POTASSIUM INTERACTIONS WITH THE (Na^+, K^+)-ATPase

by

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ABSTRACT

The asymmetric interactions of K^+ with the (Na^+,K^+)-ATPase were investigated using inside-out red cell membrane vesicles. The K^+-stimulated hydrolysis of the pseudo-substrate p-nitrophenylphosphate was examined using vesicles from human red cells and the interactions of K^+ with the Na^+-ATPase was examined using vesicles from sheep red cells that are genetically dimorphic with respect to their K^+ levels and sodium pump activity.

It was observed that cytoplasmic K^+, i.e. extravesicular K^+, stimulates p-nitrophenylphosphatase and, at very low ATP concentrations ($\leqslant$2.0 micromolar), inhibits the low-K^+ but not the high-K^+ sheep Na^+-ATPase. Extracellular K^+, i.e. intravesicular K^+, alone has no effect on p-nitrophenylphosphatase unless cytoplasmic Na^+, i.e. extravesicular Na^+, plus micromolar concentrations of ATP and a small amount of cytoplasmic K^+ ($\geqslant$0.2 millimolar) are present, in which case extracellular K^+ stimulates p-nitrophenylphosphatase. Extracellular K^+ also stimulates high-K^+ sheep Na^+-ATPase (ATP$\geqslant$0.2 micromolar) but has no effect on low-K^+ sheep Na^+-ATPase, at least at ATP concentrations up to 2.0 micromolar.

A regulatory role of ATP, in the range of 0.02 to 2.0 micromolar, on K^+ as well as on Na^+ interactions with Na^+-ATPase was also studied. The results indicate that ATP modulates the effects of extracellular K^+ and cytoplasmic Na^+ on Na^+-ATPase of high-K^+ sheep but not low-K^+ sheep red cells.

From the results, it is proposed that cytoplasmic K^+ interacts with the dephosphoenzyme and that extracellular K^+ interacts with the phosphoenzyme form of (Na^+,K^+)-ATPase; ATP modulates the release of K^+ from the dephosphoenzyme.

RESUME

Les interactions asymmétriques du K^+ avec le (Na^+,K^+)-ATPase ont été examinées en utilisant les vésicules inversées de membranes de cellules rouges. L'hydrolyse du pseudo-soustrat p-nitrophénilphosphate stimulée par le K^+ a été examinée en utilisant des vésicules de cellules rouges humaines et les interactions du K^+ avec le Na^+-ATPase ont été examinées en utilisant des vesicules de cellules rouges de moutons qui sont génétiquement dimorphiques par rapport a leurs viveaux de K^+ et l'activité de la pompe de sodium.

Il a été observé que le K^+ cytoplasmique, i.e. le K^+ extravesiculaire, stimule le p-nitrophénilphophatase et, à de très basses concentrations d'ATP ($\leqslant$2.0 micromolaire), inhibe le Na^+-ATPase de moutons bas-K^+ mais non de moutons haut-K^+. Le K^+ extracellulaire, i.e. K^+ intravésiculaire, tout seul n'avait aucun effet sur le p-nitrophénil-phosphatase à moins que le Na^+ cytoplasmique, i.e. Na^+ extravésiculaire, en plus que des concentrations micromolaires d'ATP et un peu de K^+ cytoplasmique ($\geqslant$0.2 millimolaire) sont présents, ces conditions permettant une stimulation du p-nitrophénilphosphatase par le K^+ extracellulaire.

Le K^+ extracellulaire stimule aussi le Na^+-ATPase des moutons haut-K^+ (ATP$\geqslant$0.2 micromolaire) mais n'as aucun effet sur le Na^+-ATPase des moutons bas-K^+, au moins à des concentrations d'ATP jusqu'à 2.0 micromolaire.

Un rôle régulateur de l'ATP, dans le domaine de 0.02 a 2.0 micromolaire, sur les interactions du K^+ ainsi que du Na^+ avec le Na^+-ATPase a aussi été étudié. Les résultats indiquent que l'ATP module les effets du K^+ extracellulaire et du Na^+ cytoplasmique sur le Na^+-ATPase des cellules rouges de moutons haut-K^+ mais non de moutons bas-K^+.

De ces résultats, il est proposé que le K^+ cytoplasmique interagit avec la forme déphosphoenzyme et que le K^+ extracellulaire interagit avec la forme phosphoenzyme du (Na^+,K^+)-ATPase; l'ATP module la décharge du K^+ du phosphoenzyme.

TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_{259}	absorbance at 259 nm
A_{280}	absorbance at 280 nm
ADP	adenosine diphosphate
anti-L	anti-LK antibody
ASP	aspartate
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
[γ-^{32}P]-ATP, ATP^{32}	adenosine triphosphate labelled with ^{32}P in the gamma position
ATPase	adenosine triphosphatase
Ca^{2+}-ATPase	Ca^{2+}-stimulated adenosine triphosphatase
F_1-ATPase	F_1-adenosine triphosphatase
Na^+-ATPase	Na^+-stimulated adenosine triphosphatase
(Na^+,K^+)-ATPase	Na^+- plus K^+-stimulated adenosine triphosphatase
CTP	cytosine triphosphate
1,3 D^{32}PG	1,3 diphosphoglycerate (with ^{32}P)
DPN	diphosphopyridine nucleotide
E	enzyme
E_1	first form of enzyme
E_2	second form of enzyme
E_1(ATP)	E_1 with loosely bound ATP
E_1· ATP	E_1 with tightly bound ATP
E· K	E with bound K^+
E_1. K	E_1 with bound K^+

$E_2 \cdot K$	E_2 with bound K^+
E - P	phosphoenzyme
$E_1 \sim P$	first (higher enrgy) form of phosphoenzyme
E_2 - P	second (lower energy) form of phosphoenzyme
$E_1 \sim P$ (ADP)	$E_1 \sim P$ with bound ADP
E_2 - P· K	E_2 - P with bound K^+
Mg· E_2 - P	E_2 - P with bound Mg^{2+}
$(Mg)_2 \cdot E_2$ - P	E_2 - P with two bound Mg^{2+}
EDTA	ethylene diamine tetraacetate
GA3P	glyceraldehyde 3-phosphate
GA3PD	glyceraldehyde 3-phosphate dehydrogenase
Gram.	gramicidin.
HK	high K^+
$K_{0.5}$	concentration of a ligand required for half-maximal activity
K_{cyt}	cytoplasmic K^+, i.e. extravesicular K^+
K_{ext}	external K^+, i.e. intravesicular K^+
K_d	dissociation constant for enzyme-ligand complex
K_i	dissociation constant for enzyme-inhibitor complex
K_m	Michaelis-Menten constant
LK	low K^+
LYS	lysine
Na_{cyt}	cytoplasmic Na^+, i.e. extravesicular Na^+
Na_{ext}	external Na^+, i.e. intravesicular Na^+
3-O-MFPase	3-O-methylfluorescein phosphatase
3PG	3-phosphoglycerate
3PGK	3-phosphoglycerate kinase

pNPP	p-nitrophenyl phosphate
pNPPase	p-nitrophenylphosphatase
Rb_{cyt}	cytoplasmic Rb^+, i.e. extravesicular Rb^+
SDS	sodium dodecyl sulphate
SER	serine
SH	sulphydryl group
THR	threonine
Tris	tris (hydroxymethyl) aminomethane
val	valinomycin
ρ	density

1. INTRODUCTION

1.1 MAIN CHARACTERISTICS OF THE SYSTEM

The active transport of sodium and potassium across the cell membrane serves to maintain electrochemical gradients for these cations and has a major role in many physiological functions including maintenance of cell volume, absorption processes in tissues such as kidney and intestine, and excitability in nerve and muscle.

In 1957, Skou (1) demonstrated the existence of a Na^+ plus K^+-stimulated, Mg^{2+}-dependent adenosine triphosphate phosphohydrolase (E.C.3.6.1.3), or (Na^+, K^+)-ATPase, in crab nerve. This enzyme is now considered to be the biochemical basis for active transport of Na^+ and K^+. Three years later the activity was demonstrated and its properties described in human red cell membranes, the one mammalian tissue in which the sodium pump has been well characterized (2). Furthermore, studies with resealed red cell ghosts have shown that optimal ATPase activity is observed with Na^+ present intracellularly and K^+ externally and that the side-specificity for the enzyme is the same as that for the sodium pump (3-6). Measurements of the stoichiometry of Na^+ pumped outwards and K^+ inward have indicated that 3 Na^+ and 2K^+ are pumped for every molecule of ATP hydrolyzed (3, 6-9). The enzyme system is specifically inhibited by cardiac glycosides such as ouabain (10) at the external surface (11, 12). There is some evidence that Li^+ can replace Na^+ at the cytoplasmic surface (13-16), though with much less efficiency, whereas a number of cations can replace K^+ at the external surface, with an effectiveness of $K^+ > Rb^+ > NH_4^+ > Cs^+ > Li^+$ (17), at least at physiological levels of ATP.

ATP can be competed for kinetically by other nucleotides with an effectiveness of ATP ADP dATP CTP ITP UTP (18,19), although these other nucleotides, except for CTP (160,162), will not support ion transport (20).

Early studies (21-23) with the enzyme have shown that a phosphorylated intermediate of the enzyme is formed upon incubation with Mg^{2+}, Na^+, and [γ- ^{32}P] ATP, and that the level of this phosphoenzyme is greatly diminished by further addition of K^+. Much work has since been done in order to establish the role of this phosphoenzyme in the reaction sequence of the (Na^+, K^+)-ATPase and has yielded an interesting though still incomplete picture of the chemical mechanism responsible for the exchange of Na^+ and K^+ across the membrane.

The following is a more detailed review of the properties of the system, dealing first with the structure and then the function of the enzyme.

1.2 STRUCTURE OF THE ENZYME

i) Polypeptide Composition

The sources of purified enzyme used in structural studies are tissues with particularly high ion transport activity such as kidney, rectal gland of dogfish shark, brain, and electrophorus electric organ. Electron microscopy after purification of this intrinsic enzyme system in membrane-bound form or after solubilization by detergents shows particles of 80 Å in diameter with 35-55 Å projections, possibly representing glycoprotein (24). The purest preparations have turnover rates of about 9,000 min^{-1} (25).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified preparations of (Na^+, K^+)-ATPase (24, 26-29) have revealed only two major protein components: a large polypeptide of close to 100,000 daltons and a smaller glycoprotein containing 13-16% carbohydrate and of 47,000-57,000 daltons, although the latter may be overestimated.

The large subunit represents 57-72% of the total protein and is phosphorylated by ATP (29-33) and is thus termed the catalytic subunit. It is thought to span the membrane (34, 35) and perhaps to form a channel through which ions pass (36). Partial digestion of the large chain with trypsin in the presence of various ligands has revealed the existence of a Na^+ form and a K^+ form of the enzyme and suggests that Na^+- and ATP - dependent reactions on the one hand, and K^+- dependent reactions on the other, occur in different conformations of the chains (37-39).

The 36 sulfhydryl (SH) groups on the large chain have been classified into three groups based on differences in their reactivity with N-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid) (40, 41): i) 12 SH groups at the surface of the enzyme, of which one (at the ATP binding site) is vital for activity; ii) at least 14 SH groups in a more hydrophobic environment, of which one (not in the active centre) is vital for activity; iii) at most 10 SH groups at the core of the enzyme, since they are only exposed by use of detergents. Separate SH groups are believed to be involved in phosphorylation and dephosphorylation activities (34,42) since differences in their reactivity are observed in the presence of ATP and Na^+ or K^+ and may reflect changes in conformation induced by these ligands.

