Na\textsuperscript{+},K\textsuperscript{+}-ATPase EXPRESSION IN HIGH-K\textsuperscript{+} AND LOW-K\textsuperscript{+} SHEEP RED BLOOD CELLS

by

(©) Rajan Dhir

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ABSTRACT

The aim of this study has been to elucidate isoform expression of Na\textsuperscript{+},K\textsuperscript{+}-ATPase of the sheep red blood cell and to determine whether isoform expression is different in red cells of sheep of the high-K\textsuperscript{+} (HK) and low-K\textsuperscript{+} (LK) genotype. Both of the above objectives have been addressed by examining the protein and mRNA expression of the sodium pump in red cell precursor-rich bone marrow compared to sheep brain and kidney, the former by Western, the latter by Northern analysis. In the case of the $\alpha$ subunit, isoform-specific cDNA probes from rat tissue were used. For the $\beta_1$ subunit, sheep-specific and for $\beta_2$, rat-specific cDNA probes were used. The results indicate that in bone marrow of both high K\textsuperscript{+}-(HK) and low-K\textsuperscript{+} (LK) animals, as in kidney, only one isoform ($\alpha_1$) of the catalytic subunit is detected, whereas three forms ($\alpha_1$, $\alpha_2$ and $\alpha_3$) are detected in brain as reported by others. The expression of the mRNA of the $\beta_1$ subunit of the Na\textsuperscript{+}-pump in sheep red cells of both genotypes is very low. $\beta_2$, though present in brain as reported by others, could not be detected in either the kidney or red cells.
Notre étude de la Na⁺,K⁺-ATPase de mouton avait deux objectifs distincts: Le premier, de déterminer quel isoenzyme était exprimé chez les érythrocytes, le second de déterminer si les érythrocytes de génotype HK ou LK exprimaient des isoenzymes différents. Pour ce faire, les niveaux d'expression des ARN messager et des polypeptides spécifiques ont été mesurés par analyse de type Northern et Western dans la moelle osseuse, le cerveau et le rein de mouton. Pour détecter la sous-unité α, une sonde d'ADNC spécifique de rat a été utilisée. Pour détecter les sous-unités β1 et β2, des sondes spécifiques d'ADNC de mouton et de rat ont été utilisées, respectivement. Dans la moelle osseuse de mouton de génotype HK et LK ainsi que dans le rein, seule la sous-unité α1 est exprimée, tandis que trois sous-unités (α1, 2 et 3) sont détectées dans le cerveau tel que décrit précédemment. Chez les moutons des deux génotypes, le niveau d'expression de la sous-unité β1 est très bas dans le compartiment érythrocytaire. La sous-unité β2, bien que détectée par d'autres dans le cerveau, ne put être détectée dans le rein ou l'érythrocyte.
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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

i) Demonstration that the catalytic subunit of the red cell Na⁺,K⁺-ATPase is similar to the kidney enzyme, comprising only the α1 isoform.

ii) Demonstration that the genetic dimorphism of the sodium pump of high-K⁺ (HK) and low-K⁺ (LK) sheep red blood cells is not associated with differential isoform expression.
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ABBREVIATIONS

ADP adenosine diphosphate
ATP adenosine triphosphate
bp base pair
BSA bovine serum albumin
cDNA complementary deoxyribonucleic acid
cpm counts per minute
Ci Curie
dATP deoxyadenosine triphosphate
dCTP deoxycytidine triphosphate
DEPC diethyl pyrocarbonate
dGTP deoxyguanosine triphosphate
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphate
dpm disintegrations per minute
dTTP deoxothymidine triphosphate
EDTA ethylenediaminetetraacetate
EGTA ethylenebis (oxyethylenenitrilo) tetraacetate
HK high-K⁺
kb kilobase pairs
kDa kilodaltons
LK low-K⁺
M molar
mRNA  messenger ribonucleic acid
MOPS  3-(N-morpholino)propanesulfonic acid
PAGE  polyacrylamide gel electrophoresis
Pi    inorganic phosphate
RNA   ribonucleic acid
rpm   revolutions per minute
SDS   sodium dodecyl sulfate
SSC   standard saline citrate
tRNA  transfer RNA
TBS   tris buffered saline
TCA   trichloroacetic acid
TGG   tris glycyglycine
UV    ultraviolet
CHAPTER 1

INTRODUCTION
A. The Na\textsuperscript{+}, K\textsuperscript{+}-ATPase: definition and function

The Na\textsuperscript{+}, K\textsuperscript{+}-ATPase is an integral membrane protein which uses the energy of ATP hydrolysis to transport Na and K ions, unidirectionally, across the animal cell plasma membrane. In addition, it serves as the pharmacological receptor for cardiac glycosides such as digitalis and ouabain, which are clinically important in the treatment of heart disease. These drugs are specific inhibitors of the sodium pump and thus are also invaluable in the study of the enzyme’s reaction mechanism.

In general, most animal cells have high internal concentrations of K\textsuperscript{+} and low concentrations of Na\textsuperscript{+} relative to the external medium. This electrochemical gradient, generated and maintained by the active transport of Na\textsuperscript{+} and K\textsuperscript{+} ions, is of important physiological significance and serves as the source of energy for various cellular processes. Because of the generally higher passive permeability of the cell membrane to K\textsuperscript{+} compared to Na\textsuperscript{+}, diffusion of these ions, most notably K\textsuperscript{+}, down their concentration gradient creates a membrane potential, inside negative. In turn, changes in membrane potential directly effect certain homeostatic balances such as intracellular anion concentrations and this is involved in volume regulation in certain cells (Skou, 1988). In excitable tissues such as muscle and nerve, the sodium pump maintains the action potential. Thus, the sodium gradient,
when dissipated through the transient opening of Na\(^+\) channels, leads to an action potential (Stryer, 1988).

The concentration gradient of Na\(^+\) is also used as the energy source for the Na\(^+\)-dependent cotransport with nutrients such as sugars and amino acids into the cell through various membrane carrier proteins. Water balance and reabsorption of various compounds filtered from the blood into the kidney is also regulated by a combination of active Na\(^+\) transport and Na\(^+\) cotransport (Skou, 1988).

B. Enzyme mechanism

A summary of the reaction mechanism of the Na\(^+\),K\(^+\)-ATPase is given in Figure 1. For a more detailed description of the kinetic behaviour and conformational changes associated with this multisequence reaction the reader is referred to the following reviews: Cantley, 1981; Jorgensen, 1982; Glynn, 1985; Jorgensen and Anderson, 1988. Even though many of the details are still unknown, the mechanism formulated by the work of Albers and Post and their co-workers is generally considered as the working model.

According to this model, when the Na\(^+\) loading site is exposed to the intracellular surface the enzyme is in its E\(_1\) conformation. Accordingly, in the presence of Mg\(^{2+}\) and ATP bound at a high affinity site, three cytosolic Na\(^+\) are now accessible. Both Na\(^+\) and Mg\(^{2+}\) on the intracellular
Figure 1

The reaction mechanism of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase
$P_i \xrightarrow{} E_2 \cdot P \xrightarrow{} 3Na^+$

$E_2(2K)$

$\xrightarrow{}$ ATP

$E_2 \cdot 2K \cdot ATP$

$\xrightarrow{} 2K^+$

$E_2 \cdot ATP$

$\xrightarrow{} E_1 \cdot ATP \cdot 3Na$

$E_2 \cdot P \cdot 3Na$

$\xrightarrow{} ADP$

$E_1 \cdot P \cdot ADP(3Na)$

$E_1 \cdot ATP \cdot 3Na$

$3Na_c^+$

$(Exterior)$

$(Interior)$

Ouabain
surface are thought to promote phosphorylation by ATP (Blostein, 1979). This occurs at a specific aspartic acid residue (Post et al., 1965; Post and Kume, 1973) and is thought to lead to Na\(^+\) occlusion (Glynn and Hoffman, 1971; Glynn and Richards, 1983). In this occluded state Na\(^+\) cannot exchange with its internal or external environments. Depending on the order of addition of ATP, Na\(^+\) or Mg\(^{2+}\), different "sub-conformations" of the enzyme, all contained within the E\(_1\) conformation are postulated (Mardh and Post, 1977).

The release of ADP and a conformational transition to E\(_2\)P leads to Na\(^+\) deocclusion with Na\(^+\) loading sites now exposed to the extracellular surface. Na\(^+\) is released and this exposes extracellular K\(^+\) binding sites. It is not known whether the enzyme alternatively binds and releases Na\(^+\) and K\(^+\), possibly to the same loading sites in a "ping-pong" type reaction or whether they bind simultaneously at separate sites followed by a conformational change and release to opposite sides (simultaneous mechanism) (Garrahan and Garay, 1976) although the former mechanism is most likely (Sachs, 1986). The E\(_1\)P conformation is ADP-sensitive in the sense that the addition of ADP leads to dephosphorylation of E\(_1\)P and ATP formation (Fahn et al., 1966). Substances such as sulfhydryl reagents (Fahn et al., 1966) or chymotrypsin (Jorgensen et al., 1982) which block the conversion of E\(_1\)P to E\(_2\)P promote an ADP-ATP
Following these reaction steps, the addition of two $K^+$ catalyzes the hydrolysis of $E_2P$ by lowering the activation energy (Glynn and Karlish, 1975) and this leads to $K^+$ occlusion in $E_2$ (Post et al., 1972). $Mg^{2+}$ is also thought to be released at this point (Fukushima and Post, 1978). This is supported by the fact that $Mg^{2+}$ is released at the same time as the inhibitor vanadate which is a phosphate analogue believed to trap divalent cations (Smith et al., 1980). ATP binding to a low affinity site (compared to the high affinity site of $E_1$) accelerates the $E_2$ to $E_1$ conformational change and $K^+$ release into the cell interior (Karlish et al., 1978).

It is not known whether two molecules of ATP bind to separate sites with different affinity or a conformational transition of $E_2$ to $E_1$ changes the affinity of ATP bound to a single site. Thus, when the enzyme operates at low concentrations of ATP, ATP is unable to saturate its low affinity binding site. Under this condition the release of $K^+$ is rate-limiting (Post et al., 1972). In fact, in the absence of $K^+$ and presence of $Na^+$ the slow turnover of the pump is referred to as its $Na^+$-ATPase activity (Neufeld and Levy, 1967).

Studies suggesting the existence of two major conformational states involve largely tryptic analysis (Jorgensen, 1975; Jorgensen, 1977; Koepsell, 1979; Castro
and Farley, 1979) or measurements of fluorescence changes using various structural probes (see below). For example, in the $E_1$ ($Na^+$ induced) conformation, tryptic cleavage removes first a twenty amino acid N-terminal peptide from the catalytic subunit, followed by slow cleavage at a site 750 amino acids from the C-terminal. In contrast, tryptic cleavage releases a peptide 550 amino acids from the C-terminal in the $E_2$ ($K^+$ induced) state.

Fluorescence changes associated with shifts in conformation are measured by using fluorescent nucleotide analogues such as formycin triphosphate (Karlish et al., 1978), intrinsic tryptophan fluorescence (Karlish and Yates, 1978) or molecules such as fluorescein isothiocyanate (FITC) which reacts covalently with the catalytic subunit of the pump in the ATP binding site (Karlish, 1980). In general, the rate of conformational transitions is believed to be reflected by the rate of the fluorescence change measured with each particular probe.

C. Subunit structure and membrane topography

Part (a) of Figure 2 taken from Rossier et al. (1987) shows a general model of the $Na^+\cdot K^+$-ATPase and its two protein subunits originally discovered by sodium dodecyl sulfate (SDS) gel electrophoresis of the purified enzyme. There is a larger catalytic $\alpha$ subunit and a smaller glycosylated $\beta$ subunit. Molecular weight estimates of the
Figure 2 a&b

A general model of Na\textsuperscript{+},K\textsuperscript{+}-ATPase structure (Rossier et al., 1987)
Cytoplasm

$\beta$-subunit

Cytoplasm

$\alpha$-subunit

$\beta$-subunit

$\alpha$-subunit

MgATP $\rightarrow$ MgADP $\rightarrow$ P_i

$2 \text{K}^+$

Ouabain

$3 \text{Na}^+$

(b)
two subunits have come from a variety of different techniques, including analytical ultracentrifugation, gel filtration chromatography, Western blot analysis and more recently from the cDNA clones. From the latter the molecular weight of the α subunit is 112 kDa (Kawakami et al., 1985), while that of the unglycosylated β subunit is 35 kDa (Shull et al., 1986).

Over 70% of the α subunit is exposed to the interior and this surface contains the binding sites for Na⁺ and ATP, as well as the phosphorylation site. The extracellular surface of α contains the K⁺ loading site and the binding site of inhibitory cardiac glycosides. The majority of the β subunit is external to the cell membrane and it contains three potential sites for N-linked glycosylation. The migration rate of purified β subunit on SDS gels shows considerable species and even tissue-specific heterogeneity due presumably to variation in the extent of glycosylation (for review see Sweadner, 1989).

Part (b) of Figure 2, also taken from Rossier et al. (1987), shows a model for the membrane insertion of the two types of subunits. This type of model is based largely upon hydropathy analysis of primary structure deduced from the cDNA clones. The β subunit spans the membrane only once, has a cytoplasmic N-terminal and an extracellular C-terminal. The α subunit is thought to have six to eight hydrophobic membrane spanning domains (Kawakami et al.,
The first cytoplasmic loop (#1) is thought to contain the potential Na$^+$ binding site while the potential phosphorylation (#4) and ATP binding sites may reside in the second cytoplasmic loop (#5) (Jorgensen, 1982; Shull et al., 1985). The second extracellular loop (#2) may contain the potential K$^+$ loading site in addition to the ouabain binding site (Shull et al., 1985). The orientation of the C-terminal with respect to the inner or outer membrane surface is unknown.

