) | 1.21 ± 0.02 | 1.79 ± 0.04 | 0.58 ± 0.04 |
| -ATP, 37°C (7 hr) | 0.79 ± 0.01 | 1.58 ± 0.04 | 0.79 ± 0.04 |
| +ATP. 37°C (16 hr) | 1.52 ± 0.04 | 1.84 ± 0.01 | 0.32 ± 0.04 |
| -ATP, 37°C (16 hr) | 1.07 ± 0.01 | 1.66 ± 0.09 | 0.59 ± 0.09 |

measured. As shown, there is an ATP-dependent decrease in activity following incubation of the vesicles with ATP replete lysate. Thus, there was a 26% loss after 7 hours and a 46% loss after 16 hours of incubation. In the absence of ATP there was little change in activity over the 16 hour incubation. In fact, a slight increase in activity was observed. In contrast, when vesicles derived from reticulocytes were incubated in lysate derived from mature red blood cells in the presence or absence of the ATP regenerating system, there was no loss in $Na^+$-dependent glycine transport after 16 hours incubation at 37° (Table 12). In this experiment, a loss in activity was observed with reticulocyte lysate, even in the absence of ATP. However, the loss was much greater (100%) in the ATP-replete compared to ATP-deplete system.

As described in Chapter 1, there is an ATP dependent proteolytic system in rabbit reticulocytes (72,73). This raises the possibility that this proteolytic system plays a role in the loss of membrane transport during reticulocyte maturation. I tested the sheep vesicle plus lysate system for ATP-dependent release of ninhydrin-positive material. The results shown in Table 13 suggest that there was ATP-dependent proteolysis occurring in the reconstituted vesicle system. The results suggest that proteolysis may be an important underlying factor in the loss during maturation of certain membrane transport systems. However, these results do not rule out the possiblity that the ninhydrin-positive material may be due to other products of metabolism.

## Table 12
## The Effect of Reticulocyte and Mature Red Blood Cell Lysates on $Na^+$-Dependent Glycine Transport Following Incubation With Vesicles

The incubation of the reconstituted vesicle system was essentially the same as described in Table 10, except, 0.1 ml IOV (100% IOV) derived from 40% reticulocytes, 0.15 ml of red blood cell lysate (made from either mature erythrocyte or 70% reticulocytes) and 0.020 ml of the ATP depleting or generating system were used. In the ATP depleting or generating system, sodium azide was omitted, creatine phosphate was 1.8 mM, ATP was 11.9 mM, DNP was 0.1 mM, 2-deoxy-glucose was 20 mM and there was 4.2 units of CPK. The 16 hour incubation was terminated as described in Table 10, except that the pH of the solution used was 7.7, and 10 mM instead of 5 mM $MgCl_2$ was used. The IOV were then incubated 45 minutes at 37°C. The $Na^+$-dependent glycine transport activity (1 minute flux) was determined as described in Section 2.6.B.2. The intravesicular spaces were determined by allowing $^{86}Rb$ to equilibrate for 15 minutes at 37°C in the presence of the ionophore valinomycin (20 μM). The intravesicular spaces for -ATP (mature cells), +ATP (mature cells), -ATP (reticulocytes) and +ATP (reticulocytes) were 6.53 ± 0.01, 17.7 ± 0.2, 8.45 ± 0.01 and 16.1 ± 1.4, μl of vesicle space/ml vesicle, respectively.

| Incubation Conditions | Source of Cell Lysate | $^{14}$C-Glycine Uptake (pmole/µl vesicle space | | |
|---|---|---|---|---|
| | | $-Na^+$(a) | $+Na^+$(b) | b-a |
| -ATP, 37°C | Mature Cells | 63.4 ± 0.9 | 69.3 ± 1.9 | 5.9 ± 2.1 |
| +ATP, 37°C | Mature Cells | 27.8 ± 0.4 | 35.0 ± 0.4 | 7.2 ± 0.4 |
| -ATP, 37°C | Reticulocytes | 68.7 ± 0.4 | 72.5 ± 0.8 | 3.8 ± 0.9 |
| +ATP, 37°C | Reticulocytes | 35.2 ± 2.6 | 32.1 ± 0.3 | 0 |