Amino acid analyses of the purified enzyme (27) indicate an N-terminal glycine and 47% non-polar residues. Similarities with respect to the amino-acid composition of (Na^+, K^+)-ATPase, Ca^{2+}-ATPase, mitochondrial F_1-ATPase and soluble ATPase from S. faecalis have been noted (43, 44). Amino acid residues identified at the active site are an aspartate, believed to be the phosphorylation site in the sequence (THR or SER)-ASP-LYS (45), and an arginine (46) and tyrosine (47) which have been identified at the ATP binding site.

The smaller glycoprotein has no known function but always co-purifies with the large subunit (25, 48). The two are crosslinked by dimethylsuberimidate (27) and ethylacetimidate (49) and must exist close together in the membrane. The glycoprotein is rich in hydrophobic amino acids and contains glucosamine (27, 50), galactosamine (27), glucose, galactose, mannose, fucose (50) and sialic acid (27, 50). It has been shown to bind Concanavalin A to the carbohydrate moiety (51). The mass ratio of large to small polypeptide varies from 1.7 to 2.8 and suggests 1 or 2 small subunits per large subunit in the native enzyme (24, 27, 28, 52, 53). An examination of the effects of SDS on the kidney microsome preparation suggests that the native (Na^+, K^+)-ATPase may be a much larger complex that is dissociated by detergents and warns against possible artifactual results due to the use of detergents (54).

Antibodies against brain (55) or kidney (56-58) preparations inhibit ATPase and ion transport by binding at the cytoplasmic surface and presumably inhibiting changes in conformation since they inhibit reactions involving ATP+ Na^++ K^+ but have little or no effect on reactions involving either Na^+ or K^+ (59).

SDS denaturation of the enzyme does not block its ability to induce formation of inhibitory antibodies. In contrast, antibodies against large polypeptide will bind to the enzyme without inhibiting its activity. This suggests that inhibitory antibodies may be raised against lipoprotein components or a complex of polypeptides in the preparations (59). It has been reported, however, that antibodies to the glycoprotein inhibit (Na^+, K^+)-ATPase activity (60, 61) and strengthens arguments in favor of this chain being a subunit of the complex. Differences in the enzyme inhibitory effects of antibodies raised against purified enzyme from different sources and tested on the same preparation or the same antibody tested with different preparations suggest differences in structure of (Na^+, K^+)-ATPase from different sources (62).

The number of catalytic sites in the enzyme complex have been determined by ^3H-ouabain binding, ATP binding and by phosphorylation from ATP (25). There is one mole of ouabain or ATP bound per 175,000 to 285,000 g of protein (28, 53, 63) and it may be concluded that one molecule of ouabain or ATP binds to a dimer of two large chains, each associated with one or two small chains. Evidence for a dimeric structure is supported by an earlier study using radiation inactivation, which also gave a molecular weight of 250,000 daltons (64), which is twice the highest estimate of phosphorylation, i.e. one mole of ^{32}P-bound per 130,000 g of protein (65, 66).

The difference between numbers of phosphorylation sites on the one hand, and numbers of ouabain or ATP binding sites per enzyme unit on the other, may be due in part to experimental discrepancies but may reflect differences in binding and phosphorylation properties of a dimeric enzyme, each subunit consisting of one large catalytic subunit of 100,000 daltons and 1 or 2 small glycoprotein subunits of less than 57,000 daltons (52, 67, 68 and ref. 25).

Proof of the identity of these purified enzyme preparations with sodium pump activity has been evidenced in their ability to transport Na^+ and K^+ after reconstitution into vesicles. (Na^+, K^+)-ATPase purified from electric organ (69), brain (70), kidney (71), heart (71), and rectal gland (72) have all been reconstituted with lipids and have shown $^{22}Na^+$ and $^{86}Rb^+$ transport that is inhibited when ouabain is incorporated into the vesicles. The stoichiometries (70-72) are reported to be close to the ratio of 3 Na^+/2 K^+ per ATP molecule hydrolyzed as in red cells (3, 6-9), although difficulties in measuring the initial rates of the very rapid cation fluxes in the small, highly active vesicles limit the accuracy of these measurements.

ii) Lipid Requirements

Purified (Na^+, K^+)-ATPase is associated with roughly an equal weight of lipid (73) that seems to be enriched in phosphatidylserine (74, 75). Studies on restoration of activity of lipid-depleted, inactive enzyme have also shown an important role for phosphatidylserine (76-84), although only a minor fraction of the phosphatidylserine in the membrane appears to be required (85, 86).

Phosphatidylglycerol (76, 83), phosphatidylinositol (74, 81, 82, 87), phosphatidic acid (74, 84), and sulphatides (88) also seem important for enzyme activity. In fact, any of the acidic phospholipids, especially phosphatidylserine, are capable of restoring almost full activity (48, 74, 89). The enzyme can still function, but at a much lower rate, in their absence (90). Whether these lipids are required specifically for enzyme activity or non-specifically for restructuring of the membrane is not clear (89), especially since (Na^+, K^+)-ATPase reconstituted in only phosphatidylcholine can pump ions (91). The fatty acid chains must be fluid, possibly to allow changes in conformation of the enzyme (76, 89, 92-99). Cholesterol is needed for membrane stability (100-102) and may interact at the cytoplasmic surface of the enzyme (103).

The finding that the partial reactions (see below) are less sensitive than the overall reaction to phospholipase A (104-106) and phospholipase C (106-108) digestion suggests that the lipid bilayer helps more in the integration of the reaction steps (e.g. changes in conformation) than expression of individual steps.

1.3 TRANSPORT CHARACTERISTICS

i) Na^+- K^+ Exchange

Under physiological conditions, the sodium pump system catalyses the transport of three Na^+ ions from the cytoplasmic surface and two K^+ ions from the external surface of the red cell per molecule of ATP hydrolyzed to ADP and Pi at the cytoplasmic surface (3, 6-9) with an efficiency of about 70% (109) based on studies of ATP synthesis during reversal of the pump (see below).

Similar stoichiometries for Na^+ transport have been observed in other tissues (110-112) including nerve and muscle. Because of the apparent inequality in the transport of Na^+ and K^+, efforts have been made to determine to what extent, if any, the pump is electrogenic (113-115). Electrogenicity has been demonstrated, albeit to a smaller degree, even in red cells (116). The possibility of a variable stoichiometry remains since in squid axons under certain conditions, less Na^+ ions relative to K^+ ions are transported when the cytoplasmic Na^+ concentration is lowered (117-119).

In Na^+-rich media, the enzyme shows sigmoidal activation of Na^+-K^+ exchange by external K^+ (120-123) acting at two sites of equal affinity (Km = 0.4-0.5 mM) (123). At lower or zero external Na^+ however, K^+ affinity is greater and its activation effect is less sigmoidal (120, 121, 124) and is due to both competition and a regulatory effect of external Na^+ (113, 125-127). Na^+ activation of Na^+-K^+ exchange involves what may be three cytoplasmic sites of equal affinity (Km = 0.19 mM) (128, 129) and is competed for by K^+ ($K_{0.5}$ for inhibition of 9 mM) (128, 130).

The cytoplasmic Na^+ sites are independent from the external K^+ sites under physiological conditions (120, 128, 131, 132). This suggests independent binding of Na^+ and K^+ with transport occurring when both are bound (113). Although these sites exist simultaneously, a matter of debate has been whether Na^+ and K^+ exchange binding groups simultaneously in an occluded region within the membrane (see ref. 133 and 134) or whether each site, carrying its appropriate cation, has alternate access to both surfaces of the membrane (see ref. 135).

A recent study (136) of pump kinetics in red cells supports simultaneous Na^+- K^+ exchange, although the Na^+ and K^+ affinities appeared to be interdependent.

ii) Other Modes of Transport

Four other modes of Na^+ and/or K^+ transport have been demonstrated (114) and will be described in turn.

REVERSAL

By arranging Na^+ and K^+ gradients steeper than normal and a lower than normal (ATP)/(ADP)·(Pi) ratio in red cells, it is possible to make the pump run backwards and synthesize ATP at the expense of downhill cation movements (109, 137-140). The rate of synthesis is roughly proportional to the external Na^+ concentration (137), has a $K_{0.5}$ = 0.3 M for cytoplasmic K^+, presumably the K^+ release sites (141), and is inhibited by external K^+ with a $K_{0.5}$ for inhibition of 1.3 mM, similar to the Km for K^+ activation of Na^+- K^+ exchange described above (123). The stoichiometry has not been precisely determined, but probably involves two to three K^+ ions per molecule of ATP synthesized (137).

Na^+- Na^+ EXCHANGE

Red cells in high-Na^+, K^+-free media show a ouabain-sensitive exchange of cytoplasmic and external Na^+ ions (129, 142-145). A similar exchange is also seen in squid axon (120, 145) and muscle (121, 146, 147).

Although the red cell stoichiometry is approximately 1:1, there is a marked asymmetry in the affinities for Na^+ having a $K_{0.5}$ = 10-15 mM for cytoplasmic Na^+ and a sigmoid activation curve (to over 150 mM) for external Na^+ (122, 142, 143) showing three sites with similar affinities (Km = 31 mM) in red cells (128), but different affinities in nerve (120). External K^+ inhibits with a $K_{0.5}$ for inhibition of 1 mM and allows Na^+- K^+ exchange by presumably binding to its activation sites on the pump (143, 144). Stimulation by cytoplasmic K^+ of Na^+- Na^+ exchange (122) and Na^+- K^+ exchange (122, 130, 148) suggests a role for internal K^+(113).

The Na^+- Na^+ exchange is linear with ADP concentration (tested up to 0.3 mM) (149) and ATP seems to be required (144, 150) though it may not be hydrolyzed (6, 150). Experiments with squid axons (151, 152) and muscle (153) also indicate ADP dependence of the Na^+- Na^+ exchange and that Na^+- K^+ and Na^+- Na^+ exchange can occur simultaneously in the presence of enough ADP, at least in the case of the squid axon (154). The ADP dependence of the Na^+- Na^+ exchange has led to the suggestion that it may be associated with the Na^+-dependent ATP-ADP exchange activity (see Section 1.4) (149). The full extent of the similarities between these two activities remains to be determined.

K^+- K^+ EXCHANGE

In resealed red cell ghosts containing little Na^+, a 1:1 exchange of K^+ is seen (155) and is believed to occur under physiological conditions about 20% of the time i.e. 80% Na^+- K^+ exchange and 20% K^+- K^+ exchange (156). The affinities for K^+ are asymmetric, having a $K_{0.5}$ for cytoplasmic K^+ of about 10 mM (155) and a $K_{0.5}$ for external K^+ of about 0.25 mM (156) i.e. an asymmetry in the reverse direction of that for Na^+ during Na^+- Na^+ exchange. Cytoplasmic Na^+ inhibits K^+- K^+ exchange and allows Na^+- K^+ exchange to occur (155). K^+- K^+ exchange requires cytoplasmic Pi (156), does not hydrolyze ATP nor require ADP (145) though it requires nucleotides in a non-phosphorylating role since the β-γ-methylene analog of ATP can substitute (157). The Pi requirement suggests a reversible phosphorylation of the enzyme by K^+ and Pi as indicated by studies of ^{18}O exchange between Pi and H_2O (158, 159) (see Section 1.4).

UNCOUPLED Na^+ EFFLUX

A ouabain-sensitive efflux of Na^+ into Na^+- and K^+- free media is detected in red cells or resealed ghosts and is not coupled to ion influx (124, 142, 143, 160, 161). Cytoplasmic Na^+ also binds to high affinity sites and stimulates hydrolysis with a K_m for ATP of about 1 uM (162) and a stoichiometry of two to three Na^+ ions per ATP molecule (113, 160) suggesting that this Na^+-stimulated ATPase activity (Na^+-ATPase) is the enzymic basis for uncoupled Na^+ efflux.