It is possible that there may be a third subunit of the Na$^+$,K$^+$-ATPase referred to as the gamma subunit or the "proteolipid component". This small molecular weight membrane bound protein (7600 kDa) copurifies with the α and β subunit of the pump (Reeves et al., 1980). Partial amino acid sequence data of the purified protein indicate that it contains 68 amino acids and two distinct structural domains (Collins et al., 1987). The hydrophilic amino terminal is extracellular and contains aromatic amino acid residues which may be involved in ouabain binding. The C-terminal is largely hydrophobic and thought to span the membrane. The amino acid sequence is distinct from either α or β and therefore gamma is not a breakdown product of these two subunits. The strongest evidence that gamma is a functional part of the pump is that it can be labeled along with the α and possibly β subunits by a ouabain photoactive derivative and therefore it may form part of the ouabain
binding site (Forbush et al., 1978). The cDNA sequence of the gamma subunit has only recently been determined.

D. Pump assembly and β subunit glycosylation

The function of the β subunit is presently unknown. Both the α and β subunits are translated on membrane bound ribosomes and are inserted into the rough endoplasmic reticulum (ER) co-translationally, but with different insertion kinetics (Geering et al., 1985). It is thought that subunit assembly occurs after membrane insertion (Tamkun and Fambrough, 1986) and the β subunit, in the lumen of the ER, may direct the assembly as a possible "receptor" for the emerging α subunit. Evidence for the latter suggestion is not conclusive although the time course for α insertion lags behind that of β.

The β subunit is core glycosylated in the ER and subsequent glycosylation is performed while in the Golgi, en route to the cell surface. The insertion of the β subunit is dependent on endogenous signal recognition particles (SRP) and the β subunit lacks a cleavable N-terminal signal sequence. Studies using deletion mutants of various β subunit constructs show that only the hydrophobic membrane spanning region of the protein is required for insertion (Kawakami and Nagano, 1988). The extracellular carboxy terminus is not required.

1Mercer, R. unpublished
E. Evidence for the existence of catalytic subunit isoforms

During the 1970's evidence began to accumulate to support the notion of two or more isoforms of the catalytic subunit of the Na⁺-pump. This evidence was based largely upon affinity differences of a variety of tissues to inhibition by cardiac glycosides such as ouabain. Originally, it was demonstrated that the ouabain binding profiles of tissues such as brain are considerably more complex than that of kidney (Erdmann and Schoner, 1973). The results that followed, however, were confusing as the experiments were performed with varying amounts of external K⁺ and there is a complex relationship between K⁺ concentration and cardiac glycoside binding.

In the late 1970's it was clearly demonstrated that the ouabain inhibition profiles of rodent brain are biphasic, exhibiting both a high affinity and a low affinity (Marks and Seeds, 1978). However, only a monophasic kinetic profile, corresponding to the low affinity form was observed with rat kidney. In addition, these investigators showed that Scatchard plots of equilibrium binding of tritiated ouabain to brain tissue could be resolved into two components with affinity constants resembling those obtained from the inhibition profiles. Again, in kidney there was only a single site. Much of the earlier work was carried out on rodent (rat)
tissues; much less is known of other species. However, more recently it has become clear that the differences in ouabain sensitivity reflect, for the most part, species differences with respect to interaction with the kidney-type (α1) isoform of the enzyme (see Discussion).

a) Electrophoretic mobility

In 1978 and 1979, high resolution SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of partially purified brine shrimp (Peterson et al., 1978) and brain enzyme (Sweedner, 1979) demonstrated two protein subunits. These appeared as doublets after electrophoresis, with molecular weights between 100-110 kDa. In brain one component α⁺ migrated with a lower mobility and was believed to be slightly higher in molecular size as the designation α⁺ suggests. Its presence correlated with the putative form that exhibits a higher affinity for ouabain. Alpha, the characteristic kidney type had a slightly lower molecular weight than α⁺.

Evidence supporting the foregoing came also from studies on the active site phosphorylation of the brine shrimp (Peterson et al., 1978), rat (Sweedner, 1979) and ferret (Ng and Akera, 1988) enzymes from [γ-³²P]ATP. The partially purified pump was phosphorylated, subjected to SDS-PAGE and exposed to autoradiography. Two bands corresponding to α and α⁺ can be seen from tissues such as
brain. Using this technique it can be shown that lower concentrations of ouabain in the incubation medium are required to block the phosphorylation of rat $\alpha^+$ compared to $\alpha$, supporting the view that $\alpha^+$ is indeed the form with higher ouabain affinity, at least in the rat. In general, the affinity for ouabain ($K_d$) of the rat $\alpha$ form is approximately 0.9 $\mu$M while that of rat $\alpha^+$ is 100 $\mu$M (Urayama and Nakao, 1979). In the ferret, the affinity difference is much less (approximately 10-fold) (Ng and Akera, 1987).

b) Western blot analysis

Both polyclonal sera and monoclonal antibodies have been prepared against the catalytic subunit of the Na$^+$-pump (Sweedner and Gilkeson, 1985; Urayama et al., 1989). Some are isoform specific, while most show cross reactivity with the other isoform(s). This is not surprising when considering the high degree of amino acid sequence homology (deduced from the respective cDNAs) between $\alpha$ and $\alpha^+$. Thus, it is feasible that the antigenic determinants recognized by the antibodies can be either shared or distinct and no set criteria govern the specificity of the antiserum produced. With Western blot analysis, therefore, two distinct bands can be seen corresponding to $\alpha$ and $\alpha^+$ or just $\alpha$, depending upon the tissue studied. It should be mentioned that three forms of
the catalytic subunit of the sodium pump have recently been cloned from a rat brain cDNA library (Shull et al., 1986). Two isoforms correspond to $\alpha$ and $\alpha^+$, while the third isoform ($\alpha_3$) had been previously unidentified. That the $\alpha^+$ band is in fact a combination of two forms has been confirmed in recent reports. Accordingly, the aforementioned $\alpha^+$ band comprises two isoforms now termed $\alpha_2$ and $\alpha_3$. In the following discussion, the $\alpha^+$ designation is used in the context of studies carried out prior to the observation that $\alpha^+$ represents $\alpha_2$ and/or $\alpha_3$.

c) Presence of catalytic subunit isoforms in tissues of other species

Based again on studies of ouabain binding kinetics and Western blot analysis, evidence for the existence of two isoforms of the pump in dog (Charlemange et al., 1987; Maixent et al., 1987), rat (Sweedner and Farshi, 1987) and ferret (Ng and Akera, 1988) heart tissues have been reported. These results, again based upon ouabain binding profiles and Scatchard analysis of $[^3H]$ouabain binding, suggest multiple binding sites with different affinities. The high and low affinity sites correlate well with corresponding results on the positive ionotrophic effects of digitalis on heart muscle contractility in dogs (Adams et al., 1982). Low concentrations of the drug produce a biphasic stimulation on the force of ventricle contraction
by inhibiting the Na\(^+\),K\(^+\)-ATPase, presumably due to drug interaction with the two forms of the catalytic subunit.

Using kinetic and immunological analyses similar to those discussed above, catalytic subunit isoforms have also been detected in rat adipocytes and skeletal muscle (Lytton et al., 1985a) and other rat tissues such as retina (Specht and Sweadner, 1984). Although the exact correlation between these isoforms and those present in brain remains to be established, evidence is beginning to accumulate to support the notion that isoform expression may in fact be relevant to Na\(^+\)-pump regulation and serves to fine-tune its activity. Thus, suggestions of this nature are due to various structural and functional differences between the two isoforms as discussed below. It should also be noted that although to-date only one isoform of the \(\beta\) subunit (\(\beta_1\)) has been detected immunologically, a putative isoform termed \(\beta_2\) has been identified in rat brain by cDNA cloning (Vasallo et al., 1989).

F. Structural and kinetic differences between catalytic subunit isoforms

a) N-terminal amino acid sequence

From sequence analysis of the first 14 amino acids of the purified rat kidney \(\alpha\) isoform and the purified axolemma \(\alpha^+\) form, Lytton (1985) concluded that they are sufficiently different to be products of different genes. Of the first
fourteen amino acids, five are different and this is only true if the sequences are aligned in such a way to show maximum homology. This work was important in that it ascertained that the doublets apparent on SDS gels (phosphoenzyme formation) or on Western blots were not identical ie. the one was not formed from the other by either proteolytic breakdown or post-translational modification. These data, along with the results of subsequent cDNA cloning experiments provided the major evidence that the two bands were, in fact, distinct protein isoforms.

b) Probes of molecular structure

i) Trypsin

Even though the α and α⁺ (α⁺ and α3) isoforms have identical 1-D peptide maps (using trypsin and chymotrypsin for proteolysis), they do differ in their sensitivity to trypsin digestion (Sweadner, 1979). During a time course for trypsin digestion, the α isoform is more readily digested and this may indicate differences in tertiary structure. Whether the differences are between α and α⁺ and/or α and α3 remains to be determined (see below).

ii) Sulfhydryl modifying and cross-linking reagents

Alpha⁺ (α2 and/or α3) is more reactive with Cu²⁺-o-phenanthroline, a reagent which catalyzes the oxidation of
protein sulfhydryl groups (Sweadner, 1979). In this experiment, the preferential loss of $\alpha^+$ as a higher molecular weight cross-linked aggregate suggests that it has more potentially reactive $-SH$ groups than does $\alpha$. Similar experiments have been performed to test the reactivity of both isoforms with other sulfhydryl modifying agents such as N-ethyl[\$^3H\$]maleimide (Urayama and Nakao, 1979; Sweadner, 1979). The brain enzyme is more sensitive to inhibition by this reagent than kidney and brain $\alpha^+$ incorporates more radioactive label than does kidney $\alpha$. Again, this suggests a difference in tertiary structure.

Another apparent distinction between the kidney $\alpha$ and brain-specific $\alpha^+$ isoforms is the specific inactivation of the $\alpha^+$ form by pyrithiamine (Matsuda et al., 1984). Pyrithiamine is an antagonist of thiamine action and inhibits Na$^+$,K$^+$-ATPase activity by reacting with $-SH$ groups, producing disulfide bonds and causing structural changes in the protein. Preincubation of the enzyme with this compound decreases the active site phosphorylation of $\alpha^+$ by [\$^{32}\$P]ATP without affecting the $\alpha$ isoform. In addition, it changes the ouabain sensitivity profile of brain from biphasic to monophasic and decreases the reactivity of $\alpha^+$ to N-ethylmaleimide. These results suggest that pyrithiamine specifically inactivates the $\alpha^+$ isoform due to a difference in exposure of $-SH$ groups, consistent with a difference, albeit minor, in tertiary
c) Kinetic differences between catalytic subunit isoforms

Studies on basic kinetic properties of the isoforms of the Na\(^+\)-pump reveal a few apparent differences. Although rat kidney and axolemma forms exhibit half-maximal stimulation of activity at similar concentrations of Na\(^+\) and K\(^+\), the rat axolemma forms have a slightly higher affinity for ATP (Sweedner, 1985).

Further evidence for a functional difference between isoforms is the apparent effect of insulin on the \(\alpha^+\) isoforms in adipocytes and skeletal muscle. Insulin appears to stimulate the activity of \(\alpha^+\) in adipocytes, an effect abolished by ouabain at concentrations required to inhibit only \(\alpha^+\) (Lytton et al., 1985b). Stimulation of the \(\alpha_1\) form has not been observed. At 5 mM external K\(^+\), Lytton reported that the \(K_{1/2}\) values for Na\(^+\) in rat adipocytes are 17 mM and 52 mM for \(\alpha\) and \(\alpha^+\) respectively (Lytton, 1985). In the presence of insulin, the \(K_{1/2}\) for Na\(^+\) for the \(\alpha^+\) forms was decreased to 33 mM. This increase in Na\(^+\) affinity was reflected by an apparent stimulation of \(\alpha^+\) pump activity by insulin. To date, these are the only reports of kinetic differences.

G. Alpha subunit primary structure deduced from cDNA sequencing
cDNA cloning of the Na⁺,K⁺-ATPase has provided valuable new information on the enzyme's primary structure and allows for more precise identification of amino acid sequence variations between alpha subunit isoforms. This structural information may provide certain clues to understanding potential functional differences between isoforms such as Na⁺ affinity and differential cardiac glycoside sensitivity.

The primary structure of the sheep kidney α subunit was "amongst the first" to be deduced from its corresponding cDNA (Shull et al., 1985). Its full length clone (pαNKAl) was isolated from a sheep kidney cDNA library screened with oligonucleotide probes derived from the amino acid sequences of a variety of tryptic fragments. It contains an open reading frame of 3060 base pairs (bp) and thus represents a sequence of 1016 amino acids. Hydropathy analysis of this clone suggests eight major hydrophobic membrane spanning regions ranging in size from 20-30 amino acids. It also seems that most of the subunit is cytoplasmic with short extracellular regions (refer to Figure 2). The hydrophilic lysine-rich N-terminal is highly charged and thought to be involved in ion binding and events that influence cation occlusion and the E₁/E₂ conformational transition. Several acidic amino acid residues are found in this area and may form part of a cation channel.
Following the cloning of the sheep kidney $\alpha$ cDNA, several amino acid sequences have been deduced from the cDNA's of the alpha isoform from a variety of species and tissues. The primary sequences of the $\alpha$ subunit of Torpedo californica (reported concurrently, Kawakami et al., 1985), rat brain (Schneidner, 1985), human Hela cells (Kawakami et al., 1986), pig kidney (Ovchinnikov et al., 1986) and human placenta (Chehab et al., 1987) have all been derived from their corresponding cDNAs. In general, the nucleotide sequence homology usually varies between 75-80\%, while the amino acid homology is over 80\%. This strong homology suggests that the alpha isoform in each species has arisen from a common ancestral gene. In addition, when the protein sequences are aligned and compared substituted amino acids often occur in the same region.

Further comparison of the cDNA clones for the $\alpha$ isoform reveals that they are of a similar length, representing 1016 amino acids for the sheep and pig kidney pumps, up to 1023 amino acids for the Hela cell enzyme. They also show certain common, and most likely functional domains. For example, the conserved sequence Cystine-Serine-Aspartate-Lysine usually occurs around aspartate 369, the postulated phosphorylation site. In addition, peptide fragments in a region corresponding to amino acids 498-508 contain a lysine residue (Lys 501) which reacts specifically with fluorescein 5'-isothiocyanate (FITC).
This area is thought to form part of the ATP binding site. In addition, other P-type ion translocating ATPases such as the Ca\(^{2+}\)-ATPase show these regions of conservation (Shull et al., 1985).