**Table 13**
**Ninhydrin-Positive Material Released During Incubation of a Reconstituted Vesicle Plus Hemolysate System**

The results of Table 13 show the concentration of ninhydrin-positive material in the supernatant after incubating the IOV with reticulocyte lysate and either the ATP depleting or regenerating system. The ninhydrin-positive material is determined as described in Section 2.9. The experiments described correspond to the $Na^+$-dependent glycine transport (Table 10) and the 7 hour incubation of the ATP-dependent sodium transport (Table 11).

| Experiment | Amount of Ninhydrin-Positive Material | | |
|---|---|---|---|
| | Control 0° | -ATP 37° | +ATP 37° |
| $Na^+$-Dependent Glycine Transport (Table 10) | 5.0 mM | 5.7 mM | 12.2 mM |
| ATP-Dependent Sodium Transport (Table 11) | 5.6 mM | 6.8 mM | 12.0 mM |

# 6 DISCUSSION

This investigation has been concerned with three aspects of membrane transport changes associated with sheep red blood cell maturation. The first part of the study involved experiments aimed at elucidating the basis for maturation-associated changes in $Na^+,K^+$-pump activity in sheep red blood cells, namely the apparent loss in total activity and, more importantly, the change in the kinetic properties of the pump. The second question concerned the mechanism(s) underlying the apparent loss in membrane transport, namely whether the loss of activity is an energy-dependent process. The third aspect of the work concerned the development of a membrane system amenable to direct assessment of possible intracellular factors involved in maturation-associated changes in membrane transport. To this end membrane vesicles (IOV or ROV) were characterized with respect to $Na^+$-dependent glycine transport with special emphasis on the question of the symmetry or asymmetry of this transport system. The symmetry was determined by examining the $K_m$ for both glycine and sodium and the ability of both types of vesicles to accumulate glycine against its concentration.

## 6.1 Changes in $Na^+$-ATPase and $Na^+,K^+$-Pump During Maturation of Sheep Reticulocytes

From the results presented in Chapter 3 and in other studies (Introduction 1.2.C.2 Cation Transport), it appears that the $Na^+,K^+$-pump of reticulocytes from

HK and LK sheep are similiar, but differ from those of either mature HK or LK red blood cells. As described earlier the $Na^+,K^+$-pump of the reticulocyte resembles the $Na^+,K^+$-pump of the mature LK red blood cell with respect to their behaviour at very low ATP, whereby the $Na^+$-ATPase of the isolated membranes is markedly sensitive to inhibition by potassium. In contrast, the $Na^+,K^+$-pump of the reticulocyte resembles the $Na^+,K^+$-pump of the mature HK erythrocyte with respect to its response, at physiological ATP levels, to different concentrations of intracellular potassium. Although the enzymic basis for these difference are not understood, it is possible that they may reflect differences in interactions with ATP. Thus, ATP at low affinity regulatory site(s) appears to stimulate the release of $K^+$ which becomes bound to the enzyme during the dephosphorylation step of the $Na^+,K^+$-ATPase reaction sequence (302-305). As suggested previously, it is thus plausible that the basic difference between the pumps may reside in their interactions with ATP at such site(s) (306).

Since the foregoing studies were carried out with reticulocytes produced in response to anemic stress, the question remained whether the differences in characteristics between these immature 'stress' cells and normal mature cells reflect true maturation-associated changes, or the replacement of normal cells by stress cells with different pump characteristics, i.e. whether cell replacement or cell maturation caused these apparent maturation-associated changes in the $Na^+,K^+$-pump. Indirect evidence for changes associated with cell maturation has been obtained from density fraction-

ated cells where, supposedly, the lightest (least dense) cells are the youngest and the heaviest (most dense) cells are the oldest. In these studies, the total activity of the $Na^+$-ATPase (246,247) and the $Na^+,K^+$-pump (243-245) were higher in the lightest fraction compared to the heavier fractions of cells; in addition, the response to increasing concentrations of $K^+$ changed from reticulocyte-like to mature-like with respect to the $Na^+,K^+$-pump (LK cells) and $Na^+$-ATPase (HK cells). Nevertheless, the possibility still remained that during severe anemic stress, the animal may be producing a cell different from the 'normal' erythrocyte, one that increases in density faster than 'normal' cells and thus may have a greater density than a 'normal' erythrocyte of the same age.