External K^+ inhibits at a high affinity site and the ratio of Na^+-K^+ exchange/uncoupled Na^+ efflux decreases with decreasing order of nucleotide binding affinity (160) determined from studies of (Na^+, K^+)-ATPase activity (18, 19). Low concentrations (< 5mM) of external Na^+ inhibit the uncoupled efflux at a high affinity site(s) (113, 162). Higher concentrations increased ATP hydrolysis and may be associated with a Na^+-Na^+ exchange that hydrolyses ATP in the absence of ADP, although this has not yet been directly demonstrated (162).

Thus, the evidence in favor of these five modes of ion transport being mediated by the Na^+-K^+ pump is based on their sensitivity to ouabain and/or similarities in the interactions of Na^+ and/or K^+. Further evidence is that an antiserum to a partially purified kidney preparation inhibits the (Na^+, K^+)-ATPase and all of these modes except for reversal, which has not been tested (57).

These modes of transport are illustrated in Figure 1. In summary, the main features which emerge from these studies are (113, 114, 163):

i) The outward movement of Na^+ is associated with a transfer of phosphate from ATP to the enzyme;

ii) the inward movement of Na^+ is associated with the transfer of phosphate from the phosphoenzyme to ADP;

iii) the inward movement of K^+ is associated with the transfer of phosphate from the phosphoenzyme to water; and

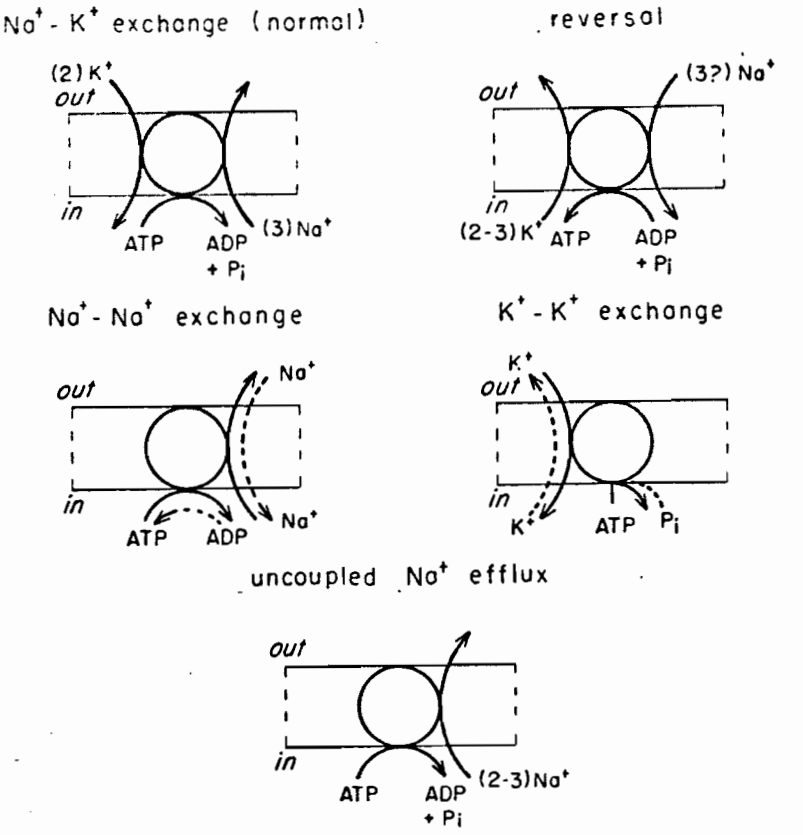

FIGURE 1: THE FIVE MODES OF BEHAVIOUR OF THE Na^+ PUMP IN INTACT CELLS OR RESEALED GHOSTS (MODIFIED FROM REF. 163)

·The solid and broken arcs represent events that are coupled. The horizontal lines represent the surfaces of the membrane and the circle represents the pump (see text for explanation).

iv) the outward movement of K^+ is associated with the transfer of inorganic phosphate to the enzyme.

Once the phosphoenzyme has been formed from cytoplasmic Na^+ or K^+ its fate depends on the composition of the external medium: if K^+ is present, phosphate is transferred to water and K^+ enters; if Na^+ is present and K^+ is not, the phosphoenzyme transfers its phosphate to ADP and Na^+ enters; if neither Na^+ nor K^+ is present in the external medium, then the phosphate is transferred slowly to water and the slow overall hydrolysis of ATP is accompanied by a slow uncoupled efflux of Na^+.

1.4 REACTION STEPS

The reaction steps in the hydrolysis of ATP by the (Na^+, K^+)-ATPase have been studied mostly in broken membrane preparations derived from active tissues such as kidney, brain, and electric organ as well as from relatively inactive red cell membranes, the mammalian system in which ion transport has been best characterized. These 'open' membrane preparations allow the direct accessibility of substrates to the catalytic site. Using [γ-^{32}P] ATP, it had been observed that Na^+, in the presence of Mg^{2+}, catalyzes the phosphorylation of the enzyme (E) and that dephosphorylation of the phosphoenzyme (E-P^{32}) is greatly accelerated by the subsequent addition of K^+ (21-23). Although the existence of an E-P intermediate in the normal operation of the (Na^+, K^+)-ATPase has been questioned (17, 164), experiments carried out at steady state (165, 166) and during the transient phases of the reaction gave credence to its role.

Thus rapid-mixing experiments in the presence of Na^+, K^+, Mg^{2+} and micromolar ATP have shown that the pre-steady state rise in E-P is followed by a lag in Pi release (165, 167) and thus directly demonstrate an E-P intermediate during the hydrolysis of ATP. Other evidence for the phosphoenzyme intermediate will be presented in the following section, which deals with experiments aimed at understanding the order of addition and release of substrates and products and the sequence of the partial reaction steps in the hydrolysis of ATP. The discussion will cover first the reactions leading to the formation of E-P and then the reactions associated with its breakdown.

i) Phosphorylation Reactions

Phosphorylation requires Na^+, Mg^{2+}, and ATP. Na^+ activates hydrolysis at the external surface of inside-out vesicles i.e. the cytoplasmic surface of cells (168, but see ref. 169) where it presumably binds to its three loading sites (Km =0.19 mM) (128, 129, 170). Li^+ can substitute for Na^+ (171) by activating hydrolysis at the cytoplasmic Na^+ sites (170). ATP can bind to the enzyme in the absence of Mg^{2+} and can phosphorylate it upon subsequent addition of Mg^{2+} and Na^+ (18, 172) with a K_d= 0.12-0.29 uM (18,19,173), an apparent K_m for phosphorylation of 0.31-3.6 uM (165,175), and an apparent K_m for Na^+-ATPase of 0.1-2 uM (175, 176). There has also been evidence for Mg^{2+}-ATP as the true substrate (177, 178) but the issue has not been settled. From studies with other nucleotides, the order of affinity for the ATP binding site is ATP> ADP dATP> CTP> ITP> UTP (18, 19). Na^+ and ATP appear to bind randomly (113, 179, 180).

The phosphorylation reaction is not readily reversible, as measured by (^{14}C) ADP-ATP exchange, unless low Mg^{2+} or high Na^+ concentrations or inhibitors (such as N-ethyl maleimide, oligomycin, Ca^{2+}, or BAL-arsenite) are present (181-184). These observations are interpreted in terms of the formation of a second, lower energy form of the phosphoenzyme (E_2-P) as illustrated in equation 1:

$$E_1 \underset{Mg^{2+},\ Na^+}{\overset{ATP}{\rightleftharpoons}} E_1 \sim P + ADP; \quad E_1 \sim P \xrightarrow{Mg^{2+}} E_2 - P \quad (1)$$

Thus, the first step of equation 1 represents phosphorylation by Na^+, Mg^{2+} and ATP. The second step is Mg^{2+}-dependent (but see ref. 185) formation of the lower energy E_2-P that is blocked by inhibitors and by high concentrations of Na^+. $E_1 \sim P$ is sensitive to ADP (which will reverse its formation to produce ATP) and insensitive to K^+ whereas E_2-P is sensitive to K^+ and insensitive to ADP. The lower energy of E_2-P compared to $E_1 \sim P$ is also suggested by observations of ^{18}O exchange between Pi and H_2O in the presence of K^+ and Mg^{2+} (158, 159) which favor the formation of E_2-P and demonstrate the ease of Pi transfer between H_2O and this form of the phosphoenzyme. Quercitin, a bioflavonoid which inhibits several types of ATPase activities, has also been shown to inhibit the (Na^+, K^+)-ATPase, probably by preventing the $E_1 \sim P \rightarrow E_2$-P transition (186).

Support for a reaction sequence involving at least three forms of E-P has been suggested from the following studies. In one study, [γ-^{32}P] ATP was allowed to bind to the enzyme in the presence of Mg^{2+} (without phosphorylating it); this was followed by a "chase" of Na^+ and nonradioactive ATP, both with and without ADP. The effect of ADP was to decrease the rate of E-P^{32} formation without affecting the rate of breakdown and strongly suggests that the ADP-sensitive form of phosphoenzyme ($E_1 \sim P$) preceeds the ADP-insensitive form (E_2-P). In their studies with the brain enzyme, Tonomura and his colleagues (165, 187) observed that in the presence of K^+, the turnover of phosphoenzyme, measured as the ratio of hydrolytic activity to the level of phosphoenzyme (v/E-P) at steady-state, was faster than the rate constant for E-P disappearance measured upon addition of EDTA plus K^+. They found also that K^+ caused an initial rapid synthesis of ATP, and that E-P was initially insensitive to ADP. These and other results (188-193) can be interpreted (113) within the framework of the above reaction sequence as evidence for an $E_1 \sim P$ with bound ADP ($E_1 \sim P$ (ADP)) i.e. insensitive to ADP since it is still bound, and that K^+ can displace its equilibrium towards the E_1 form with tightly bound ATP ($E_1 \cdot$ ATP), as illustrated in equation 2:

$$E_1 + ATP \underset{Mg^{2+}}{\overset{Mg^{2+}}{\rightleftharpoons}} E_1 (ATP) \xrightarrow{Na^+} E_1 \cdot ATP \underset{K^+}{\overset{Na^+}{\rightleftharpoons}} E_1 \sim P(ADP) \rightleftharpoons E_1 \sim P + ADP;$$
$$E_1 \sim P \xrightarrow{Mg^{2+}} E_2 - P \qquad (2)$$

Thus, K^+ increased the ratio v/E-P by shifting $E_1 \sim P^{32}$ (ADP) towards $E_1 \cdot ATP^{32}$. In fact, they observed an increase in ATP^{32} released from phosphoenzyme following the EDTA-K^+ "chase", although the time relationship between the two events is unclear from their data (Figure 3 of ref. 187). The K^+ effect is not due to competition for Na^+ (140 mMNa^+ and 0.6 mM K^+ in these studies) and may involve its high affinity site, but this is still not understood.