H. cDNA cloning provides the first evidence for a third isoform of the \(\alpha\) subunit

Although the biochemical evidence for the existence of \(\alpha\) subunit isoforms has been previously discussed, a genetic basis for their production had not been established until recently. Three distinct forms of the \(\alpha\) subunit of the Na\(^{+}\),K\(^{+}\)-ATPase have been recently cloned from a rat brain cDNA library (Shull et al., 1986). The most abundant cDNA, with an open reading frame of 1020 codons, was shown to represent an \(\alpha^+\) form after comparison of the N-terminal amino acid sequence to that previously described by Lytton. The second most abundant cDNA was identical in sequence to the rat kidney form cloned in the same study and thus represented the \(\alpha_1\) isoform. It contained an open reading frame of 1023 codons. Lastly, the cDNA of lowest abundance, representing 1013 codons, had not been previously identified at the protein or cDNA level and its sequence was distinctly different from either \(\alpha\) or the \(\alpha^+\) mentioned above. It was similar enough, however, to be considered an isoform and was termed \(\alpha_3\).

The amino acid sequence homology between \(\alpha_3\) and either
α1 or α2 is in the range of 85% and the regions most conserved are the potential phosphorylation and ATP binding sites and the hydrophobic membrane spanning domains. The major sequence differences of the rat brain isoforms lies in the N-terminal region and in the region between the phosphorylation and ATP binding sites. Since the publication of a cDNA sequence of a third isoform, a number of laboratories have sequenced and confirmed its presence in rat brain cDNA libraries (Herrera et al., 1987; Schneider et al., 1988). Northern analysis (see later) indicates that it is expressed predominantly in brain and at very low levels in certain other tissues.

The expression of the rat brain α3 form at the protein level has only been demonstrated recently (Urayama and Sweekner, 1988; Lowdnes et al., 1988; Hsu and Guidotti, 1989). Partial amino acid sequence analysis of the purified brain enzyme reveals a formic acid cleavage fragment which corresponds to the α3 isoform. Antibodies raised to this fragment recognize a protein designated as the α3 isoform which is distinct from either α1 or α2 on Western blots. With sufficiently high resolution gels, it is shown that the mobilities of the three isoforms on SDS gels follows the following order: α1 > α2 > α3.

There is some confusion in the literature regarding the nomenclature of the various isoforms. It is now generally agreed that α1 refers to the well characterized
kidney $\alpha$ form, $\alpha^+$ is referred to as $\alpha_2$ and $\alpha_3$ is the additional form present in brain. Since expression of all three isoforms has been demonstrated in brain, it is likely that the higher molecular weight band of the doublet observed on SDS gels of purified brain enzyme and on Western blots is, in fact, a combination of $\alpha_2$ and $\alpha_3$ protein. This adds an additional level of complexity to the biphasic ouabain inhibition profiles seen in brain as $\alpha_3$ exhibits a high sensitivity (0.13 $\mu$M) much like $\alpha_2$ (Urayama and Sweadner, 1988).

I. $\beta$ subunit primary structure deduced from cDNA sequencing

In the past few years the primary structure of the $\beta$ subunit of the $\text{Na}^+,\text{K}^+$-ATPase has also been successfully determined from its cDNA sequence. The amino acid sequences of the following $\beta$ subunits are now known: T. californica (Noguchi et al., 1986), sheep kidney (Shull et al., 1986), human Hela cell (Kawakami et al., 1986), pig kidney (Sverdlov et al., 1987), rat kidney (Young et al., 1987) and brain (Mercer et al., 1986). The sheep kidney $\beta$ subunit contains 302 amino acids and residues 1-33 are intracellular, 34-59 are membrane spanning and the rest is extracellular. Like the sheep kidney $\alpha$ subunit, the first in frame ATG codon lies within a nine base pair consensus sequence for initiation.

Nucleotide interspecies sequence homology for the $\beta$
subunit is not as extensive as that which is observed for the α subunit. For example, the amino acid sequence homology between human HeLa cell and T. californica β1 subunits is 61% (Kawakami et al., 1986). Quite unexpectedly, there is a region which is strongly conserved in the 3' untranslated region of each cDNA; it is proposed that this region is involved in controlling the post-transcriptional processing of β subunit mRNAs (Young et al., 1988).

The β subunit cDNA's isolated from the rat kidney and brain libraries vary in the length of their 5' and 3' untranslated regions, an effect likely due to the use of two separate ATG initiation codons in the 5' end and three potential polyadenylation sites in the 3' end (Young et al., 1988). The coding region for all the cDNA's are identical and the 5' untranslated region contains two conserved boxes which may serve as recognition sequences for various transcription factors which control the site and frequency of transcription initiation (Young et al., 1988).

Recently, Vasallo et al. (1989) identified a putative isoform of the β subunit (termed β2) in rat tissues by cross-hybridization with the original β subunit cDNA (now refered to as β1). For simplicity, this terminology will now be adopted. Several lines of evidence suggest that the β2 cDNA codes for a β subunit isoform. For example, it
exhibits 58.3% amino acid sequence homology to rat β1, a similar molecular mass (approximately 34,000 kDa) and hydropathy profile. In addition, like β1, it also contains one potential transmembrane domain and similar conserved residues of potential N-linked glycosylation.

**J. A genetic basis for the study of differential ouabain sensitivity**

The availability of α and β subunit cDNA clones has provided a genetic tool to study the structural basis for differences in interaction of cardiac glycosides with the Na⁺-pump. In addition to the ouabain affinity differences between the α₁ and α₂ isoforms of the rodent, there exist species differences in α₁ ouabain sensitivity. In fact, it is now clear that differences in ouabain sensitivity are primarily relevant to differences in sensitivity of α₁ isoforms of various species. Thus, the rodent α₁ pump is referred to as ouabain insensitive while α₁ isoforms from other species are generally ouabain-sensitive. For example, the sensitivity of the human α₁ isoform to inhibition by ouabain is 1000 times more than that of the corresponding α₁ isoform from mouse and rat.

The marked inter-species and isoform sensitivity differences have led to several studies concerned with the structural basis for ouabain interaction with the Na⁺,K⁺-ATPase. As mentioned before, preliminary analysis with
ouabain photoaffinity analogues has determined that such compounds covalently modify both the \( \alpha \) and \( \beta \) subunits suggesting that ouabain may interact with extracellular regions of both \( \alpha \) and \( \beta \) (Hall and Ruoho, 1980).

The first direct demonstration that the \( \beta \) subunit is not directly involved in mediating the species differences in ouabain affinity came from the work of Takeyasu et al. (1987). They co-transfected the \( \beta \) subunit from a ouabain sensitive species (chicken) into ouabain-resistant mouse Ltk\(^-\) cells (ouabain insensitive) and demonstrated the expression of a hybrid Na\(^+\)-pump which was ouabain insensitive. They concluded that the ouabain binding site is located on the \( \alpha \) subunit and not affected by the \( \alpha \)-\( \beta \) interaction.

That the \( \alpha \) subunit is involved in mediating species difference in ouabain sensitivity has been reported also by Kent et al. (1987). This group transfected a full-length murine \( \alpha_1 \) cDNA isolated from a pre-\( \beta \) cell library into monkey CV-1 cells and selected for resistance against concentrations of ouabain up to \( 10^{-4} \) M. The results indicate that the introduction of a ouabain insensitive \( \alpha \) subunit into sensitive recipient cells confers ouabain resistance and implies that the newly introduced protein is capable of forming hybrids with \( \beta \) subunit from the recipient cells to produce a functional ATPase molecule. The presence of murine \( \alpha_1 \) DNA in the monkey genome was
confirmed by Southern analysis.

The earlier cDNA cloning study of the sheep kidney enzyme suggests that the extracellular junction between the membrane spanning domains H3 and H4 of the α subunit may be involved in ouabain sensitivity (Shull et al., 1985). This region contains a Trp residue which has previously been implicated in the ouabain binding site (Fortes, 1977). It is also relatively hydrophobic (ouabain is a steroid with an unsaturated lactone ring) and shows very little homology to the Ca$^{2+}$-ATPase, which does not bind ouabain. By comparing amino acid sequence differences between rat kidney and brain ouabain insensitive and sensitive forms, namely differences in α and α$^{+}$ isoforms, the junction between hydrophobic regions H1 and H2 has also been proposed to be involved in the binding site (Shull et al., 1986). It is possible that the ouabain molecule may interact with a number of sites on the Na$^{+}$-pump.

To define the exact region of the α1 subunit responsible for determining the species differences in ouabain sensitivity, structure/function analysis was carried out by constructing chimeric cDNA molecules comprising putative ouabain-sensitive and -insensitive α1 regions (Price and Lingrel, 1988). By expressing various vector constructs into ouabain sensitive Hela cells, these authors demonstrated that the amino acid residues involved were in the N-terminal half of the molecule, more
specifically, the first extracellular domain between membrane spanning regions H1 and H2. Further, by the use of site-directed mutagenesis they demonstrated that a substitution of glycine for arginine at the junction between H1 and the amino terminus of the extracellular region and asparagine to aspartate at the carboxy terminus/H2 junction is sufficient to generate ouabain insensitivity.

K. Genomic organization of the $\alpha$ subunit isoforms

Information regarding the chromosomal organization of the Na$^+$,K$^+$-ATPase genes is important in defining their promotor regions and sites of potential interaction with transcription factors. First, a human genomic library constructed from leukocyte DNA was screened with sheep $\alpha$1 and rat $\alpha$1, $\alpha$2 and $\alpha$3 cDNA probes (Shull and Lingrel, 1987). Four classes of clones were isolated (each 20-25 kb in length) and by Southern analysis, two, aA and aB, were shown to represent the genes for the $\alpha$1 and $\alpha$2 isoforms. The third and fourth class of clones, aC and aD, hybridized to the sheep $\alpha$1 probe, but not to any of the rat isoform specific ones. Although these clones exhibit fairly strong amino acid homology (approximately 85% and 70% for aC and aD respectively) to each isoform, they are not thought to be the genes for any of the Na$^+$,K$^+$-ATPase isoforms, but rather for a related ATPase. A similar independent study
with genomic libraries from human placenta and brain have identified five classes of clones (Sverdlov et al., 1987). Three classes are thought to be isoform specific, while the other two are either pseudogenes or code for a related pump.

Kent et al. (1987) used mouse-hamster somatic cell hybrids and restriction fragment length polymorphisms to map the chromosomal localization of the α isoform genes. They showed that in the mouse the α1 gene mapped to chromosome 3, α2 and β to chromosome 1 and α3 to chromosome 7. The β1 subunit gene was not physically linked to α2. In another study in which in situ hybridization was carried out on human chromosomes, the alpha subunit of a human placenta cDNA localized to the short arm of chromosome 1 (Chehab et al., 1987). A more extensive analysis carried out by Yang-Feng et al. (1988) has mapped the human α1 and α2 isoforms to chromosome 1 and α3 to human chromosome 19. In this study the β1 subunit gene also mapped to chromosome 1.

L. Tissue-specific expression of α and β subunit mRNAs

With the availability of isoform-specific cDNA clones, a number of studies have been concerned with the tissue-specificity of the Na⁺,K⁺-ATPase α and β subunits in various tissues by Northern analysis (Young and Lingrel, 1987; Sverdlov et al., 1988; Schneider et al., 1988;
Emanuel et al., 1987; Herrera et al., 1987; Orlowski and Lingrel, 1988) and in situ hybridization (Schneider, 1988). In these experiments either full length cDNA probes or the N-terminal regions which exhibit the least sequence homology between the isoforms were used. There are, however, conflicting results from the different laboratories regarding the size of the mRNA species detected. In addition, the question of the existence of small amounts of a specific isoform in certain tissues has been controversial (see below) due possibly to cross-hybridization at lower stringency washing conditions. However, according to the studies cited above, the rat α1 cDNA hybridizes to one mRNA species 3.7-4.5 kb in size. The rat α2 cDNA hybridizes to two mRNA species, 3.4-4.5 kb and 5.3-6.0 kb, while α3 hybridizes to a single 3.7-4.5 kb mRNA.

Young and Lingrel (1987) have examined the pattern of α1 isoform mRNA expression in a variety of rat tissues. They show that while α1 mRNA exists in rat kidney, brain, heart, adipose, muscle, stomach, lung and liver, the intensity of the signal varies considerably. As expected, the message is most abundant in kidney, whereas much weaker signals are observed in liver. Emanuel et al. (1987) have reported that the α1 isoform is expressed in all rat tissues examined and in cell lines derived from the rat central nervous system. This ubiquitous expression of the
$\alpha_1$ mRNA has also been demonstrated by Herrera et al. (1987) and Orlowski and Lingrel (1988). Thus, the $\alpha_1$ form of the Na$^+$-pump is expressed at the mRNA level in all rat tissues, although the physiological relevance of this is not known.

$\alpha_2$ mRNA expression is more limited in tissue distribution than $\alpha_1$ and the intensity of the higher molecular weight mRNA species varies with respect to that of the lower. Young and Lingrel (1987) report a predominance of $\alpha_2$ message in brain and muscle, but only weak signals in lung, stomach and, surprisingly, kidney. The work done by Schneider et al. (1988) reports the expression of $\alpha_2$ in heart, spinal cord and diaphragm in addition to brain and muscle. However, unlike the studies from Lingrel's laboratory, other groups failed to demonstrate the presence of $\alpha_2$ mRNA in kidney (Schneider et al., 1988; Emanuel et al., 1987; Herrera et al., 1987).

Like $\alpha_2$, $\alpha_3$ mRNA expression is also more restricted in tissue distribution. It is apparent predominantly in brain, with lower levels of expression in fetal and adult muscle and in spinal cord. In situ hybridization analysis with $35S$-labelled RNA probes has shown that $\alpha_3$ is expressed predominantly in neural cells and is abundant in frozen sections of both fetal and adult brain (Schneider et al., 1988).