In order to eliminate cell replacement as a basis for the apparent differences between immature and mature cells, in vitro culture (267) of sheep reticulocytes was used in the present investigation to study maturation. This allowed cells to mature in vitro for periods up to 14 days. At specific intervals, samples were removed and examined for total activity and kinetic characteristics, namely, response to $K^+$ at both low ATP levels ($Na^+$-ATPase activity using membranes prepared from HK cells) and physiological ATP levels (ouabain-sensitive $Rb^+$ transport using intact LK cells). As shown in Chapter 3, the total $Na^+$-ATPase and pump activities, particularly the latter, (Figures 7 and 5) declined rapidly. In contrast, the change in kinetic response to $K^+$ was either small, or in most instances, not apparent. These in vitro results suggested that cell maturation is the basis for the loss in total

activity. It was realized that the failure to observe the kinetic changes would not be surprising if these changes occurred slowly, so that their appearance during in vitro culture could be limited by the ability of the cells to survive for prolonged period in vitro. Moreover, due to the heterogeneous cell population, it is possible that either premature death or inactivation of the oldest cells could obscure the appearance of the maturation-associated changes.

In order to circumvent this problem, an in vivo experiment was devised whereby the activity of newly formed cells could be followed in density-fractionated blood during recovery from anemic stress. Thus, new cells produced during severe anemic stress were identified by taking advantage of the HbA-to-HbC switch (283-286) and the reversion back to HbA during recovery. This switching occurs in response to severe anemic stress so that the 'stress' reticulocytes are identified by their HbC content. A similar approach was used by Lee, Woo and Tostesin (243) in their studies of maturation of the $Na^+,K^+$-pump in LK sheep reticulocytes; they labelled the newly formed red blood cells with $^{59}Fe$. The results of the present study show that the loss in activity ($Na^+$-ATPase) and the change in $K^+$ response profile do, in fact, occur during maturation of the HbC-containing cells. These cells appeared in increasingly dense fractions following several weeks of in vivo maturation.

Whatever its molecular basis, the genetic difference between mature HK and LK cells is manifested as a selective change during maturation which proceeds

differently in the two types of animals. This change involves loss of pump activity during maturation of both type of cells. Two interpretations are possible. One is that the reticulocyte-type pumps become either mature LK or HK pumps. The L antigen, present on immature and mature LK cells (307), may be involved in the maturation-associated changes in red cells of LK animals, since its interaction with specific isoimmune antisera (anti-L) markedly stimulates the pump and $Na^+,K^+$-ATPase activities on LK cells (307). Moreover, the anti-L stimulated component has the properties of the reticulocyte pump; i.e., it is HK-like when pump activity is assayed at high ATP concentration (308), but LK-like when its membrane $Na^+$-ATPase activity is assayed at very low ATP concentration (309). Thus,the immune reaction appears to reverse, at least to some extent, the change observed during maturation of LK cells.

The second interpretation is that immature HK and LK cells have both reticulocyte pumps as well as either mature HK or LK pumps, respectively. During maturation, there is a selective loss of the reticulocyte type only, resulting in mature cells with either HK or LK pumps.

## 6.2 The Role of Metabolic Status in Maturation - Associated Losses in Membrane Transport

During maturation and aging the activities of many membrane transport systems and cellular enzymes are dramatically decreased. These changes have been shown indirectly by comparing activities in young and old populations of erythrocytes and, more directly, by

following the activity in newly formed cells as they mature and increase in density during in vivo maturation (see, for example, ref. 243). Loss in transport activities has also been observed during in vitro maturation of reticulocytes. Thus, for example, amino acid transport carried out by neutral exchange (208) and electrogenic $Na^+$-dependent glycine uptake (235) as well as nucleoside transport (185) are rapidly lost after only a few days in culture; sodium pump activity is also decreased, particularly during maturation of sheep cells of the low potassium genotype (253,259). However the mechanism underlying these changes is unknown. The possible role of metabolic energy in these maturation and aging-associated functional losses has been examined as a first step toward gaining some insight into this process.