There is also evidence for the relative distribution of forms of E-P between $E_1 \sim P$ and E_2-P. An elaborate rapid-mixing technique with reaction times down to 3 milliseconds allowed an examination of the phosphorylation reactions at close to physiological conditions (120 mM Na^+, 10 mM K^+, and up to 0.3 mM [γ-^{32}P] ATP at 21°) (194-196), in contrast to the use of micromolar ATP concentrations and colder temperatures in the studies described above. The time course of dephosphorylation of $E\text{-}P^{32}$ after addition of ADP indicated a rapid breakdown, presumably due to formation of ATP from $E_1 \sim P$, and a slower hydrolysis of what may have been E_2-P. Dephosphorylation after addition of K^+ also showed two phases, presumably due to the rapid hydrolysis of E_2-P and slower breakdown of $E_1 \sim P$. If K^+ was present before the addition of ATP, only a slow, ADP-sensitive phase was seen and suggests that under steady state conditions, almost all the E-P is present as $E_1 \sim P$. Chelating Mg^{2+} further reduced the rate of dephosphorylation and confirmed its importance for the $E_1 \sim P \rightarrow E_2$-P transition.

Other studies under similar conditions but at micromolar ATP concentrations have indicated a burst of Pi released when K^+ was present during phosphorylation (167, 197 and see ref. 198) and suggest that only a small amount of low energy E-P (E_2-P) is present (relative to $E_1 \sim P$) under steady-state conditions.

Phosphoenzyme isolated either as $E_1 \sim P$ or as E_2-P and treated with proteolytic enzymes yields peptides with similar electrophoretic behavior (18, 199) and suggests that the various forms of phosphoenzyme differ in conformation rather than chemical structure. It should be recalled also that, as mentioned in Section 1.2 above, experiments with partial tryptic digestion (37-39) have also indicated Na^+ and K^+ forms of the dephosphoenzyme and these may represent E_1 and E_2 respectively.

ii) Dephosphorylation Reactions

In the absence of K^+, Na^+-stimulated ATP hydrolysis (Na^+-ATPase activity) is observed and is associated with a slow breakdown of phosphoenzyme (175, 176, 200-202, but see ref. 203). As mentioned above, K^+ addition stimulates dephosphorylation, presumably by combining with E_2-P. K^+ binds at the intravesicular surface of red cell inside-out vesicles i.e. external surface of cells (168), presumably at its two loading sites with a Km = 0.4-0.5 mM (123). The order of effectiveness of K^+ and its congeners in stimulating dephosphorylation is $Tl^+ > Rb^+ > K^+ > Cs^+ > NH_4^+ > Li^+$ (17).

At very low (micromolar) ATP concentrations, although K^+ and its congeners promote the breakdown of phosphoenzyme, they inhibit overall ATP hydrolysis by slowing the rate of rephosphorylation of the enzyme (175, 176, 200-202). This was directly demonstrated in a series of experiments (202) on the effects of Li^+ and Rb^+. Concentrations of Li^+ and Rb^+ that gave equal rates of dephosphorylation of E-P (formed with Na^+, Mg^+, and ATP^{32} and stopped by adding a Mg^{2+} chelator) gave different rates of hydrolysis and levels of $E\text{-}P^{32}$ resynthesis when sufficient Mg^{2+} was then added to allow a subsequent cycle of phosphorylation. Rb^+ was more inhibitory than Li^+. Similarly, when Rb^+ and Na^+ were added before Mg^{2+} and ATP^{32}, the $E\text{-}P^{32}$ level rose sharply and then fell to the same level as when Rb^+ was added after the enzyme had been phosphorylated the first time. These findings are consistent with the conclusion that inhibition is due to slow dissociation of Rb^+ from recently dephosphorylated enzyme. This reaction is illustrated (with K^+) in equation 3:

$$E_2\text{-}P + K^+ \rightleftharpoons E_2 - P\cdot K \rightleftharpoons E_2\cdot K + Pi;\ E_2\cdot K \rightleftharpoons E_1\cdot K \rightleftharpoons E_1 + K^+ \quad (3)$$

Experiments with a rapid-mixing technique have also indicated a rate-limiting step subsequent to dephosphorylation (194).

In the experiment described above, if more Mg^{2+} and ATP were added to the mixture, release of the bound ion was accelerated. It is the lack of this accelerating role of ATP which is believed to be responsible for K^+ inhibition at low ATP concentrations.

Other evidence for ATP regulation of the release of K^+ (or its congeners) from $E_2 \cdot K$ is from studies of K^+ inhibition of formycin nucleotide binding (204-206) and tryptophan fluorescence (207) which suggest that ATP at a regulatory site promotes K^+ release, thus increasing the rate of the forward step as follows: $E_2 \cdot K \xrightleftharpoons{ATP} E_1 \cdot K \rightleftharpoons E_1 + K^+$. Nonetheless, half of the enzyme is suggested to be in the $E_2 \cdot K$ form at steady state under optimal conditions of ATP hydrolysis (207). Direct evidence for an occluded enzyme-Rb complex formed in the absence of Na^+ and ATP and which is sensitive to the subsequent addition of ATP has been reported recently (208).

The regulatory effect of ATP is consistent with K^+ inhibition of ATP binding (18, 173, 202, 209-214) to a second, low affinity site (18, 162, 215-217). The high affinity of K^+ (K_i = 0.2 mM) (18) for inhibition of ATP binding suggests that external K^+ sites are involved and is consistent with its effects on dephosphorylation of E_2-P (168). However, the order of effectiveness of K^+ and its congeners in inhibiting ATP binding (18) is different from the order (given above) for their effectiveness in stimulating dephosphorylation and suggests that there may be a difference in the ease with which K^+ is released <u>after</u> it has bound to E_2 - P or that a site other than the K^+-activation site may be involved as well.(113). ATP-promoted K^+ release allows increased Na^+ binding as well (210,218). Thus, ATP has a regulatory role (increasing the Na^+/K^+ affinity ratio) other than its catalytic role in phosphorylation. The effect of ATP on Na^+ binding is synergistic in that Na^+ also facilitates ATP binding (180,219).

It should be noted that irreversible inhibitors such as beryllium, fluoride, and dicyclohexylcarbodiimide have been used in the presence of phosphorylating agents and non-phosphorylating analogs to study Na^+ and K^+ affinities and indicated that phosphorylation has little effect on Na^+ binding (220) but is necessary for K^+-binding to its high affinity sites (221-223) and supports Na^+ binding to E_1 and K^+ binding to E_2-P. Evidence for the K^+ discharge site was also suggested (Km = 0.14 M) (223).

iii) Other Properties

REVERSAL: Na^+, K^+-stimulated ATP synthesis

Since reversal of the pump coupled to ATP synthesis has been demonstrated (see section 1.3), step-by-step reversal of the ATPase reaction sequence with ATP synthesis from Pi serves to further prove the proposed mechanism for ATP hydrolysis.

Phosphoenzyme chemically similar to the one formed by $Na^+ + Mg^{2+} +$ ATP (224) has been formed from ouabain-exposed enzyme in the presence of $Pi + Mg^{2+}$ (225) or $Pi + Mg^{2+} + K^+$ (226). Phosphoenzyme can also be formed in lower yield in the absence of ouabain and presence of Pi, Mg^{2+}, and K^+ or its congeners and is chemically identical to E_2-P (224, 227), thus demonstrating the reversibility of equation 3 (above). If K^+ is excluded, large yields were obtained, but the product was insensitive to K^+ and ADP and is thought to represent a side reaction of the enzyme as described by equation 4 (see ref. 227):

$$Pi + Mg^{2+} + E \rightleftharpoons Mg \cdot E_2\text{-}P; \quad Mg \cdot E_2\text{-}P + Mg^{2+} \rightleftharpoons (Mg)_2 \cdot E_2\text{-}P \quad (4)$$

Addition of a very high Na^+ concentration ($K_{0.5}$=0.6 M) and a Mg^{2+} chelator made the E-P sensitive to both K^+ and ADP i.e. resulted in a mixture of $E_1 \sim P$ and E_2-P (224, 228). Alternatively, Pi incorporation in the presence of a low concentration of Na^+ directly formed E_2-P (228, 229). Na^+ may have displaced K^+ bound to the enzyme during its purification. Addition of a high Na^+ concentration followed by ADP allowed synthesis of ATP and demonstrated the reversibility of the ATPase reaction (equations 1-3 above). Pi incorporation in the presence of K^+ did not permit synthesis of ATP. Na^+ is required at a low affinity site, presumably its discharge site, and its binding allows the conformational change from $E_2\text{-P} \rightarrow E_1 \sim P$ (228, 229 and see ref. 230). It should be noted that the ATP synthesis observed in these studies and confirmed by another group (231) resulted from a single turnover of enzyme as opposed to multiple turnovers in the reversal experiments described earlier with intact red cells (see section 1.3).

This two-step reversal of ATP hydrolysis offers strong evidence for the $E_1 \sim P$ and E_2-P conformations of the enzyme as intermediates in the normal operation of the (Na^+, K^+)-ATPase.

CARDIAC GLYCOSIDE INHIBITION

As mentioned earlier, cardiac glycosides such as ouabain and strophanthidin are specific inhibitors of the (Na^+, K^+)-ATPase (10) and bind at the external surface (11, 12).

Although a large amount of work has been devoted to the study of the glycoside-enzyme interaction (see ref. 113), the main observations have been that ligands which stabilize the E_2 or E_2-P conformation (Mg^{2+}, Mg^{2+} + Pi, and Mg^{2+} + Na^+ + phosphorylating agents) increase the rate of binding and lower the dissociation constant whereas ligands which stabilize E_1 or $E_1 \sim P$ (Na^+ and/or nonphosphorylating analogs) decrease the rate of binding and raise the dissociation constant (232-233).

K^+ reduces glycoside binding (172, 225, 234-241) at a high affinity site (Ki = 0.2 mM), possibly involving only one K^+ ion (236, 237) and Na^+ stimulates in the presence of ATP (see above) at a high affinity site (237, 242). Studies with intact red cells and ghosts have demonstrated a reduction of ouabain binding by external K^+ (243-245) and an increase of binding by cytoplasmic Na^+ (246). These results are consistent with the interpretation of glycoside inhibition of dephosphorylation of phosphoenzyme (E_2-P) or phosphorylation of dephosphoenzyme (E_2) (247), the forms of the enzyme that interact with external K^+ and thus external glycosides as well.

Although (Na^+, K^+)-ATPase from most tissues seems to have a single ouabain binding site (248, 249), there have been studies on brain preparations (94, 239, 248) and on Cs^+ and K^+ reduction of ouabain binding to red cells (223, 250) reporting at least two sites that may show negative cooperativity.

1.5 MODEL FOR THE ATPase AND PUMP

The steps in equations 1-3 (Section 1.4) can be linked in a cycle as shown in Figure 2 to illustrate the sequence of the steps during the course of ATP hydrolysis as well as the asymmetry of the ligand interactions. Although there is no general agreement on a single model for the ATPase and pump, the model in Figure 2 summarizes several of the important features of both (see Sections 1.3 and 1.4).

The solid lines indicate the course of the reaction under physiological conditions i.e. in the presence of Na^+, K^+ and high ATP concentrations. E_1 and E_2 are the two conformations which bind cytoplasmic and external ligands, respectively. Starting with free enzyme (E_1), ATP is hydrolyzed to form the high energy conformation $E_1 \sim P$. In a Mg^{2+}-dependent step, the phosphoenzyme changes conformation to a second, lower energy form (E_2-P) from which Na^+ is released to the external surface i.e. the Na^+ translocation step. Although three Na^+ are considered to be transported per molecule of ATP hydrolyzed, experiments with red cell inside-out vesicles at low ATP concentrations and varying amounts of cytoplasmic Na^+ have indicated that at very low levels of Na^+, the ratio of Na^+ pumped per mole of ATP hydrolyzed is decreased markedly (170) i.e. the stoichiometry of Na^+ transported to ATP hydrolyzed becomes variable and that without enough Na^+ bound, ATP may be hydrolyzed without translocation of Na^+ (presumably by breakdown of $E_1 \sim P$).