Studies with $\beta_1$ subunit mRNA expression show that cDNA clones isolated from a rat kidney and brain library
hybridize to at least four specific mRNA's. They all vary in size, although the particular size which is seen is tissue dependent (Young et al., 1987). The two largest mRNA species of the β1 subunit (2.7 and 2.3 kb) are present in all tissues examined and are most abundant in brain and kidney, while the smaller two species are occasionally seen in certain tissues. Northern analysis with cDNA probes derived from the 5' and 3' ends of the β1 probes suggest that the difference in size between the mRNA species is due to varying lengths of the 5' untranslated region. It is also interesting to note that ouabain-resistant Hela C+ cell lines show DNA sequence amplification and overproduction of both α and β subunit message (Mercer et al., 1986). The functional significance of this, if any, is not known.

Overall, the pattern and intensity of expression of α and β subunit mRNA varies with the particular rat tissue investigated. Whether differential, developmental and tissue specific isoform expression is relevant to specialized functions of the different isoforms and serve to fine-tune the activity of the Na+-pump remains to be determined.

M. The Na+,K+-ATPase of genetically distinct sheep red blood cells: High-K+ (HK) and low-K+ (LK) polymorphism

Mature sheep red blood cells provide a unique and
potentially very interesting system for studying the molecular basis for structural and functional differences in \( \text{Na}^+, \text{K}^+ \)-pump behavior. In populations of sheep, there are two types of red blood cells which are morphologically and biochemically indistinguishable except for their intracellular cation concentrations and certain kinetic aspects of their \( \text{Na}^+ \)-pumps. LK (low-\( \text{K}^+ \)) cells have a cytosolic concentration of \( \text{K}^+ \) (\( \text{K}_c \)) in the order of 15 mM and HK (high \( \text{K}^+ \)) cells have higher concentrations of about 85 mM (Kerr, 1937). In contrast, the cytosolic concentrations of \( \text{Na}^+ \) (\( \text{Na}_c \)) are reversed in both cases with LK cells having 85 mM \( \text{Na}_c \) and HK cells having 15 mM. Thus, the sum of the internal cation concentration is the same in both cases as is the external (plasma) cation concentration of 150 mM \( \text{Na}^+ \) and 5 mM \( \text{K}^+ \). The cytosolic cation concentrations of the HK cell, as opposed to LK, most closely resembles that of the typical animal cell.

This difference between HK and LK cells is genetically determined and inheritance is governed by simple Mendelian principles. The LK genotype (homozygous LL) is dominant over that of HK (MM) and thus heterozygous animals (LM) are phenotypically LK (Evans, 1954). The end result is that the red cells of approximately 2/3 of all sheep are LK and those of the remaining 1/3 are HK. On average, heterozygous LK cells also have \( \text{K}_c \) concentrations that are slightly higher than their homozygous counterparts.
(Rasmussen and Hall, 1966). It should be noted that this HK/LK dimorphism is also seen in the red blood cells of a variety of species such as cattle, goats, bears and dogs, although human red blood cells have internal cation concentrations that are typically high-K⁺.

**N. Functional differences in the Na⁺,K⁺-pumps of HK and LK red blood cells**

**i) Active and passive transport activity**

Originally it was noted that HK cells have 4- to 6-times the active transport activity of LK cells. This work, carried out by Tosteson and Hoffman in 1960, comprised the measurement of both ouabain-sensitive and-insensitive Na⁺ and K⁺ transport. In addition, this classic paper showed that the leak permeability of the red cells to K⁺ was three times higher in LK than HK cells. The passive permeability to Na⁺ is the same in both cases. The authors concluded that even though HK cells have higher active transport activity, their lower leak fluxes counterbalance this effect to maintain a similar total internal cation concentration at steady state. This allows for volume control and may explain the fact that HK and LK cells have approximately the same cell volume. In addition, other investigators observed greater cation transport in HK cells as measured by other parameters such as Na⁺-ATPase activity (Whittington and Blostein, 1971) and
Thus, HK cells have a greater number of functional Na\textsuperscript{+}-pump molecules than do LK cells (Joiner and Lauf, 1975). In such experiments, the number of pump sites per cell can be measured directly by quantifying the number of \textsuperscript{3}Houabain molecules bound at equilibrium. It can be shown that while HK cells have approximately 120 pump molecules per cell, LK cells have about 50. The number of pump sites is also slightly higher in homozygous LK cells than in heterozygous ones. Although one might assume that the higher transport activity and K\textsuperscript{+} content of HK cells is due to the greater number of pump sites, this does not appear to be entirely true, since there are well-established kinetic differences in the properties of the sodium pumps of the two types of cells, which probably also affect the cation transport activity measurements (see section below).

ii) Internal cation activation kinetics

The most prominent kinetic difference between HK and LK cells resides in the observation that in LK cells, the K\textsubscript{1/2} for K\textsuperscript{+} as a competitor for the three cytosolic Na\textsubscript{load}ng sites is much greater than in HK cells (Hoffman and Tosteson, 1971). In LK cells, this results in K\textsubscript{C} being a potent inhibitor of the interaction of Na\textsuperscript{+} at its intracellular loading sites. In this same study it was
shown that in LK cells, varying intracellular K⁺ down to 1 mM by replacement with Na⁺ increases the K⁺ influx several fold, albeit to rates still lower than observed in HK cells. On the other hand, similarly increasing Kᵦ to values over 30% of the total Na⁺K concentrations (i.e. over 30 mM if the total cation concentration is 100mM) completely abolished K⁺ influx activity, again demonstrating the inhibitory effect of Kᵦ. Since altering the internal cation concentrations does not affect the apparent affinity of the pump for external K⁺ (Kₒ), it was concluded that the inhibitory effect is not at the external surface. The results by Hoffman and Tosteson (1971) showed also that external activation kinetics of Kₒ in the presence of Naₒ show a similar K₁/₂ for maximum stimulation in both HK and LK cells. Thus, the effect of potassium is likely to be at the internal cation-loading site(s), suggestive of a difference in conformational state between the two types of pumps.

iii) Ouabain binding kinetics

The rate of ouabain binding to HK pumps is significantly faster than binding to LK pumps and this is thought to be related to the higher rate of turnover of HK pumps which is, in turn, probably due to the lower inhibitory effect of Kᵦ (Joiner and Lauf, 1975). This view is consistent with the experimental observation that the
rate of ouabain binding in LK cells can be increased by replacing intracellular K⁺ with Na⁺ (Joiner and Lauf, 1978). The difference may also be due in part to the greater number of HK pumps/cell when compared to LK. This factor likely acts in conjunction with the kinetic effect to contribute to the overall result.

iv) Kinetic differences between HK and LK reticulocyte pumps

The above discussion is limited to what is known regarding the kinetic and quantitative differences between HK and LK mature red blood cells. The situation, however, becomes more complicated when HK and LK reticulocytes are also considered. In comparison to mature red cells, reticulocytes have higher pump fluxes, high intracellular K⁺ and differences between HK and LK retics are not apparent. Thus, sheep reticulocytes have typical HK cation concentrations and only during cell maturation does the HK/LK divergence become apparent. In addition, when cells are preloaded with varying amounts of K⁺ and their ouabain-sensitive K⁺-influx is measured at physiological ATP, HK and LK reticulocytes have a kinetic profile resembling mature HK cells (Dunham and Blostein, 1976). In contrast, HK and LK reticulocyte pumps resemble LK pumps when the enzyme is tested at very low ATP concentrations. This behavior is apparent when K⁺ effects on Na⁺-activated ATP
hydrolysis are tested. Thus, inhibitory effects of K\(^+\) (KCl \(\geq 5\) mM) on the Na\(^+-\)ATPase activity of both HK and LK reticulocyte membranes are observed, resembling the behavior of mature LK cells. The profile is distinctly different from the Na\(^+-\)ATPase of mature HK cells in which case a stimulation of Na\(^+-\)ATPase activity is observed at potassium concentrations up to 5 mM (Blostein et al., 1974). The above observation indicates clearly a kinetic difference between pumps of HK and LK cells.

0. Aim of this Project

In the past a considerable amount of effort has focused on the behavior of active Na\(^+\) and K\(^+\) transport by the sodium pump using the red blood cell as a model system. This holds true particularly for studies of the different modes of ion translocation and side-specificity of ligand effects. On the other hand, more active tissues such as brain and kidney have been a focus of study of enzyme structure and kinetics. In spite of this, the question remains as to the nature of the Na\(^+\),K\(^+\)-ATPase isoform(s) expressed in the red cell. Therefore, the first aim of this project has been to establish whether or not the red cell sodium pump resembles kidney (\(\alpha\)) or brain (\(\alpha^+\) as well as \(\alpha\)) with respect to its structure by analysis of mRNA expression.

The red blood cell system, however, provides certain
obstacles in the study of the molecular basis of mRNA expression. Part of the difficulty lies in isolating functional RNA from red cells young enough to express specific sodium pump message. Earlier attempts have failed to detect Na\textsuperscript{+},K\textsuperscript{+}-ATPase specific mRNA in a cDNA library constructed from human reticulocyte poly (A)\textsuperscript{+} mRNA (Chehab et al., 1987) or sheep reticulocytes (Dunham, P.B., personal communication). It appears that at this stage of red cell maturation, the cells are devoid of sodium pump message even though the enzyme protein in reticulocytes is higher than in the mature erythrocyte (Grafova and Blostein, 1988). Therefore, the experimental approach in my study has been to isolate Na\textsuperscript{+},K\textsuperscript{+}-ATPase specific mRNA from red cell precursors present in the bone marrow tissue of anaemic HK and LK sheep.

In light of the foregoing, a second question that was addressed concerned the molecular basis for the genetic polymorphism observed for HK and LK red cell sodium pumps. With regards to a potential functional role for differential isoform expression, a first step is to examine the possibility that the kinetic differences between HK and LK pumps may be due to differential expression of isoforms of either the catalytic alpha subunits or possibly beta isoforms. Therefore, the second aim of this project has been to examine the possibility of differential expression of isoforms of the catalytic as well as beta subunits of
the sodium pump at the protein and mRNA level in HK and LK red cells.
CHAPTER 2

MATERIALS AND METHODS
A. RNA extraction

i) Solid tissues

Sheep brain and kidney tissues were obtained from a freshly sacrificed animal, immediately frozen on dry ice and then later transferred to a -70°C freezer. RNA extraction was performed according to the method of Chirgwin et al. (1979), with minor modifications as follows. Briefly, the frozen tissue was first cooled in liquid N₂, then thoroughly disrupted and homogenized in a stainless steel Waring blender containing 5.0 ml of guanidinium isothiocyanate solution per gram of original tissue. This solution, prepared fresh each time, consisted of 4 M guanidinium isothiocyanate, 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, pH 8, 10 mM 2-mercaptoethanol and 2% Sarkosyl (N-lauryl sarcosine). The homogenized mixture was then added to an equal volume of redistilled phenol (ultrapure, BRL) preheated to 55°C (hot phenol) and previously saturated with 0.1 M sodium acetate, pH 5.2 and containing also 0.1% 8-hydroxyquinoline. One-half volume of 0.1 M sodium acetate, pH 5.2 and chloroform/isoamylalcohol (24:1) was subsequently added and the RNA was extracted with constant shaking at 55°C for 10 minutes. The extraction mixture was cooled in a bath of dry ice and ethanol and centrifuged at 5000 rpm at 4°C for 10 minutes to separate the aqueous and organic phases. The upper aqueous phase, containing nucleic acid material was recovered and re-
extracted with phenol/chloroform until no protein was visible at the interface.

ii) Cell suspensions

To obtain bone marrow tissue enriched in nucleated precursor cells, HK and LK sheep were made anaemic by the following procedure: one litre of peripheral blood was taken by jugular phlebotomy on three consecutive days and then on day 5 and day 8. On day 13, the animals were given general anesthetic and bone marrow samples were taken by aspiration from the iliac crest. The marrow was collected in sterile tubes, centrifuged at 3000 rpm for 10 minutes and the plasma removed. The packed cells were frozen in a dry ice/ethanol bath and transferred to a -70°C freezer for storage. To extract the RNA the frozen cells were added directly to 4.0 M guanidinium isothiocyanate, preheated to 55°C. The cells were lysed and an equal volume of hot phenol was very quickly added and then one-half volume of chloroform/isoamylalcohol. The extraction was performed as described above.

To precipitate the RNA, 5.0 M NaCl (final concentration, 0.2 M) was added to the aqueous phase, now free of protein and DNA contamination, along with two volumes of 99% ethanol. The mixture was cooled at -20°C overnight or in a dry ice/ethanol bath for 15 minutes. The ethanol-precipitated RNA was collected by centrifugation at
10,000 rpm at 4°C for 20 minutes and the pellet dried under vacuum. The RNA was dissolved in an appropriate volume of diethyl pyrocarbonate (DEPC)-treated H₂O and absorbance readings were taken at 260 and 280 nm to determine its concentration and purity, assuming a value of 0.022 absorbance units at 260 nm for RNA at a concentration of 1 µg/µl and an absorbance ratio (260/280) of 2 represented pure RNA.

B. Poly (A)⁺ selection of mRNA

Poly (A)⁺ selection of mRNA from preparations of total RNA is essentially that developed by Aviv and Leder (1972) with minor modifications. Oligo(dT)-cellulose (Pharmacia) was first equilibrated in sterile loading buffer in 10 ml disposable polystyrene columns. The loading buffer consisted of 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8, 0.05% SDS and 0.5 M NaCl. The RNA sample was heated to 65°C for 5 minutes, diluted with an equal volume of twice concentrated (2X) loading buffer and then briefly cooled on ice. The sample, adjusted to 0.5 M NaCl, was applied to the column and the flow adjusted to approximately 1 drop/minute. The eluate was collected, re-heated to 65°C, cooled and re-applied to the column for a second round of poly (A)⁺ selection. After applying the sample, the column was washed with 10-15 ml of loading buffer to remove most of the poly (A)⁻ RNA. The poly (A)⁺ RNA was then eluted
with 3 ml of sterile elution buffer comprising 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, pH 8 and 0.025% SDS. The RNA was recovered by ethanol precipitation as described above, dissolved in DEPC-treated H₂O and absorbance readings were taken. The poly (A)⁺ RNA was stored at -70°C.