$Na^+$-dependent glycine transport and sodium pump sites, estimated by $^3H$-ouabain binding, are particularly advantageous systems for studying aging-associated losses in membrane functions. $Na^+$-dependent glycine transport is easily amenable to quantitative assays in intact cells whose metabolic status have been perturbed. Thus, any dissimilarities between fed and starved cells due to differences in their electrochemical cation gradients and $Na^+,K^+$-pump can be eliminated by assaying the transport activity with gramicidin D added to collapse the gradients and ouabain to inhibit the pump.

Moreover, in contrast to amino acid exchange systems in which influx of labeled amino acid shows trans-stimulation, any difference in the intracellular

amino acid pools of fed and starved cells should not affect the initial rate of $Na^+$-dependent $^{14}C$-glycine influx (235,294). Similarly, our estimates of sodium pump sites were carried out under conditions (2-3 hour incubation) designed to ensure saturation of specific ouabain binding sites in both fed and starved cells.

These experiments show that maturation- and aging-associated losses in these membrane functions do not reflect non-specific cell degeneration. By using in vitro culture of sheep reticulocytes, it is observed that the losses of both $Na^+$-dependent glycine transport and sodium pump sites are modulated by the energy status of the cells. This does not appear to hold true for maturation-associated changes in all functions. Thus, activity of the cytoplasmic enzymes glucose-6-phosphate dehydrogenase, hexokinase, and phosphoglucose isomerase decrease to similar extents in fed and starved cells. The extent to which losses of other membrane functions or cytoplasmic components are energy dependent remains to be determined. It will also be important to determine whether and to what extent the loss of function is associated with the loss of the putative functional component.

When the results from Figure 5 which shows the loss of ouabain-sensitive $^{86}Rb$ flux during in vitro culture and Figure 14, depicting the loss of specific $^3H$-ouabain binding sites during in vitro culture, are compared, it appears that the $^{86}Rb$ flux decreases twice as fast as the loss of $^3H$-ouabain binding sites. However, it will be imperative to corroborate these results with an experiment measuring both these parameters concomitantly.

Although the observed losses in transport functions are much greater in metabolically replete than in starved cells, this study shows that the loss of activity still occurs in starved cells (compare, for example, Figure 11 and Table 6), usually to a much greater extent during the first phase (0 - 17 hours) than the subsequent (17 - 41 hours) phase of culture. A number of factors may influence the kinetics of transport loss in starved cells, including (i) the rate and extent to which energy (ATP) is diminished and (ii) the extent to which the putative degradative or removal (or both) process is active at the onset of the culture period. Thus, different preparations of reticulocyte-rich suspensions were used for each separate experiment. These suspensions vary in the state of maturation of the cells and are often obtained from different anemic animals.

The effect of starvation can be reversed, at least partially. Thus in an experiment (Table 4), in which $Na^+$-dependent glycine transport was measured during <u>in vitro</u> culture of fed and starved reticulocytes, 16.5-hour starved cells were washed free of the ATP-depeleting medium and reincubated a further 24 hours in a substrate replete medium. The results show that after 16.5 hours the fed cells had 19.9% of the original $Na^+$-dependent glycine transport, while the starved cells had 64.5%. When one aliquot of the starved cells was fed the activity decreased to 28.7% of the original activity, compared to a decrease to 40.6% of the original activity in the continuously starved cells. Thus, the degradative system appears to be still present and amenable to activation upon energy regeneration. It is

of interest to note, however, that although ATP was increased 3-fold during the refeeding period (16.5 to 40.5 hours), the actual ATP concentration remained relatively low, suggesting that the energy requirement for this membrane transport loss is also low. This is also apparent from Figure 11, which indicates that the loss in activity was only prevented when the ATP levels were very low.