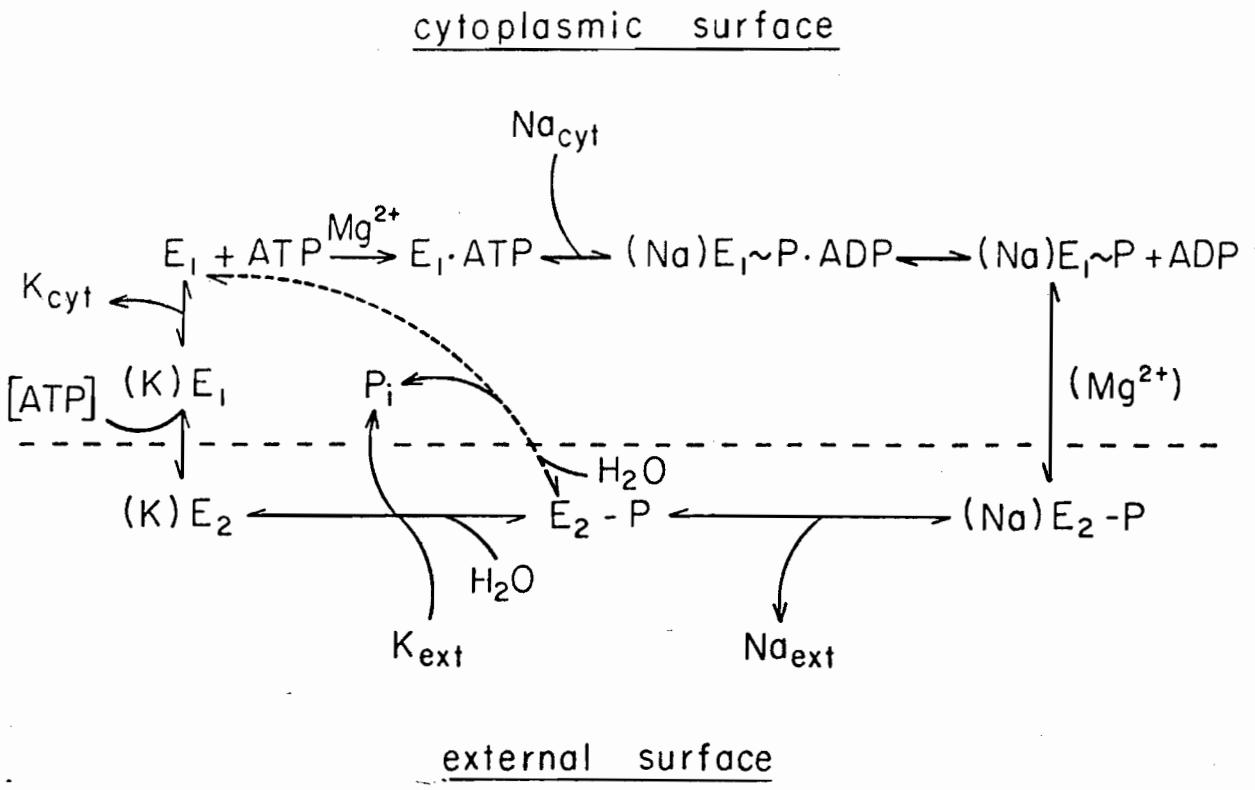

FIGURE 2: MODEL FOR THE (Na^+, K^+)-ATPase AND PUMP

(see text for explanation)

Presumably E_2-P can be hydrolyzed in the absence of external Na^+ and K^+ to form E_1 (broken line in figure 2) and represents the Na^+-ATPase with uncoupled Na^+ efflux. In the presence of ADP and external Na^+, E_2-P is not hydrolyzed and reverts back to E_1 resulting in regeneration of ATP (ATP-ADP exchange) and Na^+-Na^+ exchange.

In the presence of external K^+, K^+ binds to E_2-P and accelerates dephosphorylation to form an $E_2 \cdot K$ complex. At low ATP concentrations, this complex is occluded and results in inhibition (of Na^+-ATPase) due to its rate-limiting breakdown (see Section 1.4). In the presence of higher ($\geq 2\mu M$) ATP concentrations, ATP (in brackets) accelerates release of K^+ via a second, low affinity site, allowing the enzyme to return to its original conformation ($E_2 \cdot K \rightarrow E_1 \cdot K \rightleftharpoons E_1 + K^+$) as K^+ is translocated. There is no evidence that the ATP bound at this low affinity site is the same molecule that is hydrolyzed in the next cycle of the pump when cytoplasmic Na^+ is present. In the absence of cytoplasmic Na^+ and presence of K^+ and Pi, the K^+ translocation step is reversed (K^+ - K^+ exchange) and the enzyme is phosphorylated (E_2-P) from Pi.

It has been suggested recently (251-253) that the (Na^+, K^+)-ATPase shows half-of-the-sites reactivity as has been described for alkaline phosphatase (254). According to this scheme, (Na^+, K^+)-ATPase exists as a dimer, each half consisting of one large and one or two small chains (see Section 1.2).

Each half proceeds through the entire reaction cycle chemically, mechanically, and energetically coupled in a "flip-flop" mechanism, 180° out of phase with the other half i.e. while one half of the dimer is proceeding through phosphorylation steps, the other half is proceeding through dephosphorylation steps. Although this scheme is far from being proved for the (Na^+, K^+)-ATPase, it does account for several important discrepancies mentioned earlier with regards to both ion transport and the reaction scheme presented above (Figure 2):

i) The model in Figure 2 would predict consecutive translocation of Na^+ and K^+ whereas transport studies favor simultaneous translocation (see Section 1.3). According to half-of-the sites reactivity (251), each half of the dimer is involved in the consecutive translocation of Na^+ and K^+, but because the halves are 180° out of phase, the entire pump complex would translocate Na^+ and K^+ simultaneously.

ii) Several studies have suggested two ATP and ouabain binding sites of different affinities per (Na^+, K^+)-ATPase site. In a dimeric enzyme, each half would contain an ATP binding site and a ouabain binding site (on the large chain) and would show negative cooperativity i.e. binding of the first ligand (ATP or ouabain) to a high affinity site on one half would decrease the binding affinity for a second ligand on the other half. Both sites could be filled under appropriate conditions and would display different affinities. The low affinity regulatory site for ATP would be the binding site on the half that is phosphorylated.

iii) The observations of Tonomura and his colleagues described in Section 1.4 suggesting tightly and loosely bound ATP can be accounted for (see ref. 163) as high and low affinity sites on different halves of the dimer, of which the former continued to phosphorylate the enzyme after the reaction had been terminated. Furthermore, the increased turnover rate measured as the ratio v/E-P relative to the rate constant for dephosphorylation induced by K^+ may not be due to an effect of K^+ on the $E_1 \cdot ATP \rightleftharpoons E_1 \sim P$ (ADP) equilibrium as suggested by Tonomura and his colleagues but rather that in the absence of K^+, the halves of the dimer are independent and are both phosphorylated, giving a higher level of phosphoenzyme; the initial formation of ATP upon K^+ addition may be due to K^+ competition with ATP present at its low affinity site before it had a chance to phosphorylate.

iv) The role of Mg^{2+} in specifically inhibiting the ATP-ADP exchange reaction and thus stimulating the $E_1 \sim P \rightarrow E_2$-P transition has been criticized (185) since Mg^{2+}-ATP and Mg^{2+}-ADP have also been shown to inhibit this exchange (185, 255). These results can be reconciled with a half-of-the-sites reactive enzyme (256) in which Mg^{2+}-nucleotides bind to the low affinity site and inhibit exchange by favoring the forward reaction. Furthermore, this inhibition is relieved by K^+ competition via its high affinity sites (257), in agreement with its effect on ATP binding at the low affinity ATP site.

The objective of this thesis is to examine the interactions of K^+ with the enzyme since these seem to be crucial to the regulation of activity. Two experimental approaches are taken and will be considered in turn in the next two sections.

1.6 PHOSPHATASE ACTIVITY

The (Na^+, K^+)-ATPase also catalyzes the hydrolysis of other organic phosphates, e.g. acetyl phosphate, carbamyl phosphate, unbelliferone phosphate and p-nitrophenyl phosphate (pNPP), in a K^+-dependent reaction that is believed to involve the terminal steps of the ATPase reaction (113). K^+ activates at a low affinity site(s) in the presence of Mg^{2+}, although higher concentrations of either ion are required when the other is raised (258-262). Mg^{2+} is required to stabilise the complex, but will inhibit at too high a concentration by binding at its low affinity site (263, 264).

Na^+ alone promotes a minimal phosphatase activity (265-267). Na^+ inhibits at high K^+ concentrations, but activates at low K^+ concentrations (259, 262, 265) i.e. high Na^+/K^+ ratio.

ATP inhibits phosphatase activity in the absence of Na^+ (260, 266, 268, 269) by decreasing the affinity for substrate and K^+ (262, 268). Both competitive (266, 268, 270) and non-competitive (260, 270) inhibition by ATP have been reported and suggest that ATP binding at a low affinity site is competitive with K^+ and non-competitive with substrate.

In the presence of Na^+, however, ATP stimulates phosphatase activity by increasing the affinity for K^+ (267, 268, 271-274) and renders the reaction sensitive to oligomycin and N-ethylmaleimide (266-268, 275). The effect of ATP is biphasic in that micromolar concentrations stimulate whereas high concentrations inhibit the phosphatase (270, 276).

Experiments on beryllium inactivation (221, 277 as described in Section 1.4) have indicated that the affinity for K^+ is increased in the presence of both Na^+ and ATP or phosphatase substrates and suggests that phosphorylation is required to expose the high affinity K^+ sites (222). A phosphoenzyme intermediate during acetyl phosphate hydrolysis has been demonstrated (266, 278-280). pNPP can also phosphorylate a serine residue if exposed over long periods at high acidity (pH 5.0) in the presence of K^+ or at neutral pH in the presence of Na^+ (281) and phosphorylation in the presence of ouabain has also been noted (282), though this may not be related to the phosphatase. If a phosphoenzyme intermediate is formed during hydrolysis of pNPP, it is less reactive than E_2-P since less water oxygen is incorporated from pNPP hydrolysis than from hydrolysis of acetyl phosphate or ATP (283). Several groups have been unable to find a p-nitrophenol-ATP or pNPP-ADP exchange (279, 282, 284).

An early model for the phosphatase (267) postulated a new form of phosphoenzyme that could be formed from Na^+ and/or K^+ and that could be doubly phosphorylated in the presence of more reactive agents e.g. ATP, and assumed that the phosphorylated enzyme is more effective as a phosphatase.

Other models explain Na^+ effects in the absence of K^+ as being due to allosteric regulation (265, 283, 285). Although a doubly phosphorylated intermediate can be made more attractive in view of the half-of-the-sites reactivity model, later evidence for the $E_2 \cdot K$ complex (202) led to the suggestion that phosphatase substrates phosphorylate this complex to form E_2-P which could also be formed in the presence of Na^+ and ATP after dephosphorylation by K^+. This would account for the stimulation by Na^+ and ATP based on the reaction mechanism for the ATPase as well as the requirement for Mg^{2+} in order to stabilize E_2 (263, 264). Similarly, the sidedness of Na^+ and K^+ effects have been suggested in a model (259) in which K^+ alone acts at the cytoplasmic Na^+ sites whereas Na^+ and ATP phosphorylate the enzyme at these sites and expose the higher affinity, external K^+ sites.