C. Plasmid purification

Full length sheep kidney α1 (Shull and Lingrel, 1985) and β1 subunit (Shull et al., 1986) cDNA clones were gifts from Dr. J. Lingrel, University of Cincinnati College of Medicine. The α1 cDNA spanned 3.65 kb and was contained in pBr322. The β1 subunit cDNA was received as such and had been contained originally in pBr322 and grown in host HB101. Bacteria infected with plasmids containing full length isoform-specific rat cDNA clones were gifts from Dr. R. Mercer, Washington University School of Medicine. α1 and α3 were contained in the plasmid pGem4, while α2 was in Bluescript. Rat full length β2 cDNA was kindly provided by Dr. R. Levenson, Yale University School of Medicine.

The cDNA inserts were isolated from the plasmids as follows: bacteria infected with specific plasmids were grown overnight at 37°C on agar plates prepared by dissolving 15.0 g of agar (Difco) in 1.0 l of LB broth (10 g bacto tryptone (Difco), 5.0 g bacto yeast extract (Difco) and 10 g NaCl/litre H₂O) containing 20 μg/μl tetracycline (sheep α1) or 100 μg/μl ampicillin (rat α1, α2 and α3). A
single colony was then inoculated into 5.0 ml LB broth containing the specific antibiotics (see above) and was grown overnight at 37°C. The overnight culture was then added to 500 ml sterile brain/heart infusion (Difco) and incubated 5-6 hours at 37°C with constant shaking, after which 100 mg chloramphenicol was added to amplify the growing plasmid and the incubation was continued overnight.

The bacteria were harvested by centrifugation at 5000 rpm at 4°C for 5 minutes, washed with ice cold 10 mM Tris-HCl, pH 7.5 and re-suspended in 10 ml of sterile H$_2$O. Bacterial lysis was initiated by the addition of 2 ml of 10 mg/ml lysozyme (Sigma, 50,000 units/mg protein) in 0.25 M Tris-HCl, pH 8.0. One ml of 10% SDS was added and the suspension was mixed and left at room temperature until lysis was complete. The lysate was centrifuged at 15,000 rpm at 4°C for 1 hour to remove chromosomal DNA and bacterial residue. The supernatant was then extracted three times with redistilled phenol, saturated with 1.0 M Tris-HCl, pH 7.6 and containing 0.1% 8-hydroxyquinoline. The plasmid DNA was then precipitated from the aqueous phase by the addition of an equal volume of ethanol, air-dried, re-dissolved in sterile water and stored at -20°C.

In certain instances it was necessary to remove RNA contamination from the plasmid preparations. The RNA was salt-precipitated by the addition of 5.0 M NaCl to a final concentration of 3.0 M and collected by centrifugation at
10,000 rpm. The plasmid DNA in the supernatant was precipitated with 70% ethanol and dissolved in 5.0 ml of 10 mM Tris-HCl, pH 7.6, containing 5 mM EDTA, pH 8.0. RNase digestion was carried out as follows: 1.0 ml of 10 mg/ml RNase (Sigma, 250,000 units/mg protein) was added to the plasmid DNA and incubated for 1 hour at 37°C. 1.0 ml of 20 mg/ml proteinase K (Sigma, 10-20 units/mg protein) was then added and the mixture incubated for 2 hours at 55°C to remove residual traces of RNase. The plasmid DNA was re-extracted with phenol as described above, precipitated with ethanol and dissolved in sterile water. Absorbance reading at 260 and 280 nm were taken to determine concentration and purity, assuming a 260/280 ratio of 1.8 for pure DNA.

D. Restriction digests of the plasmid preparations

Sheep α1 subunit cDNA comprising the entire coding region was isolated from pBR322 by digestion of plasmid DNA with Pst I. All restriction digests were carried out according to manufacturer's instructions (BRL or Pharmacia). Since there is an internal Pst I site, two 1.8 kb fragments are produced. Similarly, N- and C-terminal fragments, 500 and 600 bp in length and comprising both coding and non-coding regions were produced by digestion of the recovered full-length cDNA (see below) with Sal I and Bgl II, respectively.

Rat α2 was recovered from Bluescript by digestion of
plasmid DNA with Eco R1. This produced a 3.6 kb α2 cDNA and the 3.0 kb vector. Plasmids containing rat α1 and α3 were first linearized with Nae I and Sca I, respectively. Further digestion of the linearized α1 plasmid with Sma I produced the 3.2 kb cDNA insert and 2.9 kb vector. Similarly, the linearized α3 plasmid could be further separated from its cDNA insert by further digestion with EcoR1.

**E. DNA gel electrophoresis and electroelution**

Following restriction digestion, the cDNA inserts were separated from their parent vectors by electrophoresis in 0.9% agarose gels containing 40 mM Tris-HCl, pH 7.6, 30 mM sodium acetate and 1.0 mM EDTA, pH 8.0. Tracking dye (0.4% bromophenol blue, 50% glycerol and 1.0 mM EDTA, pH 8.0) was added to the samples and electrophoresis was carried out for 5-7 hours at 80-90 volts. After electrophoresis, the gels were stained with 10 μg/μl ethidium bromide, destained in H₂O for up to 1 hour and visualized under ultraviolet light.

The appropriate cDNA bands were excised from the gel and equilibrated in 25 ml of electroelution buffer (20 mM Tris-HCl, pH 7.6, 0.2 mM EDTA, pH 8.0 and 5 mM NaCl). The gel slices were loaded into an unidirectional electroelution apparatus (IBI Model UEA), covered with buffer and the DNA eluted into a high salt solution of 3.0
M sodium acetate. The applied voltage varied between 20-30 volts depending on the size of the DNA and migration of the DNA bands from the gel was monitored under ultraviolet light. The DNA was removed, two volumes of ethanol were added to the sodium acetate solution and the DNA was precipitated overnight at -20°C and collected by centrifugation for 10 minutes in an Eppendorf centrifuge. The precipitate was washed with ice-cold 70% ethanol, vacuum dried and dissolved in sterile H₂O. Mini gel electrophoresis was used to ascertain the size (by comparison to lambda DNA molecular weight markers) and purity of the recovered cDNA fragments.

**F. ³²P-labelling of the cDNA probes**

DNA was labeled to high specific activity by the method of random priming (Volgestein, 1982) with a cDNA oligolabelling kit purchased from Pharmacia as follows: 50 ng of cDNA was diluted to 31 μl with sterile water and denatured by boiling for 10 minutes. The probe was cooled on ice, briefly centrifuged in an Eppendorf centrifuge, after which the following substances were added from the kit at room temperature: 2 μl of BSA (3.0 mg/ml), 10 μl reagent mix consisting of dATP, dGTP, dTTP and p(dN)₆ oligo primers, 50 μCi of [γ-³²P]dCTP and finally 5-10 units of Klenow fragment of DNA polymerase. The labelling reaction was left overnight at room temperature and then terminated.
by addition of first 30 μl of a solution comprising 10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1.0 mM EDTA, pH 8.0 (TNE) and then 20 μl of "stop" buffer from the kit. The unincorporated nucleotides were separated from the labeled DNA fragments by a simple gel filtration procedure ie. application to a small Sephadex G-50 column and then centrifugation for 2 minutes in a table top clinical centrifuge (IEC model) at setting #4. The column was prepared as follows: one ml disposable syringes were packed with Sephadex G-50 (medium) previously swollen in TNE and then washed twice with 100 μl of TNE.

Trichloroacetic acid (TCA)-precipitable radioactivity was determined to estimate the amount of incorporated label. Briefly, 1.0 μl of isolated probe was diluted to 30 μl with H2O containing 50 μg herring sperm DNA added as a carrier, followed by 2.0 ml of ice cold 20% TCA. The mixture was left on ice for 1/2 hour and then filtered under vacuum, through a Whatman GF/C 2-4 cm filter. The filter was dried under a heat lamp for 10 minutes and then counted by liquid scintillation spectrometry. Using this procedure specific activities of up to 2 X 10^9 cpm/μg of TCA-precipitable DNA were obtained.

G. Northern blot analysis

i) RNA gel electrophoresis

One percent agarose gels were prepared by melting 1.0
g of ultrapure agarose (BRL) in 73 ml of DEPC-treated H₂O. 10 ml of 10X MOPS buffer (200 mM morpholinepropanesulfonic acid, 50 mM sodium acetate and 10 mM EDTA adjusted to pH 7.0 with acetic acid) was added along with 16.2 ml of 37% formaldehyde (final concentration 6%). The gels were poured, solidified at room temperature and used immediately or stored at 4°C. RNA samples were prepared for electrophoresis by heating for 15 minutes at 55°C in 50% formamide (deionized), 6% formaldehyde and 1X MOPS buffer. The samples were quickly cooled on ice and bromophenol blue tracking dye added. The agarose gels were covered in running buffer (1X MOPS) and the RNA samples applied to the gel with capillary pipettes. Electrophoresis was carried out in an IBI apparatus (Model MPH) for 5-7 hours with an applied voltage of 80-90 volts. Following electrophoresis, the gels were stained with ethidium bromide (10 µg/µl) for 10 minutes and destained overnight in distilled H₂O. The gels were then visualized and photographed under ultraviolet light.

ii) Transfer of RNA from gels to GeneScreen membrane

The RNA gels were trimmed and Whatman 3MM filter paper and GeneScreen nylon hybridization transfer membrane (DuPont Chemicals) were cut to sizes slightly larger than the gel. The gels were placed bottom side-up on Whatman filter paper having the ends immersed in transfer solution
(10X SSC: 1.5 N NaCl and 0.15 N sodium citrate). The nylon membrane was pre-wetted in 10X SSC and placed on the gel with care taken to remove air bubbles. Two pieces of Whatman filter paper, also pre-soaked in 10X SSC were then layered over the membrane, followed by a weighted stack of paper towels. Transfer proceeded for 2-3 days and the paper towels were replaced as they became wet. Upon completion of the transfer, the nylon membrane was washed in 5X SSC for 5 minutes to remove residual traces of agarose and then air dried at room temperature. The membrane was then baked at 80°C for two hours in a vacuum oven.

iii) Prehybridization, hybridization and autoradiography

The prehybridization and hybridization protocols for GeneScreen membrane were carried out according to manufacturer's instructions with a few minor modifications. Briefly, baked RNA blots were placed in heat-sealable plastic bags containing 10 ml of prehybridization solution: 50% formamide, 0.2% polyvinylpyrrolidone, 0.2% BSA, 0.2% Ficoll, 0.05 M Tris-HCl, pH 7.5, 1.0 M NaCl, 0.1% sodium pyrophosphate, 1.0% SDS, 10% dextran sulfate and 100 µg/ml denatured herring sperm DNA to block the non-specific binding to the membrane. Care was taken to remove air-bubbles, the bags were sealed and prehybridization was carried out overnight at 42°C with constant shaking. For
hybridization, 25-50 ng of heat-denatured DNA probe per 10 ml of solution (usually 500,000 cpm/ml) were added directly to the prehybridization buffer, mixed thoroughly and hybridization was carried at 42°C with constant agitation for 18-24 hours.

Following hybridization, the filters were washed twice at room temperature for 10 minutes per wash with 2X SSC to remove the excess DNA probe. This was followed by two further washes each for 1/2-1 hour with 2X SSC containing 1% SDS at 65°C. When more stringent washing conditions were required, the amount of SSC was reduced to 0.5 or 0.1X as indicated, while maintaining the concentration of SDS at 1.0%. Finally, the last wash was for 1 hour at room temperature with 0.1X SSC. All washes were performed with constant agitation. After washing, the membranes were blotted on Whatman filter paper and while still moist, wrapped in Saran wrap. Radioactivity was detected by exposure of the membranes to Kodac X-AR 5 autoradiographic film at -70°C, using double Dupont Cronex Lightening Plus intensifying screens.

H. Western blot analysis

i). Preparation of samples

The following protocol, basically designed to obtain a microsomal membrane-rich fraction from tissue, was applied to 0.5 g of sheep brain, axolemma (brainstem) and
kidney as well as 0.6 ml of white cells, HK and LK bone marrow. These tissues were homogenized on ice in 3.0 ml of 0.32 M sucrose containing 1mM EDTA, pH 7.4 and 10 mM imidazole-HCl, pH 7.2 using a motor-driven hand-held glass homogenizer. The homogenate was then subjected to a series of differential centrifugations. The first was for 20 minutes at 2600 rpm to remove nuclei and unhomogenized material. The supernatant was re-centrifuged for 20 minutes at 8400 rpm to eliminate mitochondria and myelin. These centrifugations were carried out in a Sorval RC-2B centrifuge (SS.1 rotor). The resulting supernatant was then ultracentrifuged at 30,000 rpm in the SW.70 rotor of a Beckman L5-65 ultracentrifuge for 1 hour and the resulting crude microsomal pellet was dissolved in 500 μl of homogenization buffer and stored at -70°C until use.

ii) SDS-polyacrylamide gel electrophoresis

Five microliters of crude microsomal membrane preparations, appropriately diluted, were prepared for electrophoresis by first adding 1.0 mM EDTA, pH 7.4 and 2.0 mM phenylmethylsulfonyl fluoride from 0.2 M and 200 mM stock solutions, respectively, followed by 20 μl of sample buffer containing the following: 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 1.25 X 10^{-3}% (w/v) bromophenol blue. Polypeptides were then separated on a 7.5% SDS-polyacrylamide gel with a 3.5% stacking gel.
according to the procedure developed by Laemmli (1970). Marker proteins purchased from Bio-Rad were run as standards and were of the following reported molecular weights: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (65 kDa) and ovalbumin (45 kDa).

iii) Transfer and immunoblotting

Following the completion of electrophoresis, the gels were equilibrated in transfer buffer containing 25 mM Tris, 0.19 M glycine and 20% methanol for approximately 30 minutes. They were then placed over a piece of Whatman 3MM paper, pre-soaked in transfer buffer and layered over the bottom pad of the transfer sandwich. Pre-wetted nitrocellulose was then placed over the gel, followed by another layer of moistened filter, care being taken to remove all air bubbles in each new layer. The top pad of the transfer sandwich was then added and the protein was transferred to nitrocellulose membrane at 100 volts for 1.0 hour in a Bio-Rad Trans-Blot apparatus.