It is not known whether the energy- or ATP-dependency is due to a direct effect of ATP per se. Furthermore, if the effect is due to ATP, it is possible that ATP exerts its effect by modifying certain membrane component(s), for example by phosphorylation, rendering it (them) susceptible to removal or degradation, or both. Alternatively, ATP may activate a degradative system such as ATP-dependent proteolysis, which is much more active in reticulocytes than mature cells (for review see reference 73). Rapoport and co-workers (310-312) suggest that the main function of this ATP-dependent proteolytic system is to degrade the mitochondria during maturation. According to this group, the ATP-dependent proteolytic system works in conjunction with the lipoxygenase enzyme to degrade the mitochondria (311). Other researchers working in this area have shown a propensity for this proteolytic system to degrade abnormal proteins (74,75). There are two theories concerning the role of ATP in this proteolytic system. According to Hershko and co-workers (313) the role of ATP in protein degradation is in the formation of conjugates of the 8 kilodalton polypeptide, ubiquitin, with cellular proteins. In contrast, Goldberg and colleagues (314) argue that ATP directly stimulates

proteolysis. In addition and in contrast to the ubiquitin-dependent system, the purified protease, purified by this group, is stimulated by other nucleotides and pyrophosphate and does not require magnesium. Recently, Speiser and Etlinger (315) have proposed a role for a protease inhibitor which can be repressed by ATP if ubiquitin is present

It may be relevant that the ATP-dependent loss in glycine transport has a pH-sensitivity profile similar to that described for the ATP-dependent proteolytic system of reticulocytes (310). It is possible, however, that this energy-modulated loss in membrane transport activity may occur in other ways. For example, Zweig et al (124) proposed several mechanisms of in vivo maturation of reticulocytes to erythrocytes. These membrane remodeling events may include endocytosis of spectrin-free regions of the membrane and their subsequent intracellular degradation or elimination through exocytosis. The proposed role of this mechanism is to eliminate mobile receptor domains, increase spectrin concentration, reduce the surface area of the erythrocyte and remove specific membrane components. Shrier and co-workers (316,317) have found that endocytosis can occur by either distinct energy-dependent or energy-independent routes. Thus, energy-modulated loss in transport activity may reflect a loss of the transporter by a mechanism whereby ATP-dependent endocytosis is a primary event. Although, Pan and Johnstone (318) have recently shown that the transferrin receptor is externalized through vesicle formation during in vitro culture of sheep reticulocytes, in this present study, a significant difference between MCHC of fed and starved

red blood cells was not apparent. This result would not be expected if membrane exocytosis occurred to a significant extent. Moreover, Lutz, Liu and Palek (319) have shown that human erythrocytes release vesicles during ATP depletion but not when the cellular ATP level is maintained. These results suggest that the release of spectrin-free vesicles is unlikely to be the basis for the loss of the two membrane transport systems investigated.

## 6.3 Transport Characteristics of Sheep Reticulocyte Membrane Vesicles

In order to directly study the possible role of cytoplasmic factors which may interact and perturb membrane transport processes during cell maturation, this investigation was directed towards preparing and elucidating the characteristics of sealed inside-out vesicles from reticulocytes. Although such preparations have been described for mature sheep red blood cells (320), until now, preparations of vesicles (IOV or ROV) or even sealed right side-out ghosts have not been described for immature red blood cells. In this aspect of the investigation I demonstrated that the procedure of preparing vesicles of uniform sidedness (inside-out or right side-out orientation), originally developed for mature human red cells (264), can be adapted to sheep reticulocytes. These vesicles have an advantage over intact cells, or even lysed and restored cells, in that there is no solute compartmentalization, interference from cytoplasmic enzymes and subtrates, or nonhomogeneity with respect to the effective intracellular solute concentration.

The experiments aimed at characterizing membrane transport in these vesicles appear to reflect, rather faithfully, the transport properties of the intact cell with respect to both $Na^+$-dependent glycine transport and $Na^+,K^+$-pump activity. Thus, as in intact cells and to a lesser extent, right side-out "ghosts", $Na^+$-stimulated uptake of glycine results in substantial net accumulation (235). The present results show that in mammalian red cells, as in avian cells (230), $Na^+$-dependent glycine transport system can not only operate kinetically in the reverse mode whereby both $Na^+$ and glycine are co-transported from cytoplasmic to extracellular surface, but can also perform osmotic work in reverse. $Na^+,K^+$-pump activity is also active in these vesicles and, assuming that the inhibition of glycine uptake by ATP is mediated by activation of the $Na^+,K^+$-pump, only inside-out vesicles should respond. The fact that net glycine accumulation in IOV, equivalent to normal net loss, is inhibited by ATP substantiates the conclusion that accumulation is occurring into the inside-out vesicles and not into the small fraction ( 20%) of vesicles which are right side-out. In addition, glycine accumulation in ROV is not inhibited by added ATP. These data indicate also that the sodium pump and amino acid transport systems are not segregated in different populations of vesicles.