Although the model invoking changes in affinity and orientation of the K^+ sites is consistent with the kinetic data obtained from broken membrane preparations, experiments in red cell ghosts indicate that only external K^+ is stimulatory (286). Therefore, the question remains whether a more complex model is relevant, e.g. one involving high andlow affinity external K^+ sites that are sensitive to changes in concentration of ligands such as Na^+ and ATP. To shed light on this problem, I have examined the sidedness of the alkali cation interactions with the phosphatase in inside-out red cell vesicles using pNPP as substrate.

1.7 GENETIC DIMORPHISM IN SHEEP

Sheep and goats are genetically dimorphic with respect to the intracellular K^+ content of their red cells. The cells of one type have high K^+ (HK) and the cells of the other type have low K^+ (LK) concentrations, the latter being dominant (287). The difference in the K^+ gradient across the red cell membrane is partly associated with the 4-fold higher rates of ouabain-sensitive Na^+ and K^+ transport (288) and (Na^+,K^+)-ATPase activity (289), as well as a somewhat greater number of ouabain binding sites (290-292) in HK than in LK sheep cells. A similar number of ouabain binding sites is observed in both types of goat red cells (321).

Different kinetic characteristics between the HK and LK enzyme system are also evident, particularly when the assays are carried out at very low ATP concentrations under which condition Na^+ activation of ATPase activity is readily apparent and is referred to simply as Na^+-ATPase. Thus, although HK membranes have a ten-fold greater Na^+-ATPase activity than LK membranes, the Na^+-dependent ATP-ADP exchange reaction is only 2.7 times greater in HK (294) and suggests that a step subsequent to ADP release is an important factor in this difference.

A particularly striking distinction between HK and LK is the interaction of K^+ with the enzyme system. This kinetic distinction between the two is shown in studies demonstrating differences in the affinities of HK and LK pump sites for intracellular K^+, LK being strongly inhibited by K^+ (131, 295). A difference in the interaction with K^+ at the external surface, though less striking, is also reported (131).

An antibody (anti-L) raised in HK sheep injected with LK red cells increases the rate of ouabain binding to LK cells (291, 293) and stimulates the pump rate (297), probably by increasing the Na^+/K^+ affinity ratio at the cytoplasmic surface (148, 296, 298-300), consistent with Na^+ and K^+ competition with the enzyme i.e. to form $E_1 \cdot Na$ and $E_1 \cdot K$ forms, respectively (296).

Studies of (Na^+, K^+)-ATPase support the difference in K^+ interactions evidenced in studies of pump activity. K^+ inhibits at the Na^+ sites of LK goat red cell membranes (299-301) and studies of Na^+-ATPase in membranes from sheep red cells assayed at very low ATP concentrations have shown that the LK activity is inhibited whereas the HK activity is activated by $K^+ \leqslant 5mM$ (294, 302, 303). With LK goat membranes, the Na^+/K^+ affinity ratio of the (Na^+, K^+)-ATPase is low compared to HK, but increased by the addition of anti-L or by increasing the pH (304). At very low ATP (0.2 μM) however, the earlier studies by Blostein et al (305) showed that anti-L increased the Na^+-ATPase of LK sheep without changing the K^+ inhibition profile. It may be that the failure to observe a kinetic change in the latter study is because higher concentrations of ATP, acting perhaps at a low affinity regulatory site(s), may be required for the change in the Na^+/K^+ affinity ratio.

Although the difference between HK and LK Na^+-ATPase has been ascribed mainly to a difference in the interaction of K^+ at intracellular sites, this has not been directly shown since all studies were done with broken membranes.

In the study of Cavieres and Ellory (296), a correction was made for incomplete occupation of external K^+ sites using a saturation function obtained from influx experiments. The investigation described in this thesis was aimed to directly examine the K^+ interactions at both the cytoplasmic and external surfaces using a system in which cation concentrations at both membrane surfaces could be controlled. Although intact red cells and ghosts are the systems in which ion transport have been best characterized, the concentrations of ligands and presence of interfering soluble enzymes cannot be controlled in red cells or even in resealed ghosts. Therefore, I have prepared inside-out membrane vesicles from HK and LK red cells which are relatively free of contaminating enzymes by a modification of the method described for human red cells (306). Results that will be presented suggest a difference in the interaction of K^+ at both membrane surfaces and of ATP as the basis for the kinetic differences observed.

2. MATERIALS AND METHODS

2.1 COLLECTION OF RED BLOOD CELLS

Blood from normal human donors or from sheep is obtained by venipuncture. Nine volumes of blood are added to one volume of 0.1 M EDTA, pH 7.4. The blood is centrifuged for 5 minutes at 4°C at 3,000 rpm in an International Equipment Co. Model B-20 refrigerated centrifuge using a 947 rotor. The plasma and buffy coat are removed by suction and the cells are resuspended and washed three times with 4 to 5 volumes of ice-cold 154 mM NaCl.

2.2 PREPARATION OF INSIDE-OUT VESICLES

Inside-out vesicles are prepared according to the method of Steck et al (306) for human blood as modified by Blostein and Chu (168). Red cells are lysed with 20 volumes of ice-cold 5mM phosphoric acid buffered with Tris base to pH 7.4, left for 10 minutes on ice, and centrifuged for 20 minutes at 10,000 x g at 4°C in a Sorvall RC-2B refrigerated centrifuge with an SS-34 fixed angle rotor. The supernatant is discarded and 10 volumes of ice-cold H_2O are added to the pellets ("ghosts") and left for 10 minutes on ice. Thirty volumes of ice-cold 5mM Tris PO_4, pH 7.4 are added and the ghost suspension is centrifuged for 20 minutes at 10,000 x g at 4°C. This procedure is repeated and the supernatant is removed. A fraction of the ghost preparation is stored on ice, when necessary, and 25 volumes of ice-cold 0.5 mM Tris PO_4, pH 7.4 are added to the rest.

The ghosts are left one hour on ice to allow inward vesiculation of the membrane. The ghosts are concentrated by centrifugation and vesicles are released from them by shearing, i.e. by passing the suspension five times through a 27 gauge needle.

Sealed inside-out vesicles are separated from broken membranes by layering 3 ml of the suspension on a solution (14 ml) of Dextran T 70 (Pharmacia) (ρ = 1.01 g/ml) made up by dissolving 4 g in 100 ml of 0.5 mM Tris phosphate, pH 7.4, containing 0.1 mM $MgCl_2$; this solution has previously been heated in a boiling water bath at 100°C for 15 minutes and cooled on ice since preliminary studies showed that unheated solutions dramatically reduced the ATPase activity of the vesicles. The preparation is centrifuged at 27,000 rpm (100,000 x G) for one hour in a Beckman L5-65 ultracentrifuge equipped with an SW-27 swinging bucket rotor. The vesicles banding at the top of the tubes are presumably inside-out (ref. 306), and are collected and slowly equilibrated with successively higher concentrations of Tris glycylglycine, pH 7.4, containing 0.1 mM $MgCl_2$ (as described in ref. 168). The final vesicle pellet is suspended in 40 mM Tris glycylglycine, pH 7.4 containing 0.1 mM $MgCl_2$ at a protein concentration of 0.5 to 1 mg/ml and stored on ice for no longer than five days.

2.3 p-NITROPHENYL PHOSPHATASE ASSAY

Unless indicated otherwise, p-nitrophenyl phosphatase, or pNPPase, is assayed at 5 mM Tris pNPP and 4 mM $MgCl_2$ in Tris glycylglycine, pH 7.4, and the chloride salts of cations added as indicated in the figure legends.

Unless indicated otherwise, the reactions are stopped after 10-15 minutes at 37^{o} by addition of a similar volume of a quench solution containing 0.2 N NaOH, 2.5% SDS, and 4 mM EDTA as described by Ottolenghi (307). Absorption of the product p-nitrophenol at 410 nm was determined. Values shown were corrected for the absorption by p-nitrophenol produced due to hydrolysis in the absence of vesicles by subtracting the absorption observed in tubes to which vesicles were added after the reaction was stopped. pNPP and p-nitrophenol used as a standard are purchased from Sigma Chemical Company. pNPP (disodium salt) is converted to the Tris salt by ion-exchange on Dowex 50x4-200R specially washed cation exchange resin purchased from Sigma Chemical Company and adjusted to 0.1 M after titration to pH 7.4 using the molar extinction coefficient for pNPP of 8,000 measured at 305 nm. Strophanthidin and valinomycin are purchased from Sigma Chemical Company.

The results of experiments typical of at least three others are shown. The values shown are the differences between the means of replicate ($\geq$3) measurements with and without alkali cations i.e. the mean value for the choline chloride baseline is subtracted. Standard errors of the differences between the means are indicated.

For experiments in which ATP or Pi were added, preliminary tests ascertained that they did not affect the choline baseline activity at all concentrations tested. Where indicated in the Legends to the Figures, linear regression analyses of double reciprocal plots of the activities observed versus added ligand or substrate were performed using a Hewlett-Packard Programmable Desk Top Computer model 9831A. All activities are expressed on the basis of milligrams of membrane protein measured.

2.4 3-O-METHYLFLUORESCEIN PHOSPHATASE ASSAY

3-O-methylfluorescein phosphatase (3-O-MFPase) is assayed under the same conditions as pNPPase but with 3-O-methylfluorescein phosphate as substrate and measuring the product, 3-O-methyl fluorescein, by following its fluorescence in a continuously recording Aminco-Bowman spectrophotofluorometer (excitation maximum 475 nm, emission maximum 525 nm) equipped with a water jacket that maintains the cuvettes at 37^{o} during the course of the assay. 3-O-methylfluorescein phosphate and 3-O-methylfluorescein used as a standard are purchased from Sigma Chemical Company. The results of experiments typical of at least 2 others are shown. The values shown are the differences between the values obtained with KCl and those obtained with choline chloride. The activities are expressed on the basis of milligrams of membrane protein measured.

2.5 NEURAMINIDASE ASSAY

Sialic acid is determined by the method of Warren (308) as described by Perrone and Blostein (11). Total sialic acid is the amount released following incubation of the inside-out vesicles with 0.2 N H_2SO_4 at 80^{o} for one hour. Neuraminidase-sensitive sialic acid is the amount released after incubation of vesicles with neuraminidase (50 ug/mg membrane protein; type lV grade, Sigma Chemical Company) in 0.2 M Tris acetate buffer, pH 5.7 for one hour at 37^{o}. N-acetyl neuraminic acid is used as a standard and is purchased from Sigma Chemical Company. The results shown are the means of duplicate determinations and the values are expressed on the basis of milligrams of membrane protein measured.

2.6 ATPase ASSAY

Na^+-ATPase activity (measured by ^{32}Pi released from [γ-^{32}P] ATP) of inside-out vesicles is measured as described elsewhere (168). One volume of vesicles is added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, NaCl, KCl, choline chloride, and [γ-^{32}P] ATP as indicated in the Figure Legends. After 1-5 minutes at 37^o, 1.9 ml of 5% trichloroacetic acid containing 2.5 mM disodium ATP (purchased from Sigma Chemical Company) and 5 mM KH_2PO_4 is added to stop the reaction. One ml of Norit A charcoal (purchased from Fisher Scientific Company) in 5% trichloroacetic acid (1.5 g Norit A charcoal/10 ml 5% trichloroacetic acid) is added and incubated for one hour on ice, with occasional mixing, to adsorb the unhydrolyzed ATP. The charcoal is removed by filtration and aliquots of filtrate are counted for determination of radioactivity. Ouabain is purchased from Sigma Chemical Company.