After transfer, the nitrocellulose was removed from the sandwich and placed in blocking solution of 3% gelatin in 10 mM Tris-HCl, pH 7.4 and 150 mM NaCl (TBS). The membranes were blocked overnight at 4°C. The solidified gelatin was melted in the microwave and the nitrocellulose was washed three times at room temperature with TBS (5
minutes/wash). The membranes were then incubated for 2 hours in a heat-sealable plastic bag with Na\(^+\), K\(^+\)-ATPase specific anti-lamb kidney polyclonal antiserum, provided by Dr. W. Ball, University of Cincinnati. The antiserum was diluted 1:4000 with 1% gelatin-TBS. After incubation with the primary antibody, the membranes were then washed twice with 1% gelatin-TBS, then twice with 1% gelatin-TBS containing 0.05% Tween 20 and then finally with 1% gelatin-TBS. Each wash was for 5-10 minutes at room temperature. Following this, the membranes were incubated for 1 hour with biotinylated anti-rabbit IgG diluted 1/1000 with 1% gelatin-TBS and then subjected to the same wash procedure as described above. The membranes were then further incubated at room temperature for 1 hour with Vectastain containing avidin and biotinylated horseradish peroxidase (Vector Laboratories) in 1% gelatin-TBS and washed once again. The immunoblots were developed with 0.1% diaminobenzidine containing 0.1% hydrogen peroxide (v/v).

I. Quantification of relative amounts of specific mRNA in Northern blots

To quantify the relative abundance of \(\alpha_1\) and \(\beta_1\) message, radioautographs were scanned using a Hoffer scanning densitometer connected to a strip chart recorder. Areas under the peaks were quantified by cutting out and weighing the tracing of each peak. For each separate
autoradiograph the weight of each peak divided by the amount (μg) of RNA analyzed was normalized to that of the kidney enzyme.

**J. Ouabain inhibition analysis of Na⁺,K⁺-ATPase activity**

Na⁺,K⁺-ATPase activities of sheep kidney and axolemma microsomes prepared as described previously were assayed as described below. Ouabain inhibition profiles of each tissue were carried out in the absence of ouabain and presence of 10⁻¹⁰ M to 10⁻³ M ouabain. Briefly, the ATPase reaction medium comprised 1 mM MgSO₄, 1mM ATP (Tris-form), 1 mM EGTA, pH 7.4, 50 mM NaCl, 5 mM KCl and 40 mM Tris-glycylglycine, pH 7.4. [γ-³²P]ATP was also included (approximately 8 X 10⁷ cpm/ml) as well as ouabain in the concentrations indicated above. Each assay was performed in triplicate at 37°C in a final volume of 200 μl comprising 160 μl medium containing the varying concentrations of ouabain and 40 μl of microsomes. It should be noted that the microsomal preparations were made permeable by treatment with 0.4 mg SDS/mg protein before assaying as this ratio was shown in preliminary experiments to maximize Na⁺,K⁺-ATPase activity.

The reaction was terminated by the addition of 0.8 ml ice-cold solution of 5% trichloroacetic acid containing 5 mM ATP and 2.5 mM NaH₂PO₄, after which 0.5 ml of 15% charcoal (Norit A) in 5% TCA were added and the mixture was
left on ice for one-half hour with occasional vortexing. The suspension was then centrifuged at 3000 rpm for 10 minutes at 4°C to remove the charcoal to which was absorbed residual unhydrolyzed nucleotide. An aliquot of the supernatant, generally 0.5 ml, was added to 5.0 ml of scintillation cocktail (Ecolume, ICN) and the radioactivity measured by liquid scintillation spectrometry (LKB RackBeta).
CHAPTER 3

RESULTS
A. Cardiac glycoside sensitivity as a means of distinguishing distinct sodium pump isoforms

i) Ouabain sensitivity profiles of sheep specific isoforms

The extent of differences among different tissues in cardiac glycoside affinity appears to vary among different species. For example, rodent isoforms show the most extreme differences, to the extent that the rat and mouse kidney enzyme is strikingly insensitive to ouabain ($K_d=100$ uM) compared to its brain counterpart ($K_d=0.1$ uM) (Urayama and Nakao, 1979; Marks and Seeds, 1978). Although the differences in apparent affinity for cardiac glycosides are now recognized to be relevant mainly to the insensitivity to ouabain of the rodent catalytic alpha isoform, differences ascribed to distinct isoforms have been observed in mammals other than rodents, namely dogs (Maixent et al., 1987) and ferrets (Ng and Akera, 1988). Thus, the question remains whether and to what extent this holds true for other species. In the present study, the first step towards characterization of the red cell isotype viz-à-viz other tissues, as well as LK versus HK red cells has involved the question of ouabain sensitivity. To this end, the initial experimental work involved analyzing the ouabain inhibition profiles of sheep brain (axolemma) and kidney tissues.

In the experiments shown in Figures 3 and 4, ouabain sensitivity of the $Na^+\!\!\,K^+\!\!\,-ATPase$ of axolemma and kidney
Figure 3

Time course of ouabain inhibition of the kidney Na\(^+\),K\(^+\)-
ATPase

Microsomal membrane preparations were prepared from
homogenates of sheep kidney tissue as described in
Materials and Methods. Microsomes were first treated with
SDS (0.4 mg SDS/mg protein) for 30 minutes at room
temperature in order to maximize Na\(^+\),K\(^+\)-ATPase activity and
then diluted to a protein concentration of 0.05 mg/ml. 40
µl were then assayed in triplicate for 20, 30 and 40
minutes in 160 µl of reaction medium consisting of 1 mM
MgSO\(_4\), 50 mM NaCl, 5.0 mM KCl, 40 mM Tris glycyglycine
(TGG), pH 7.4, 1.0 mM EGTA, pH 7.4, 1.0 mM [\(\gamma\)-\(^{32}\)P]ATP (7.7
X 10\(^7\) cpm/ml) and the indicated concentrations of ouabain.
The [\(\gamma\)-\(^{32}\)P]ATP used in these experiments was prepared by C.
Polvani.
Figure 4

Time course of ouabain inhibition of the axolemma Na\textsuperscript{+},K\textsuperscript{+}-ATPase

The Na\textsuperscript{+},K\textsuperscript{+}-ATPase assays of the axolemma enzyme with the indicated concentrations of ouabain in the reaction medium were performed as described in Figure 3.
was compared. In order to assure that the Na\textsuperscript{+},K\textsuperscript{+}-ATPase assay was performed under conditions close to equilibrium ouabain binding at each concentration of ouabain, a time course of ATP hydrolysis was carried out. Figures 3 and 4 show the $^{32}\text{P}\text{I}$ released from $[\gamma-^{32}\text{P}]\text{ATP}$ as a function of time at each concentration of ouabain tested for the kidney and axolemma enzymes, respectively. As shown, the rate of hydrolysis decreases becoming constant after 20 minutes, presumably the time taken to reach equilibrium binding. Therefore, the ouabain-sensitivity profile was obtained by plotting the rate obtained between 20 and 40 minutes (Figure 5).

In Figure 5, Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in the presence of ouabain is expressed as a percentage of the maximum observed in the absence of ouabain, versus ouabain concentrations of $10^{-2}$ to $10^{3}$ μM. Unlike brain or axolemma of the rat, in which the ouabain sensitivity profile is biphasic, both the sheep axolemma and kidney profiles are monophasic, superimposable and exhibit an identical $K_d$ (1.0 μM) for ouabain inhibition under the conditions used (50 mM Na\textsuperscript{+} and 5 mM K\textsuperscript{+}). Therefore, in sheep, there is no evidence for differences in ouabain sensitivity associated with distinct isoforms.

B. Western blot analysis of erythroblast-rich bone marrow

In order to analyze directly isoform expression of
Figure 5

Log plot of kidney and axolemma Na⁺,K⁺-ATPase activity as a function of ouabain concentration

Na⁺,K⁺-ATPase activity, measured from 20 to 40 minutes at each [ouabain] (Figures 3&4), is expressed as a percentage of the maximum activity measured in the absence of ouabain.
Kidney

Axolemma
the catalytic subunit of the sodium pump, Western blot analysis was performed with anti-lamb kidney polyclonal serum. This serum recognizes both the $\alpha_1$ and $\alpha^+ (\alpha_2$ and/or $\alpha_3$) isoforms, as well as the $\beta$ subunit. As shown in Figure 6, $\alpha_1$ and $\alpha^+$ are detected in brain and axolemma with the $\alpha^+$ ($\alpha_2$ and/or $\alpha_3$) isoform(s) having a slightly, but distinctly lower mobility than $\alpha_1$. Only the $\alpha_1$ isoform is seen in kidney. In the axolemma samples analyzed at two different dilutions, reactivity with $\alpha^+$ is somewhat greater than with the $\alpha_1$ isoform compared to brain. The $\beta$ subunit in brain and axolemma migrates as a diffuse band with the same relative mobility whereas, the migration of the kidney $\beta$ subunit is somewhat lower. This is probably due to differences in the nature and/or extent of N-linked glycosylation (for review see Sweadner, 1989).

As shown in Figure 6, the antiserum also detects the $\alpha_1$ isoform protein in HK and LK bone marrow of which the blood cell precursors are largely erythroblasts (see below). The $\alpha_1$ isoform is present also in a white blood cell-rich fraction isolated from peripheral blood of sheep. Northern analysis of $\alpha_1$ isoform expression in poly (A)$^+$ RNA of peripheral blood white cells indicated only a minute quantity of message (data not shown). The $\beta$ subunit of white cells and HK and LK bone marrow was not detected with this particular anti-serum. Although other explanations for the lack of the $\beta$ subunit in sheep erythroblasts
Figure 6

Immunoblot analysis of protein expression in sheep tissues

Membrane proteins were first diluted in sample buffer and subjected to electrophoresis and immunoblotting as described in Materials and Methods. The following quantities of membrane proteins were applied to each respective lane: white blood cell (5.0 µg); HK marrow (60 µg); LK marrow (13.5 µg); brain (5.0 µg); axolemma 1 (6.7 µg); kidney (1.0 µg); axolemma 2 (3.3 µg). Prestained protein markers were also run as standards for molecular weight comparison. The immunoblot shown was from an experiment carried out by E. Grafova.
cannot be ruled out (see Discussion), at face value these results indicate that the nature of the isoforms detected by immunoblotting correlates well with mRNA expression (see next section).

**C. RNA expression in red cell precursors of anaemic HK and LK sheep**

1) **Morphology of bone marrow from anaemic sheep**

   Figure 7a&b represent typical micrographs of the cells present in the bone marrow of an anaemic LK sheep. Following repeated phlebotomy, bone marrow was withdrawn from the iliac crest and the cells obtained were examined microscopically as described in Methods. Although most of the cells are immature and mature red cells, the majority (>70%) of the large blast cells are morphologically typical of red cell precursors.

2) **Agarose gel electrophoresis of RNA isolated from bone marrow of anaemic sheep**

   Figure 8 is a photograph of total RNA and oligo-dT-selected poly (A)$^+$ RNA from sheep tissues, separated on 1% agarose gels containing 6% formaldehyde and stained with ethidium bromide. The 28S and 18S ribosomal RNA's are readily visible and distinct in the total RNA preparations. Some ribosomal contamination is evident also in the poly (A)$^+$ RNA-enriched fractions. Some transfer RNA (tRNA)
Figure 7a

Micrograph of bone marrow obtained from an anaemic LK sheep and stained with Wright stain.
Figure 7b

Micrograph of bone marrow obtained from an anaemic LK sheep
and stained with Wright stain
Figure 8

A typical electrophoretic profile of preparations of total and poly (A)$^+$ RNA separated on agarose gels

RNA was extracted as described in Materials and Methods and after denaturation in sample buffer was subjected to electrophoresis through 1% agarose gels containing 1X MOPS and 6% formaldehyde. Electrophoresis was carried out for 6 hours at 85 volts and the gel stained with ethidium bromide (10 μg/μl) for 10 minutes and destained overnight in H$_2$O. Lanes 1 and 3 show 10 and 20 μg of reticulocyte total RNA and lanes 2 and 4 show 0.8 and 1.6 μg of reticulocyte poly (A)$^+$ RNA, respectively. Lanes 5 and 6 show 10 μg and 20 μg of LK bone marrow total RNA.
detected at the bottom of the gel in the total RNA samples is removed during the poly (A)$^+$ selection procedure.

3) Northern blot analysis

i) Catalytic isoform expression

Northern blot analysis of LK sheep bone marrow, brain and kidney probed with the full length cDNA encoding the sheep kidney $\alpha_1$ isoform indicates $\alpha_1$ is present in all the three tissues examined. Figure 9 shows the reactivity of this probe with total and poly (A)$^+$ RNA for the specified tissues. The positions of the 28S and 18S ribosomal RNA's are indicated. As shown, the intensities of the bands corresponding to 10 and 30 $\mu$g total RNA are similar to those corresponding to 1.0 and 3.0 $\mu$g of poly (A)$^+$ LK RNA, respectively, indicating a 10-fold enrichment of specific message. In other samples of marrow, message was difficult to detect in total RNA added in amounts up to 30 $\mu$g. Also shown in Figure 9 is the expression of the sheep $\alpha_1$ isoform from kidney and brain (0.14 $\mu$g of kidney and 0.8 to 1.0 $\mu$g of brain total RNA). As shown, the intensity corresponding to 0.14 $\mu$g kidney total RNA was similar to that for 3.0 $\mu$g marrow poly (A)$^+$ RNA and 0.8 $\mu$g of brain total RNA. This indicates that kidney tissue has the greatest abundance of sodium pump message, followed by brain and then bone marrow. The isoform specific expression of the sodium pump was also examined by Northern blot analysis using rat
Northern analysis showing the reactivity of sheep LK bone marrow, kidney and brain tissues with the sheep α1 cDNA probe

Total RNA was extracted and poly (A)$^+$ selected as described in Materials and Methods. After denaturation the samples were separated in a 1% formaldehyde-agarose gel. As indicated, 1.0 and 3.0 μg of poly (A)$^+$ RNA and 10 and 30 μg of total RNA from LK bone marrow, 0.14 μg of kidney total RNA and 0.8, 0.9 and 1.0 μg of brain total RNA were transferred to GeneScreen membrane and hybridized to $^{32}$P-labelled, random-primed cDNA containing sequences of the sheep α1 catalytic subunit of the sodium pump (specific activity = 3 X 10$^8$ cpm/μg TCA-precipitable DNA). The membrane was washed twice for 5 minutes/wash at room temperature with 2X SSC, followed by two washes at 65°C for 30 minutes/wash with 2X SSC, 1% SDS and finally for 1.0 hour at room temperature with 0.1X SSC. The blot was exposed overnight at -70°C.
Sheep α1 probe

10 μg total poly A+ RNA
1 μg poly A+ RNA
LK marrow
30 μg total poly A+ RNA
3 μg poly A+ RNA
0.14 μg Kidney
total RNA
0.8 μg Brain
total RNA
0.9 μg
1.0 μg
isoform specific cDNA probes. Figure 10 shows the comparative analysis of 3.0 μg of LK bone marrow poly (A)$^+$ RNA, 0.3 μg kidney total RNA and 1.3 μg of brain total RNA. The α1 isoform is expressed in all tissues, consistent with the results of Figure 4 and with the approximate size of the rat α1-specific mRNA (3.7 kb; Young and Lingrel, 1987; Orlowski and Lingrel, 1988). In contrast, α2 and α3 isoforms are apparent only in brain tissues, the former as two messages of 3.4 and 5.3 kb and the latter, 3.7 kb, as reported by others in rat tissues (Young and Lingrel, 1987; Orlowski and Lingrel, 1988). In our blots, the slightly lower mobility of α2 (3.7 kb) when compared to α1 and α3 could not be discerned from the value of 3.4 kb reported by others (see above).