In this investigation, the usefulness of IOV and ROV has been exploited in order to elucidate some basic characteristics of $Na^+$-dependent glycine transport which are difficult to explore with intact cells. Evidence for the coupled flow of $Na^+$ and solute in both

directions has been obtained in experiments with intact cells (321-323). However, adequate precision in the measurement of the coupling ratio has not been possible for fluxes occurring from the cytoplasmic to extracellular medium because of interfering substrate (see, for example, reference 323). Using IOV and ROV derived from reticulocytes it has been possible to examine concurrently the coupled $Na^+$ and glycine movements in both directions. The high anion permeability of these vesicles should effectively 'clamp' the membrane potential and ensure that the electrical driving force is not changed during these measurements. The results indicate that this ratio is 2 $Na^+$ per glycine transported in both directions. This same value was obtained for $Na^+$ :glycine co-transport in pigeon erythrocytes (223,224). It should be mentioned, however, that a 2:1 coupling ratio may not be a general phenomenon. Thus, in rabbit reticulocytes a value of 1:1 for amino acids other than glycine was obtained by Wheeler et al (227).

The other aspect examined has been the symmetry of the $Na^+$-dependent glycine transport system with respect to apparent affinities for $Na^+$ and glycine. As described early in the Introduction, Ellory, Jones and Young (201) have delineated two $Na^+$-dependent glycine transport systems in human erythrocytes, one chloride independent, the other chloride dependent, with the latter being the major glycine transporter. In Figures 10 and 17 the chloride dependent glycine transport system comprises 75% and virtually all (94%) of the total $Na^+$-dependent uptake in intact cells and IOV, respectively. It is entirely possible that the residual activity observed in cells pre-incubated in a chloride-

free medium (see Figure 10) was, in fact, due to a small amount of chloride still present.

Johnstone (231) has found that $Na^+$-dependent glycine transport in Ehrlich ascites tumour cells is asymmetric with respect to the $K_m$ for $Na^+$. She showed that the $K_m$ for efflux is an order of magnitude greater than for influx and the velocity for efflux is also several times larger than for influx. Vidaver and Shepherd (230), using avian erythrocytes, have also demonstrated asymmetry in the $Na^+$-dependent glycine transport system with the $K_m$ for glycine at 126 mM $Na^+$ to be 5 times greater for efflux than for influx and the $V_{max}$ for efflux twice that for influx.

In the present study the $K_m$ values for both glycine and $Na^+$ were determined at several concentrations of the other. Benderoff (324) reported a $K_m$ value for glycine, in intact erythrocytes, to be 0.48 mM at a $Na^+$ concentration of 145 mM. In contrast to Winter and Christensen's work with rabbit reticulocytes (205) he found no evidence for high and low affinity glycine systems in sheep reticulocytes. The present results indicate that the difference in apparent $K_m$ values obtained for IOV and ROV are of marginal significance since they differ by less than a factor of two. Moreover, it is plausible that small differences are secondary to membrane charge asymmetry. For technical reasons, namely the paucity of reticulocytes that can be isolated from even large volumes of anemic sheep blood, these kinetic experiments have the limitation that each apparent $K_m$ determination was done separately, usually on vesicles derived from a different blood sample, often from a different animal. Therefore, I have not attempted to compare $V_{max}$ values for ROV and IOV.

It may be relevant that Carruthers and Melchior (325) showed that human erythrocyte hexose transport is intrinsically symmetrical but that the interaction of the system with a low molecular weight cytosolic factor effects the appearance of an asymmetrical system.