The results of experiments typical of at least three others are shown. The values are means of triplicate determinations, unless indicated otherwise, and the standard error of the mean is indicated, unless it falls within the size of the symbols used in the Figures. The values are corrected for the baseline activity observed in the absence of NaCl and presence of KCl. Activities are expressed on the basis of milligrams of membrane protein measured.

Isotopically labelled ATP used in these experiments is prepared according to the method of Glynn and Chappel (309) as modified by Post and Sen (310). Thus, [γ-^{32}P] ATP is prepared from ADP and ^{32}Pi (New

England Nuclear Company) utilyzing the coupled reactions:

$$GA3P + {}^{32}Pi + DPN \xleftrightarrow{GA3PD} 1,3\ D^{32}PG + DPNH$$

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

The $[\gamma\text{-}^{32}P]$ ATP is purified on a Dowex-1-Cl column (200-400 mesh, 2% cross-linked; purchased from Sigma Chemical Company) of 0.6 x 1.0 cm and neutralized with solid Tris base. The ATP concentration at pH 7.4 is determined spectrophotometrically at 259 nm with a correction for non-specific ultraviolet absorption by correcting for the absorption at 280 nm as follows (311):

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

An aliquot is taken for determination of radioactivity. The specific radioactivity is 5 to 10 Ci/m mole.

2.7 $^{86}Rb^+$ AND $^{22}Na^+$ UPTAKE MEASUREMENT

Samples of vesicles are added to the upper chamber of a Millipore filtration apparatus (Millipore Corporation) containing 10 ml of ice-cold buffer of identical composition as the uptake medium but lacking radioactive tracer, and immediately filtered through filter pads (type RA, 1.2 u; Millipore Corporation) and washed with 15 ml of the same buffer. The pads are then dried and their radioactivity is determined.

The results of experiments typical of at least four others are shown. Uptake is expressed on the basis of microliters of radioactive medium taken up per milligram of membrane protein measured. Conversion of this value to nanomoles per milligram simply involves multiplying by the medium Rb^+ or Na^+ concentration.

2.8 PROTEIN MEASUREMENT

The protein concentration of membrane suspensions is determined by the method of Lowry et al (312) using crystalline bovine serum albumin (fraction V, Sigma Chemical Company) as a standard.

2.9 OPTICAL DENSITY MEASUREMENT

All spectrophotometric determinations are carried out in a Zeiss PMQ II spectrophotometer using quartz (Carl Zeiss Company or Beckman Instrument Company) or glass (Carl Zeiss Company or Helma Company) cuvettes with a 1 cm light path, and in the case of pNPPase assays, with semi-micro cuvettes (0.3 ml volume, 1 cm light path; Carl Zeiss Company).

2.10 RADIOACTIVITY DETERMINATIONS

Aliquots of aqueous samples of ^{32}Pi and filtration pads containing $^{86}Rb^{+}$ or $^{22}Na^{+}$ are placed in scintillation counting vials containing 10 ml of a 1:1 mixture of toluene and Aquasol II (New England Nuclear Company). The vials are counted in a Packard Tri-Carb model 3003 or a Searle model Delta 300 liquid scintillation spectrometer.

3. K^+-STIMULATED pNPPase OF INSIDE-OUT MEMBRANE VESICLES FROM HUMAN RED CELLS

As described in Section 1.6, (Na^+, K^+)-ATPase catalyzes the Mg^{2+}-dependent, K^+-activated hydrolysis of p-nitrophenylphosphate (pNPP). This reaction is considered to be related to the terminal K^+-dependent hydrolysis of the phosphoenzyme intermediate of (Na^+, K^+)-ATPase. Kinetic studies in a number of laboratories have led to several interpretations of the reaction mechanism. A major problem has been that in most studies broken membrane preparations were used so that reactants and ligands could not be separately controlled at the two membrane surfaces. Although resealed human red cells ghosts were used in the study of Rega et al (286), small amounts of ligands, in particular ATP and Na^+, may have been present inside the resealed ghosts.

In the present study, the sidedness of alkali cation activation of phosphatase activity was examined using inside-out vesicles prepared from human red cells according to the method of Steck et al (306). This system allows very precise control of the composition of the medium at the cytoplasmic surface (168, 170). It is also possible to 'load' the vesicles with a particular cation (see ref. 168 and 170) and then either dilute them to reduce the extravesicular cation concentration or wash them to remove virtually all of the free extravesicular cations.

Since the vesicles used in these studies are inside-out, extravesicular Na^+ and K^+, equivalent to Na^+ and K^+ at the originally cytoplasmic surface, are designated as Na_{cyt} and K_{cyt}, respectively.

Similarly, intravesicular Na^+ and K^+ being Na^+ and K^+ at the originally external membrane surface, are designated as Na_{ext} and K_{ext}, respectively.

3.1 EFFECTS OF K^+

As can be seen in Experiment A of Table 1, addition of 10 mM K_{cyt} alone stimulates pNPPase. The K^+-stimulated activity is almost completely inhibited by the addition of 25 uM strophanthidin, a lipid-soluble cardiac glycoside. In Experiment B of Table 1, vesicles preequilibrated with 10 mM K^+ i.e. approximately 10 mM K_{ext}*, and then washed free of K^+ at the cytoplasmic surface have very little pNPPase. In fact, addition of the K^+ ionophore,valinomycin, does not reduce the small amount of K^+ activation as expected if the K^+ activation were, in fact, due to K_{ext}. Thus valinomycin must have reduced K_{ext} by about two orders of magnitude (intravesicular space $\simeq$15 ul/mg; membrane protein used= 1 mg/ml). This suggests that the small activation by K_{ext} is probably due to K^+ remaining tightly bound at sites at the other (cytoplasmic) surface.

The response of pNPPase to varying K_{cyt} was tested. The $K_{0.5}$ observed in experiments with several separate preparations was about 1 mM with maximal activity at about 10 mM K_{cyt}. In the representative experiment shown in Figure 3, the $K_{0.5}$ calculated by linear regression analysis of double reciprocal plots of the data was 0.8 mM. Although other investigators have observed sigmoidal activation by K^+ (265), the relatively large variances in measurements at low K_{cyt} preclude a more precise analysis of K_{cyt} activation.

* Throughout this study, the concentration of Na^+ and K^+ at the external surface is given as that used to load the vesicles. In general, 70-80% equilibration was attained after overnight loading.

EXPERIMENT	ADDITIONS (mM)				K^+-STIMULATED
	K_{cyt}	K_{ext}	Stroph.	Val.	ACTIVITY (nmoles/min/mg)
A	10	0	-	-	0.98 ± 0.04
	10	0	0.025	-	0.06 ± 0.02
	10	0	0.250	-	0.06 ± 0.04
B	0	10	-	-	0.08 ± 0.02
	0	10	-	+	0.12 ± 0.02

TABLE 1: SIDEDNESS OF K^+ EFFECTS ON pNPPASE

Vesicles (1 mg protein/ml suspended and stored in 40mM Tris glycylglycine, pH 7.4) are concentrated to one-fifth their original volume by centrifugation. In Experiment A, 1 volume of a solution containing 44 mM $MgCl_2$ and 110 mM choline chloride is added to 10 volumes of vesicles to obtain final concentrations of 36 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 10 mM choline chloride. The vesicles are equilibrated overnight on ice followed by 25 minute at 37°. 0.005 volumes of absolute ethanol or 5 mM or 50 mM strophanthidin in absolute ethanol are added as indicated and the vesicles are incubated for 5 minutes at 37°. They are then added to 9 volumes of reaction medium in a final volume of 0.1 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 10 mM KCl or choline chloride, as indicated, and 0.005 volumes of ethanol or 25 μM or 250 μM strophanthidin, as indicated.

In Experiment B, 5-fold concentrated vesicles are added to an equal volume of 20mM KCl or choline chloride and 8 mM $MgCl_2$ to obtain a final concentration of 20 mM Tris glycylglycine, 10 mM KCl or choline chloride, as indicated, and 4 mM $MgCl_2$. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. The vesicles are then diluted 19-fold with a solution of similar composition, except that K^+ is replaced by choline, and centrifuged. The supernatant is removed and the pellet is washed without resuspension as follows: 1 ml of the same solution is added and after 2 minutes on ice, removed, and the washing is repeated three times; the vesicles are then resuspended to their original volume (after loading). One volume of vesicles is added to four volumes of reaction medium in a final volume of 0.1 ml of the same composition as in Experiment A, but with 0.0022 volumes of ethanol or 0.45 mM valinomycin in ethanol, as indicated, instead of strophanthidin.

In both experiments, the reactions are allowed to proceed for 10 minutes at 37° and then stopped with 0.2 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of KCl and presence of choline chloride (1.18± 0.01 and 0.93± 0.02 n moles/min/mg for experiments A and B, respectively).

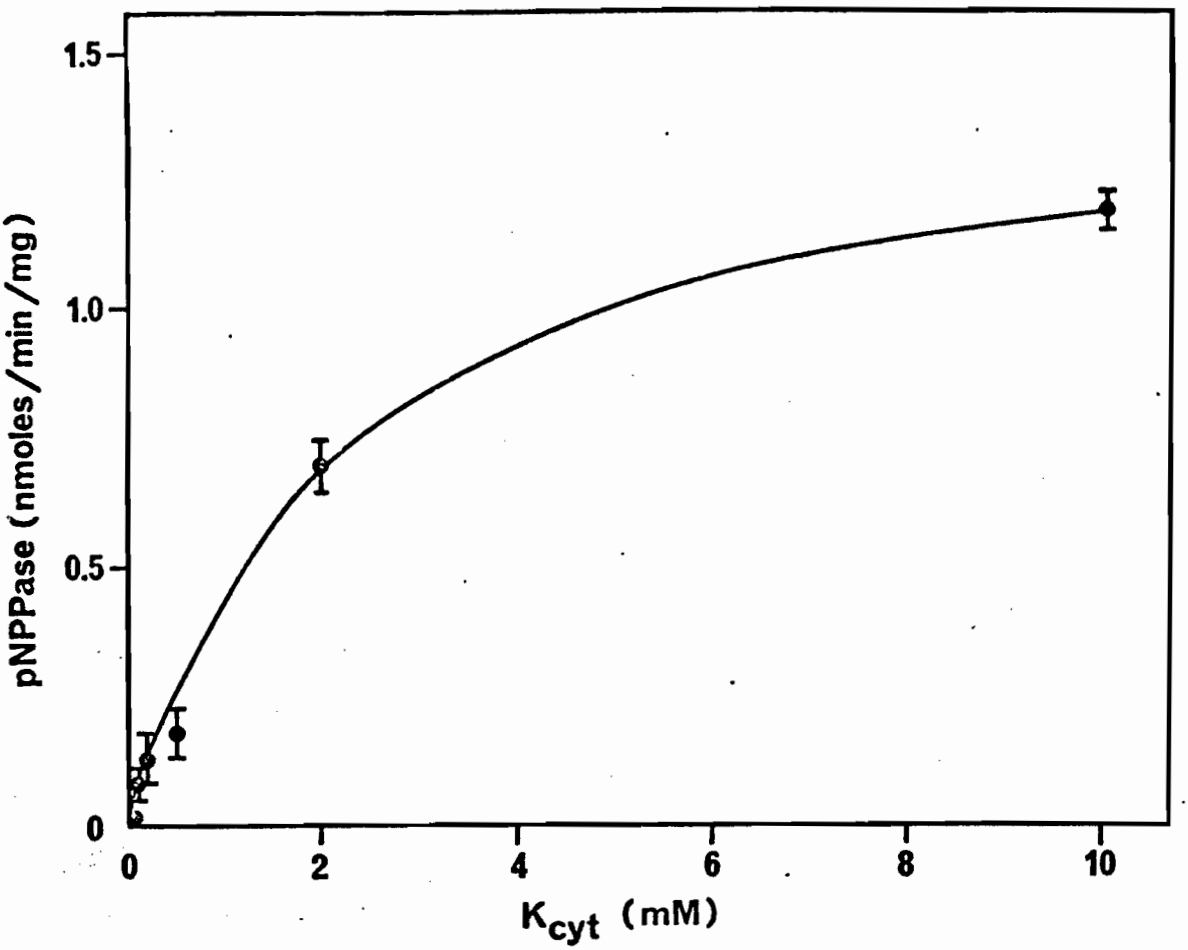

FIGURE 3: EFFECT OF VARYING K_{cyt} ON pNPPase

0.01 volumes of 1 M choline chloride and 0.04 volumes of 0.1 M $MgCl_2$ are added to one volume of vesicles to obtain final concentrations of 38 mM Tris glycylglycine, pH 7.4, 10 mM choline chloride, and 4 mM $MgCl_2$. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. They are then added to an equal volume of reaction medium in a final volume of 0.15 ml containing 19 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, KCl as indicated with choline chloride present to maintain a final chloride concentration of 10 mM. The reactions are allowed to proceed for 10 minutes at 37° and then stopped with 0.15 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of KCl and presence of choline chloride (0.82± 0.03 n moles/min/mg). The curve is drawn by eye. Linear regression analysis of double reciprocal plots of these data were carried out and the value for $K_{0.5}$ obtained was 0.8± 0.1 mM (r= 0.8893).