Northern analysis of bone marrow obtained from an HK sheep was also carried out using the rat α1 cDNA as a probe. With this particular sample from an anaemic HK animal, approximately 750 μg of bone marrow poly (A)$^+$ RNA was recovered from the selection of 15 mg of bone marrow total RNA. Since a large percentage of this total RNA is probably derived from the vast quantity of reticulocytes present in the marrow cell population we analyzed also sheep reticulocyte total RNA with the sheep α1 cDNA probe. Sodium pump-specific mRNA could not be detected (data not shown) consistent with previous results indicating the absence of sodium pump message in human reticulocyte RNA.
Figure 10

Northern blot analysis of sodium pump catalytic isoforms in LK bone marrow, brain and kidney

RNA was extracted and poly (A)$^+$ selected as described in Materials and Methods and separated in a 1% agarose-formaldehyde gel. Following electrophoresis, 3.2 µg of LK marrow poly (A)$^+$ RNA, 0.3 µg of kidney total RNA and 1.25 µg of brain total RNA were transferred to GeneScreen nylon membrane and hybridized to random-primed, $^{32}$P-labelled rat α1, α2 and α3 isoform specific probes. The specific activities of each probe were as follows: α1 (6.5 x $10^8$ cpm/µg DNA), α2 (5 x $10^8$ cpm/µg DNA) and α3 (9.5 x $10^8$ cpm/µg DNA). The membranes were washed twice with 2X SSC for 5 minutes/wash at room temperature, then twice with 2X SSC, 1% SDS for 45 minutes/wash at 65°C and finally with 0.1X SSC for 1.0 hour at room temperature. This blot was exposed overnight at -70°C.
Rat Probe: 16 S  28 S

a1

LK marrow
Kidney
Brain

a2

LK marrow
Kidney
Brain

a3

LK marrow
Kidney
Brain
(Chehab et al., 1987).

With rather large amounts of HK poly (A)$^+$ RNA analyzed (7.5, 15.0 and 22 μg), together with the indicated quantities of brain and kidney total RNA, the reactivity of the marrow samples was high relative to kidney and brain as shown in Figure 11. Figure 11a represents an overnight exposure of the autoradiogram. As shown the α1 isoform is expressed in all three tissues. Figure 11b is a shorter exposure of the same autoradiogram.

Na$^+$,K$^+$-ATPase isoform specific mRNA expression was also analyzed in HK sheep bone marrow cells using the rat specific isoform probes. Figure 12 shows the comparative pattern of expression of HK marrow (1 and 3 μg poly (A)$^+$ RNA), kidney (0.35 μg total RNA) and brain (1.5 μg total RNA). In this blot, the amounts of HK RNA were reduced compared to the experiment in Figure 11 so that the intensity of the signal obtained with the α1 probe was similar for each tissue. With the same amounts of RNA electrophoresed concurrently and probed with rat α2 and α3 under high stringency conditions, neither the α2 nor α3 isoforms were detected in either the red cell or kidney, similar to the results obtained with erythroblast-rich marrow from an ILK animal. As shown above all three isoforms are found in sheep brain tissue.

ii) β subunit expression
Figure 11a

Northern analysis of sodium pump $\alpha_1$ mRNA in sheep HK bone marrow

Preparations of total and poly (A)$^+$ RNA were separated on 1% agarose-formaldehyde gels, transferred to nylon membrane and hybridized with $^{32}$P-labelled rat $\alpha_1$ cDNA. Lanes 1, 2 and 3 represent 7.5, 15.0 and 22.0 $\mu$g of HK marrow poly (A)$^+$ RNA, respectively, lanes 4 and 5 represent 0.65 and 1.3 $\mu$g of brain total RNA, respectively, and lanes 6 and 7 represent 0.18 and 0.35 $\mu$g of kidney total RNA, respectively. The specific activity of the probe was $1.4 \times 10^9$ cpm/$\mu$g TCA-precipitable DNA. The membrane was washed twice with 2X SSC for 10 minutes/wash at room temperature, then twice with 2X SSC, 1% SDS for 1 hour/wash at 65°C and finally with 0.1X SSC for 1 hour at room temperature. The autoradiogram was exposed overnight at -70°C.
Rat α1 probe
**Figure 11b**

Northern analysis of sodium pump α1 mRNA in HK sheep bone marrow

This is the same blot as shown in Figure 11a except that the time of exposure has been reduced to 2 hours at -70°C.
Comparative pattern of mRNA expression of sheep sodium pump catalytic subunit isoforms in anaemic HK bone marrow, kidney and brain

1.0 and 3.0 μg of HK marrow poly (A)+ RNA, 0.35 μg of kidney total RNA and 1.3 μg of brain total RNA were run on a 1% agarose-formaldehyde gel, transferred to GeneScreen membrane and hybridized to rat isoform-specific cDNAs. The specific activities of each probe were as follows: α1 (2.1 \times 10^9 \text{ cpm/μg DNA}), α2 (1.8 \times 10^9 \text{ cpm/μg DNA}) and α3 (2.0 \times 10^9 \text{ cpm/μg DNA}). The membranes were washed twice with 2X SSC at room temperature for 10 minutes/wash, then twice with 0.5X SSC, 1% SDS at 65°C for 1 hour/wash and finally with 0.1X SSC for 1.0 hour at room temperature. After the first exposure the membranes were washed again with 0.1X SSC for 45 minutes at 65°C. This blot represents a 3-day exposure at -70°C.
28 S →  

18 S →  

Rat probe: \( \alpha_1 \) \( \alpha_2 \) \( \alpha_3 \)
Expression of β subunit isoform-specific mRNA of the sodium pump was analyzed using a full length sheep kidney β1 hybridization probe. Figure 13 shows the result of a Northern blot in which brain (0.13, 0.25 and 0.5 μg of total RNA), kidney (0.07, 0.18 and 0.35 μg of total RNA), LK marrow (3.0 μg poly (A)+ RNA) and HK marrow (1.0 and 3.0 μg poly (A)+ RNA) were analyzed with the β1 cDNA. As expected, the mRNA for the β1 subunit is smaller than that of the α subunit isoforms. As noted previously (Orlowski and Lingrel, 1988), two β1 mRNA species, 2.7 and 2.35 kb in size, are present in rat brain and kidney. The abundance of the β1 subunit in brain is greater than the α isoforms. This is evident from a comparison of Figures 13 and 12; in Figure 13 (β1 probe) the quantity of brain RNA analyzed was decreased in order to obtain a similar signal intensity as in Figure 12. It should be noted that in general, probes of similar specific activity were used for each Northern blot.

As shown in Figure 13, β1 subunit message could not be detected in 3.0 μg of LK marrow. This holds true even after a one-week exposure of this particular autoradiogram (not shown). In contrast, a signal, albeit relatively weak, is detected with an equivalent amount of HK marrow. β1 subunit mRNA expression is clearly of low abundance in marrow cells and the inability to see even a weak signal in LK marrow may be due to a lower enrichment of the poly
Figure 13

Northern analysis of the expression of the sodium pump β1 subunit in sheep tissues

0.13, 0.25 and 0.5 µg of brain total RNA, 0.07, 0.18 and 0.35 µg of kidney total RNA, 3.0 µg of LK poly (A)+ RNA and 1.0 and 3.0 µg of HK poly (A)+ RNA were run on a 1% agarose-formaldehyde gel, transferred to GeneScreen membrane and hybridized to 32P-labeled cDNA containing the full coding sequence of the sheep sodium pump β1 subunit. The specific activity of the probe was 1.8 X10⁹ cpm/µg of DNA. The membrane was washed twice with 2X SSC for 10 minutes/wash to remove excess probe, then twice with 2X SSC, 1% SDS at 65°C for 1 hour/wash and finally at room temperature for 1 hour with 0.1X SSC. The membrane was re-washed for 1 hour at 65°C in 0.5X SSC, 1% SDS, re-exposed and washed again with 0.1X SSC, 1% SDS for 1 hour at 65°C. This particular autoradiogram was exposed for 2 days after the final wash.
Sheep $\beta$1 probe
(A)$^+$-selected RNA and generally lower abundance of sodium pump message (see Section iii). Thus, even with the relatively more reactive marrow sample from the HK animal, a signal could not be detected with 1.0 µg of marrow poly (A)$^+$ RNA even after one-week exposure (not shown).

The expression of the putative β2 subunit of the sodium pump was analyzed using an isoform-specific rat cDNA probe. Figure 14 shows that while β2 message is clearly seen in sheep brain, it is not present in kidney or bone marrow. Since a rat probe was used, it is possible that cross-species homology differences may exist, however, the presence of β2 message in brain has been reported by others (Vasallo et al., 1989).

iii) Relative abundance of α1 and β1 subunit messages

The relative amounts of isoform specific mRNAs were quantified by scanning densitometry of the autoradiographs of the Northern blots as described in Materials and Methods. Table 1 shows the amounts of α1 and β1 mRNA levels in sheep kidney, brain, HK and LK bone marrow tissues. The scans were of autoradiographs shown in Figures 9, 10, 12 and 13 and for each autoradiograph the amounts shown were normalized to the amount in kidney. In Table 2, the amounts per µg RNA (total RNA for kidney and brain and poly (A)$^+$ RNA for bone marrow taken from Table 1) are given, normalized also to the amount in kidney.
Northern analysis of sodium pump β2 mRNA in sheep kidney, brain, and bone marrow tissues

5.0 and 10 µg of HK poly (A)+ RNA, 0.5, 1.0 and 1.5 µg of kidney total RNA and 0.75, 1.0 and 2.0 µg of brain total RNA were run on a 1% agarose-formaldehyde gel, transferred to GeneScreen membrane and hybridized to 32P-labeled rat β2 cDNA. The specific activity of the probe was 1.1 X 10^9 cpm/µg of DNA. The membrane was washed twice with 2X SSC for 10 minutes/wash, then twice with 1X SSC, 1% SDS at 65°C for 1 hour/wash and finally at room temperature for 1 hour. This blot was exposed overnight at -70°C.
Rat β2 probe

| 1 2 | HK marrow poly A⁺ RNA |
| 3 4 5 | Kidney total RNA |
| 6 7 8 | Brain total RNA |
Table 1

Quantitation of \( \alpha_1 \) and \( \beta_1 \) specific RNA in sheep brain, kidney and bone marrow
<table>
<thead>
<tr>
<th>Figure #</th>
<th>Probe</th>
<th>Tissue</th>
<th>(µg RNA)</th>
<th>Reactivity&lt;sup&gt;1&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total</td>
<td>poly(A)&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>Sheep α1</td>
<td>Kidney</td>
<td>0.14</td>
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<tr>
<td></td>
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<td></td>
<td>Brain</td>
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<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LK marrow</td>
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</tr>
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<td>1.0</td>
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<td></td>
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<td></td>
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</tr>
<tr>
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<td>Kidney</td>
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<tr>
<td></td>
<td></td>
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<sup>1</sup> In each experiment, the relative areas under the peaks of the radioautographs are normalized to that of the highest amount of kidney mRNA analyzed.
Table 2

Comparison of reactivity of $\alpha_1$ and $\beta_1$ specific mRNA in sheep kidney, brain and bone marrow
## TABLE 2

<table>
<thead>
<tr>
<th>Figure #</th>
<th>Probe</th>
<th>Tissue</th>
<th>Reactivity/μg RNA&lt;sup&gt;1&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>9</td>
<td>Sheep α1</td>
<td>Kidney</td>
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<td>Brain</td>
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<td>LK marrow</td>
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</tr>
<tr>
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<td>Rat α1</td>
<td>Kidney</td>
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<td>Brain</td>
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</tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>HK marrow</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<sup>1</sup>Data taken from Table 1. For sample analyzed at more than one concentration, average values are shown. n.d., not detected
Comparison of HK to LK marrow indicates that the enrichment of the specific poly (A)$^+$ RNA of HK marrow is approximately two-fold greater than LK marrow. In addition, compared with the kidney preparation, the mRNA expression of the $\beta_1$ subunit of HK sheep bone marrow is $\leq 10\%$ of the expression of the corresponding $\alpha_1$ mRNA. Compared to brain (Figures 9 and 12), the value is less than 3%, the latter being approximately 3-fold higher than kidney.
CHAPTER 4

DISCUSSION
The first objective of this study has been to examine the expression of the Na\(^+\),K\(^+\)-ATPase in sheep red cells and to compare it to that of other sheep tissues such as kidney and brain which are known to comprise predominantly α1 (kidney) and a mixture of α1, α2 and α3 (brain) isoforms of the catalytic subunit. For over two decades, red cells have been particularly useful for studying the transport of sodium and the relationship between sodium pump behavior and the Na\(^+\)- plus K\(^+\)- activated ATP hydrolysis. Particularly noteworthy are the studies of the different modes of the sodium pump which are observed by altering experimental conditions such as ligand concentrations (Na\(^+\), K\(^+\), ATP, ADP and P\(_i\)).