The present experiments with sheep reticulocyte membrane vesicles provide evidence that the vesicles should be useful for studies of maturation- and aging-associated changes in membrane transport function. The incubation of a 'reconstituted' system comprised of sheep reticulocyte IOV and hemolysates prepared from reticulocytes and from mature erythrocytes, in the presence and absence of an ATP-regenerating system, suggest that ATP and some other cytoplasmic factor(s) present only in reticulocytes, have an important role in maturation and aging. It is also relevant that an increase in ninhydrin-positive material was observed during these incubations. It should be mentioned, however, that the experiments with the 'reconstituted' system are somewhat flawed by the unexplained variability in the effect of ATP on the $^{14}C$-glycine and $^{22}Na^{+}$ 'baseline' values for uptake. A comparison of the data in Tables 10 and 12 indicates that ATP pre-incubation decreases the baseline values for $^{14}C$-glycine influx, whereas in the experiment shown in Table 11, ATP pre-incubation increases the baseline $^{22}Na$ influx.

## 6.4 Other Possible Mechanics Underlying Erythrocyte Maturation

In spite of extensive research into the causes of erythrocyte senescence, no definitive mechanism or

sequence of events has been established. Some investigators believe that the initial changes occur in the cytosol and then are expressed on the cell surface (35). According to Kay and co-workers (326-329) senescent erythrocytes are recognized by a "senescent antigen", a Band 3 degradation product on the cell surface. This antigen is recognized by autologous IgG antibodies which appear to aid in the removal of aged erythrocytes from the circulation. Bartosz et al (330) found that there were increased amounts of bound IgG in older bovine erythrocytes. Some of the possible causes of exposure of the cryptic "senescent antigens" are: desialyation, proteoloysis (both internal and external), and free radical reactions on both sides of the membrane. Whatever the role of these processes in the removal of senescent erythrocytes from the circulation, it appears that energy/ATP plays a critical role in the loss of some membrane transport functions.

# 7 SUMMARY

Maturation and aging-associated changes in membrane transport of sheep reticulocytes of the high-$K^+$ and low-$K^+$ genotypes were studied. Reticulocyte-enriched fractions of sheep red blood cells were obtained following repeated phlebotomy. Changes in the $Na^+,K^+$-pump of reticulocytes from animals of the low potassium genotype (LK) and in sodium-activated ATP hydrolysis ($Na^+$-ATPase) of reticulocyes from sheep of the high potassium genotype (HK) were followed during *in vitro* culture. The results indicate that there is a progressive decrease in ouabain-sensitive $^{86}Rb^+$ uptake (LK cells) and $Na^+$-ATPase activity (HK cells). *In vitro* maturation for periods up to 10 days was not associated with changes in kinetic behaviour, from that typical of reticulocytes to that typical of mature cells. However, during longer term (several weeks) *in vivo* maturation in which HbC was used as a marker of newly formed reticulocytes, kinetic changes as well as a decline in total activity were observed.

The effect of metabolic depletion on the maturation-associated loss of membrane functions has been studied by using sheep reticulocytes incubated *in vitro* at 37°C for periods up to 41 hours. ATP was either maintained with glucose, adenosine plus inosine, or depleted with 2-deoxyglucose plus arsenate. Two membrane transport systems were studied: $Na^+$-dependent glycine transport activity and the $Na^+,K^+$-pump, estimated from measurements of the number of $^3H$-ouabain binding sites per cell. Both transport systems were

decreased during maturation. However, the decrease was much less in ATP-depleted cells compared to ATP-replete cells. In addition, this energy-modulated loss in $Na^+$-dependent glycine transport is influenced by the pH of the incubation medium, with the rate of loss increasing from pH 7.0 to 7.6. It is concluded that the loss of certain functions during reticulocyte maturation is retarded by metabolic depletion.

Membrane vesicles of distinct sidedness, either inside-out or right side-out, have been prepared from sheep reticulocytes. Using these vesicles the $Na^+$-dependent glycine transport system was found to be symmetrical with respect to: (a) the $Na^+$-dependency of glycine transport, (b) the ability to accumulate glycine against a concentration gradient, (c) the stoichiometry, i.e. 2 sodium ions are transported per molecule of glycine transported and (d) the apparent Michaelis-Menten constants for sodium at varying concentrations of glycine and for glycine at varying concentrations of sodium. The only apparent difference was the effect of ATP. With inside-out, but not right side-out vesicles, ATP diminished the rate and total accumulation of glycine, an effect secondary to the activation of the $Na^+,K^+$-pump.

Using a 'reconstituted' vesicle system, comprised of IOV plus cell lysates, it was observed that in the presence of reticulocyte lysate and an ATP-regenerating system the activity of both the $Na^+,K^+$-pump and $Na^+$-dependent glycine transport decreased more rapidly with ATP present. This was not observed when lysate from mature red blood cells was used. Associated with

ATP-dependent loss of activity was an increase in the amount of concomitantly measured ninhydrin-positive material, consistent with the notion that ATP-dependent proteolysis has a role in maturation-associated loss of certain membrane functions.

## 8 CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Parts of this investigation have been reported in the following publications:

Blostein, R., Drapeau, P., Benderoff, S. and Weigensberg, A. "Enzymic Diversification of the Sodium Pump in Sheep Red Cells" (1981) in Erythrocyte Membranes 2. Recent Clinical and Experimental Advances. Editors: Walter C. Kruckeberg, John W. Eaton and George J. Brewer. Alan R. Liss Inc., New York pp. 35-51.

Weigensberg, A.M., Johnstone, R.M., and Blostein, R. "Reversal of $Na^+$-Dependent Glycine Transport in Sheep Reticulocyte Membrane Vesicles". J. Bioenerg. Biomembr. (1982) 14:335-345.

Blostein, R., Drapeau, P., Benderoff, S. and Weigensberg, A.M. "Changes in $Na^+$-ATPase of Sheep Reticulocytes". Can. J. Biochem. Cell Biol. (1983) 61:23-28.

Weigensberg, A.M. and Blostein, R. "Energy Depletion Retards the Loss of Membrane Transport During Reticulocyte Maturation". Proc. Natl. Acad. Sci. USA (1983) 80:4978-4982.

Blostein, R. and Weigensberg, A.M. "The Role of Metabolic Energy in the Maturation-Associated Loss of Reticulocyte Membrane Transport" (1984) in Erythrocyte Membranes. Recent Clinical and Experimental Advances Ed. G.J. Brewer (in press).

The author considers the contributions to original knowledge of this investigation to be the following:

1. A method of following the fate of reticulocytes released into the circulation following phlebotomy-induced anemia in sheep, whereby the HbA-to-HbC switch is used to 'mark' newly formed cells. The results showed that as reticulocytes from an anemic HK sheep mature, they increase in density, their membrane $Na^+$-ATPase activity decreases and the kinetic profile changes from LK-like to HK-like.

2. A modification of the method originally described for the preparation of inside-out and right side-out vesicles from human red blood cells was developed for the preparation of inside-out and right side-out vesicles from sheep reticulocytes.

3. The characterization of the sidedness of $Na^+$-dependent solute (glycine) transport using vesicles of distinct orientation, namely, IOV and ROV derived from reticulocytes have been studied. They were characterized with respect to: (i) the ability to accumulate glycine against a concentration gradient, (ii) the apparent Michaelis-Menten constants for $Na^+$ and glycine at various concentrations of the co-transported substrate, (iii) the stoichiometry of the $Na^+$-dependent glycine transport system.

4. The demonstration that energy depletion of sheep reticulocytes retards the loss of at least two membrane transport activities, namely $Na^+$-dependent

glycine transport and the $Na^+,K^+$-pump. Both activities were quantitated using fed and starved intact cells. $Na^+,K^+$-pump activity was estimated indirectly by measuring the number of specific ouabain binding sites per cell and glycine transport was measured under conditions to eliminate differences due to changes in the sodium electrochemical gradient. These findings were corroborated by studies with inside-out vesicles incubated with membrane-free reticulocyte lysate in the presence but not in the absence of an ATP regenerating system, the activities of the $Na^+$-dependent glycine transport system and the $Na^+,K^+$-pump decreased. In addition, when mature red blood cell lysate was substituted for reticulocyte lysate no significant difference in $Na^+$-dependent glycine transport activity was evident.It was also demonstrated that the ninhydrin-positive material increased concomitantly with the decrease in membrane transport activity in the vesicle plus reticulocyte lysate reconstituted system.

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