The advantages of the red cell system have been the relative ease of measuring ion transport in a relatively homogeneous cell suspension, the purity of membranes (ghosts) isolated from cells and the ability to regulate and distinguish between the effects of ions at internal and external membrane surfaces. Furthermore, with these cells, sealed membrane vesicles of distinct orientation, either right-side-out or inside-out can be obtained.

Information regarding Na\(^+\),K\(^+\)-ATPase structure has been obtained with tissues with pump densities orders of magnitude greater than the red cell, including the kidney and electric organ of Torpedo californica. The high activity of pure and partially purified enzyme derived from
these tissues has allowed investigators to conduct certain kinetic studies not feasible with the low activity red cell membrane. For example, the kidney enzyme has been used extensively in kinetic studies of the partial reactions of the pump by ATP having the advantage of low levels of non-specific ATP binding, hydrolysis and phosphorylation compared to the red cell system. It is only with the high specific activity enzyme that occluded states of both $K^+$ (Glynn and Richards, 1982) and $Na^+$ (Glynn and Richards, 1983) have been observed.

With regards to the foregoing, the fundamental question of whether the sodium pump of the red cell is identical to that of more active tissues, particularly the kidney has remained largely unresolved. It is not known whether the behaviour of the kidney enzyme, for example, holds true for all facets of behaviour of the red cell enzyme. Thus, to what extent do all transport modes observed in the red cell hold true for the kidney enzyme, particularly the special features of $Na^+$ efflux uncoupled to $K^+$, but coupled to anions (Marin and Hoffman, 1988)? In this study, the question was addressed by elucidating the protein and, more importantly, mRNA expression of the sodium pump in sheep red cells. I have confirmed by Western blotting earlier studies (Blostein and Grafova, 1988) using specific anti-lamb kidney polyclonal serum that the reactivity of the catalytic subunit of the red cell is
identical to that of kidney and lacks the lower mobility isoform(s) present in certain tissues such as brain. Similar results were reported earlier for dog red cells (Inaba and Maede, 1986).

A more direct approach has been to carry out Northern blot analysis in red cells sufficiently immature to have specific mRNA. For this purpose, it was necessary to extract sodium pump-specific mRNA from the bone marrow tissue of anaemic sheep and compare the pattern of expression to that of kidney and brain, the latter known to express the three isoforms of the catalytic subunit $\alpha_1$, $\alpha_2$ and $\alpha_3$. Northern analysis using a full length cDNA from sheep kidney and, more importantly, rat isoform-specific cDNA probes show that the $\alpha_1$ isoform of the catalytic subunit is expressed in all three tissues.

Since histochemical staining showed that the majority of the blast cells in marrow are red cell precursors and that a few white cells were present, it is unlikely that the contribution of sodium pump-specific mRNA of white cell precursors is of major significance. Furthermore, Northern analysis of RNA obtained from peripheral white cells indicated very low quantities of pump expression even after poly (A)$^+$ selection. Thus, it can be concluded that the catalytic subunit of the sheep red cell sodium pump is identical to that of the kidney.

In spite of the structural similarity in the
catalytic subunit, there are certain differences in the behavior of the two pumps, particularly the temperature-dependent effects on the composition of phosphoenzyme (K+-insensitive versus K+-sensitive EP). As well, it was observed that at 0°C, ATP modulates the kinetic behaviour (ratio of E-ATP:EP) of the human red cell, but not kidney Na⁺,K⁺-ATPase (White and Blostein, 1982). It is possible that the above kinetic distinctions are due to other factors such as differences in lipid environment or possible pump-associated proteins which modify Na⁺,K⁺-ATPase activity in red cells (see below). Considering the former, a number of experimental findings have suggested that long-chain fatty acids can be potential regulators of Na⁺,K⁺-ATPase activity as they are known to inhibit the activity of the sodium pump in a reversible manner (Ahmed and Thomas, 1971). In addition, it has been shown that phospholipase C treatment preferentially reduces the activity of the rat α⁺ isoform over that of α (Matsuda and Iwata, 1986), again pointing to the influences of lipid environment.

Studies described earlier have indicated that the catalytic subunit isoforms of the sodium pump may in fact be functionally distinct. However, at present, there is little knowledge of the extent to which the distinction(s) is/are relevant to the presence of catalytic isoforms. With respect to apparent differences in cardiac glycoside
affinity, most studies have been carried on rat tissues, where the differences are greatest in magnitude and little information has accumulated from work with other species. Studies with the rat and to a lesser extent, the dog and ferret isoforms have suggested that these affinity differences in cardiac glycoside inhibition may be a species-specific phenomenon. With this in mind, the question of whether ouabain affinity differences are relevant to other species such as sheep was addressed by analyzing the ouabain inhibition profiles of the Na^+,K^+-ATPase activity of sheep kidney and axolemma. The results show that the sheep α1 and α^+ isoforms are identical with respect to their inhibition by ouabain. Therefore, it is concluded that distinct ouabain inhibition profiles are not relevant to distinct forms of the catalytic subunit. It appears that, the marked differences (approximately 1000-fold) in ouabain sensitivity in the rat are due to interspecies differences in ouabain sensitivity of the α1 isoform, per se, such that rat α1 is very insensitive to cardiac glycosides.

The second major aim of this study was to analyze Na^+,K^+-ATPase expression in HK and LK sheep red blood cells. Based on earlier observations of kinetic differences between these two genotypes the question has remained whether the structural basis for these differences are due to differential expression of isoforms. In the
present study, Western blot analysis shows the presence of only the \( \alpha_1 \) isoform in anaemic bone marrow of both HK and LK sheep as mentioned above. Earlier studies failed to detect differences in LK or HK mature or immature cells (Blostein and Grafova, 1988). Northern analysis of bone marrow poly (A)\(^+\) RNA from anaemic HK and LK sheep using cDNAs for each of the respective rat catalytic isoforms indicates the presence of only \( \alpha_1 \), consistent with the result of the Western blot. Thus, it is unlikely that the genetic divergence apparent in the mature red cells of HK and LK sheep (HK and LK reticulocytes are both "HK-like") is due to the differential expression of sodium pump catalytic subunit isoforms.

The expression of the \( \beta_1 \) isoform of the Na\(^+\),K\(^+\)-ATPase was also analyzed at the mRNA level. The results indicate a weak signal in HK cells. The low levels of \( \beta_1 \) subunit expression at the mRNA level correlates well with the absence of \( \beta \) expression in HK and LK cells observed by Western blotting (Blostein and Grafova, unpublished). The failure to detect the \( \beta_1 \) subunit mRNA in LK cells is most likely due to the lower enrichment in specific poly (A)\(^+\) RNA in the LK marrow compared to the HK marrow used in these studies. Indeed, the level of specific mRNA varied considerably among different marrow samples from the same sheep and no message could be detected in poly (A)\(^+\) RNA isolated from reticulocytes (not shown). A likely reason
for the generally low amounts of β₁ subunit in both HK and LK red cells may be due to higher turnover rate of the β subunit message when compared to the α₁ message. The β subunit has been implicated in the insertion of the sodium pump into the cell membrane. However, it is not known whether it is required for functional pumping activity. Therefore, it is not unreasonable to suggest that the turnover rate may be different from that of the catalytic subunit.

Recently Vasallo et al. (1989) have reported that whereas β₁ subunit mRNA is absent from cell lines derived from the rat central nervous system, a β-like mRNA (β₂) is detected in reasonably abundant quantities. This differential expression of β₁/β₂ isoforms is also seen in other rat tissues such as spleen, thymus and lung. In these tissues there is no expression of the β₁ isoform, whereas β₂ is detected. Adult rat liver, however, does not contain either the β₁ or β₂ isoforms. Thus, in addition to the possibilities mentioned above for the general lack of β subunit message in red cells, it is possible that other β isoforms are expressed in these cells. Although the sheep tissues were analyzed with a rat cDNA probe, it is unlikely that failure to detect β₂ in bone marrow is due to low interspecies homology, since with low stringency conditions, β₂ was detected in brain as expected (Vasallo et al., 1989), but not in bone marrow. This holds true
even though the amount of bone marrow added (10 μg poly (A)⁺ RNA) compared to brain (2.0 μg total RNA) should have given a detectable signal at least based on the relative amount of α1 in the two tissues (Figure 11).

A possible clue to understanding the structural and functional differences between HK and LK red blood cells lies in the observation that immunization of HK sheep with LK red cells produces serum which when incubated with LK cells, stimulates the activity of the LK sodium pump (Ellory and Tucker, 1969). This result has led to the notion that the L-antigen may be structurally associated with the sodium pump of LK sheep red cells and serves to inhibit its activity. Anti-L, an IgG₁-type antibody (Synder et al., 1971) produced in this way is thought to cause perturbations in the conformation of the L-antigen and thus relieve its inhibitory effect on the sodium pump. Since the initial discovery of a stimulatory anti-L serum, several investigations have focused on the biochemical nature of the L-antigen and the basis for the stimulatory effect of the antiserum. HK sodium pumps are thought to be associated with the M-antigen (Rasmusen and Hall, 1966) which is detected by a anti-M complement associated hemolytic assay (Lauf and Tosteson, 1969). This serum, produced by the immunization of LK sheep with HK red cells, causes no change on the pump activity of HK cells and thus, unlike the effect on anti-L, there does not appear to be a
functional basis for the presence of the M-antigen.

Studies on the binding characteristics of anti-L and anti-M antibodies to LK and HK cells have revealed a large excess of L- and M-antigens compared to the number of pump sites in mature red cells of sheep (Lauf and Sun, 1976; Tucker et al., 1976). Estimations of the number of antigenic sites, based on equilibrium binding studies of \(^{125}\text{I}\)-labeled antiserum, indicate that homozygous HK cells (MM) have approximately 3000 M-antigenic sites while heterozygous LK cells (LM) have approximately 1500 (Lauf and Sun, 1976). In comparison, homozygous LK cells (LL) have about 1500 L-antigenic sites and in heterozygous LK cells the number is even smaller. Therefore, the number of antigenic sites exceeds the number of pump sites by an order of magnitude. However, these numbers may be misleading since polyclonal antiserum can bind to more than one determinant on a particular antigen. It is plausible also that the differences in L-antigenic and pumps sites is less in immature red cells which have a much higher pump density.

The kinetic changes in the pump activity of the LK Na\(^+\),K\(^+\)-ATPase upon incubation with anti-L serum are likely caused by alterations in ion binding affinities at the cytosolic surface (see below). As mentioned earlier, one of the main reasons for the lower pump activity of LK cells is due to the pronounced inhibitory effect of K\(_c\) acting at
intracellular sodium loading sites. Anti-L is thought to relieve this inhibitory effect and thus increase the apparent affinity for Na⁺ at its internal binding sites (Lauf et al., 1970; Glynn and Ellory, 1972). This manifests itself in an increase in the rate of pump activity. In addition, anti-L increases the rate of ouabain binding in both heterozygous and homozygous LK cells and presumably this effect is a direct repercussion of the increased rate of pump activity (Joiner and Lauf, 1975). There is no change, however, in the total amount of [³H] ouabain bound at equilibrium.

It is also interesting to note that the stimulatory effect of anti-L on sodium pump fluxes can be abolished by the treatment of LK cells with trypsin (Lauf et al., 1971). Trypsin digestion also destroys the increased rate of ouabain binding brought about by incubation with the serum (Lauf et al., 1977). In addition, anti-L does not bind to trypsinized LK cells and the L-antigen can be protected from tryptic inactivation by prior incubation with anti-L (Lauf et al., 1977). These experimental observations suggest that trypsin destroys the antibody producing determinants of the L-antigen and are consistent with the notion that the L-antigen is a protein or glycoprotein. They do not, however, exclude the possibility that the antiserum recognizes carbohydrate moieties.

The situation in which specific membrane proteins are
structurally associated with pump proteins and serve to regulate their activity is not without precedent. For example, phospholamban is a low molecular weight protein of approximately 22-27 kDa which is believed to inhibit the activity of the Ca\textsuperscript{2+}-ATPase in cardiac sarcoplasmic reticulum (SR) (Tada et al., 1975). Phosphorylation of phospholamban by Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (Le Peuch et al., 1979) or protein kinase C (Movsesian et al., 1984) is thought to alter its interaction with the Ca\textsuperscript{2+}-pump and this results in stimulation of Ca\textsuperscript{2+}-pump activity. In addition, it has been shown that specific monoclonal antibodies prepared against the cardiac SR phospholamban also stimulate the Ca\textsuperscript{2+}-ATPase (Suzuki and Wang, 1986). The monoclonal antibodies also block the phosphorylation of phospholamban by cAMP-dependent protein kinases. Much like the situation with the L-antigen, antibody binding to phospholamban is thought to alter its conformation such that it no longer can interact with the Ca\textsuperscript{2+}-pump and thus the inhibitory effect is removed.

Based on the role of phospholamban in regulating Ca\textsuperscript{2+}-ATPase activity, it is of interest to speculate that, in a generally similar manner, another relatively small protein component, the gamma subunit, interacts with and modulates the sodium pump. Although the effects of the gamma subunit on Na\textsuperscript{+}-pump activity remain to be established, it is certainly a potential candidate for pump
regulation. At present, the relationship between possible intrinsic membrane protein regulators of the sodium pump such as the L-antigen to that of the gamma subunit is unknown.

Thus, it can be concluded from the present study that the catalytic subunit of the red cell Na\(^+\),K\(^+\)-ATPase is identical to that of the kidney enzyme. In addition, the genetic dimorphism apparent in HK and LK sheep red cell sodium pumps is not caused by differential isoform expression. Lastly, unlike \(\beta 1\) subunit expression in sheep brain and kidney, the relative amounts of \(\beta 1\) expressed in HK and LK red cells are generally low.
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