The response of pNPPase to varying pNPP concentrations at 10 mM K_{cyt} is shown in Figure 4 and indicates a $K_{0.5}$ for pNPP of about 1 mM and shows that almost maximal activity is observed at 5 mM pNPP, the concentration used in all other experiments.

Although K_{ext} alone does not activate pNPPase, the possibility remains that K_{cyt} activation requires K_{ext}. As shown in Experiment A of Table 2, addition of valinomycin to a reaction medium containing 0.5 mM K^+ in order to equilibrate K^+ on both sides of the membrane does not increase the activity above that observed in the control (K_{cyt}). However, the possibility still remains that only a small amount of K_{ext} is required since it is observed that about 10% of the congener $^{86}Rb^+$ penetrated the vesicles during the 5 minute incubation in the presence of 5 mM pNPP (see Figure 15).

In an effort to counteract and/or minimize the possible effect of K_{ext}, the following experiments were carried out.

(i) In experiment B of Table 2, pNPPase is assayed for 5 minutes in the presence of 1 mM K_{cyt}. The vesicles are pre-equilibrated with 50 mM Na^+, i.e. approximately 50 mM Na_{ext}, and washed free of Na^+ at the cytoplasmic surface. A significant change in activity could not be detected, suggesting that Na_{ext} competition with K_{ext} is not occurring or is ineffective, at least under this condition.

(ii) If small amounts of K_{ext} are absolutely required, it was reasoned that the time-course of pNPPase in the presence of K_{cyt} should show a lag until sufficient K^+ penetrated the vesicles.

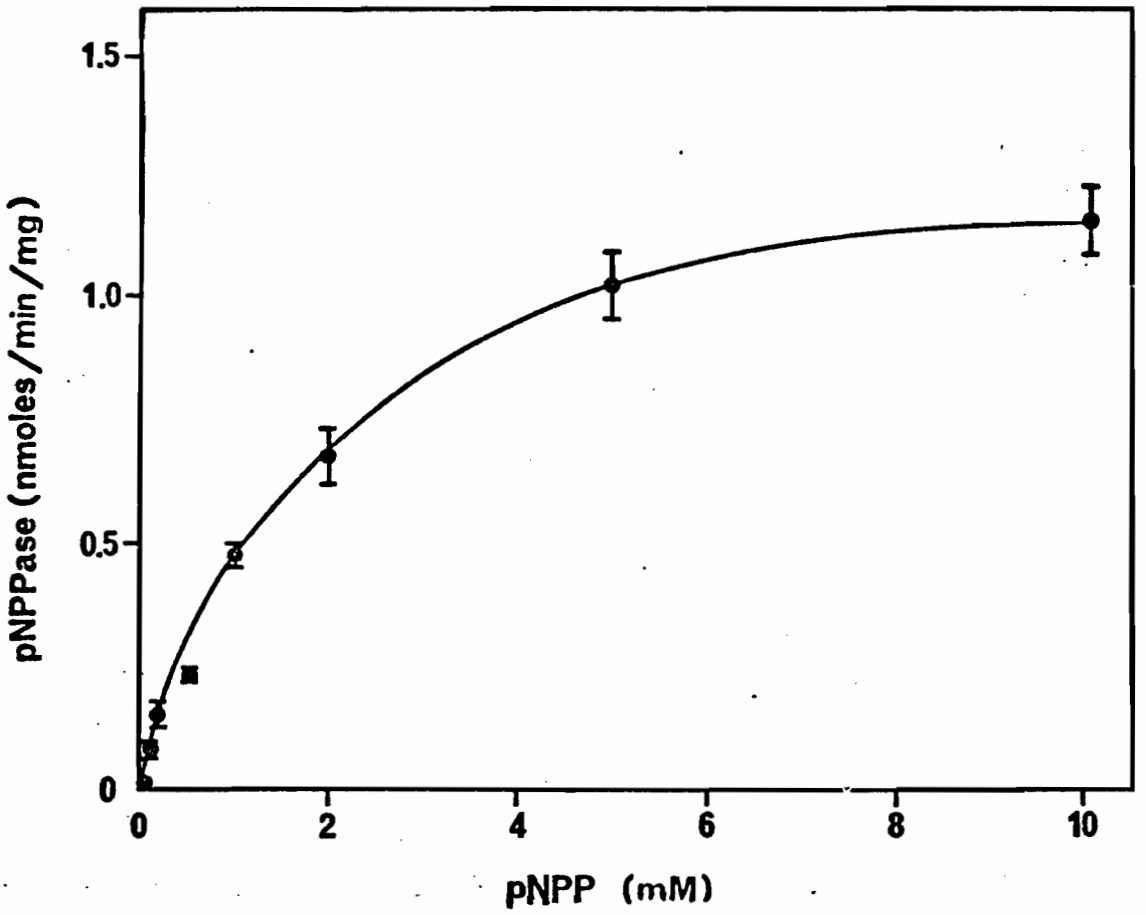

FIGURE 4: EFFECTS OF VARYING pNPP ON K^+-STIMULATED pNPPase

Vesicles are concentrated to one fifth their original volume by centrifugation and are added to an equal volume of a solution containing 8 mM $MgCl_2$ and 20 mM choline chloride to obtain final concentrations of 20 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 10 mM choline chloride. The vesicles are allowed to equilibrate overnight on ice followed by 30 minutes at 37°. One volume of vesicles is then added to 4 volumes of reaction medium in a final volume of 0.1 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 10 mM KCl, pNPP (Tris form), pH 7.4, as indicated with choline chloride present to maintain an osmolarity equivalent to that of 10 mM pNPP (Tris form), pH 7.4. The reactions are allowed to proceed for 15 minutes at 37° and then stopped with 0.2 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of KCl and presence of choline chloride (0.30±0.02, 0.36±0.01, 0.53± 0.02, 0.81±0.01, 1.13± 0.03, 1.77±0.04, and 2.49±0.05 n moles/min/mg at 0.1,0.2,0.5,1,2,5, and 10 mM pNPP, respectively). The curve is drawn by eye. Linear regression analysis of double reciprocal plots of these data were carried out and the value for $K_{0.5}$ obtained was1.2±0.1 mM (r= 0.9807).

EXPERIMENT	ADDITIONS (mM)			K^+-STIMULATED
	Na_{ext}	K_{cyt}	Val.	ACTIVITY (nmoles/min/mg)
A	0	0.5	-	0.36 ± 0.04
	0	0.5	+	0.31 ± 0.04
B	0	1.0	-	0.49 ± 0.07
	50	1.0	-	0.43 ± 0.08
	50	0	-	0.00

TABLE 2: EFFECTS OF VALINOMYCIN AND Na_{ext} ON K_{cyt}-ACTIVATED pNPPase

In Experiment A, 0.04 volumes of 0.1M $MgCl_2$ and 0.013 volumes of 1M choline chloride are added to one volume of vesicles to obtain final concentrations of 37 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 14 mM choline chloride. The vesicles are equilibrated overnight on ice followed by 20 minutes at 37°. 0.0022 volumes of 0.45 mM valinomycin in absolute ethanol is added to a fraction of the vesicles, as indicated, and all the vesicles are incubated for 10 minutes at 37°. They are then added to an equal volume of reaction medium in a final volume of 0.15 ml containing 18.5 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, KCl as indicated with choline chloride added to maintain a final chloride concentration of 17 mM, and 0.0022 volumes of 0.45 mM valinomycin in absolute ethanol, as indicated.

In Experiment B, vesicles are concentrated to one fifth their original volume by centrifugation and are added to 0.055 volumes of 1M choline chloride or NaCl, as indicated, to obtain a final concentration of 38 mM Tris glycylglycine, pH 7.4 and 50 mM choline chloride or NaCl. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. They are then washed by diluting them 19-fold with a solution of similar composition except that Na^+ was replaced by choline. Following centrifugation, the supernatant is removed and the pellet is washed without resuspension as follows: 1 ml of the same solution is added and after 2 minutes on ice, removed, and the washing is repeated three times; the vesicles are then resuspended to their original volume (after loading). The vesicles are then added to 9 volumes of reaction medium containing 40 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, KCl as indicated with choline chloride added to maintain a final chloride concentration of 38 mM, in a final volume of 0.15 ml.

Six replicates (Experiment A) or five replicates (Experiment B) are allowed to react for 5 minutes at 37° and then stopped with 0.15 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.36±0.01 and 0.23±0.00 n moles/min/mg for Experiments A and B, respectively).

As shown in Figure 5, a lag is not observed.

(iii) If the K_{ext} requirement is sufficiently small, e.g. $\leq$0.05 mM, K_{ext} may have reached its necessary level within a very short period e.g. within the first few minutes, at least with K_{cyt} = 1 mM, and a lag would not be apparent under the condition of Figure 5. Therefore, another measure of the time-course of K^+-activated phosphatase activity was carried out using the fluorescent phosphatase substrate, 3-O-methylfluorescein phosphate (313). As seen in Figure 6, the 3-O-methylfluorescein phosphatase (3-O-MFPase) activity is linear with the protein concentration in the reaction medium within the range used for these studies (up to 0.2 mg). A trace of the continuous recording of the fluorescent product 3-O-methylfluorescein is shown in Figure 7 and indicates linearity from the onset of the reaction. Furthermore, it is seen that K_{ext} alone does not activate 3-O-MFPase. However, the 3-O-MFPase does not follow Michaelis-Menten kinetics, as shown in Figure 8.

Although lack of knowledge regarding other possible kinetic differences between these two phosphatase substrates precludes conclusive interpretation of these results, the observations described above strongly argue against a requirement for K_{ext}. If pNPPase activation by K^+ is due to K_{ext} as well as K_{cyt}, less than 0.05 mM is required, i.e. there may be sufficient K_{ext} in the absence of valinomycin (see Experiment A of Table 2) due to K^+ penetration from the cytoplasmic side. This estimate of K_{ext} is based on the measured amount of alkali cation (Rb^+) that penetrates the vesicles under the conditions of the assays shown in Figure 5, Figure 7, and Table 2.

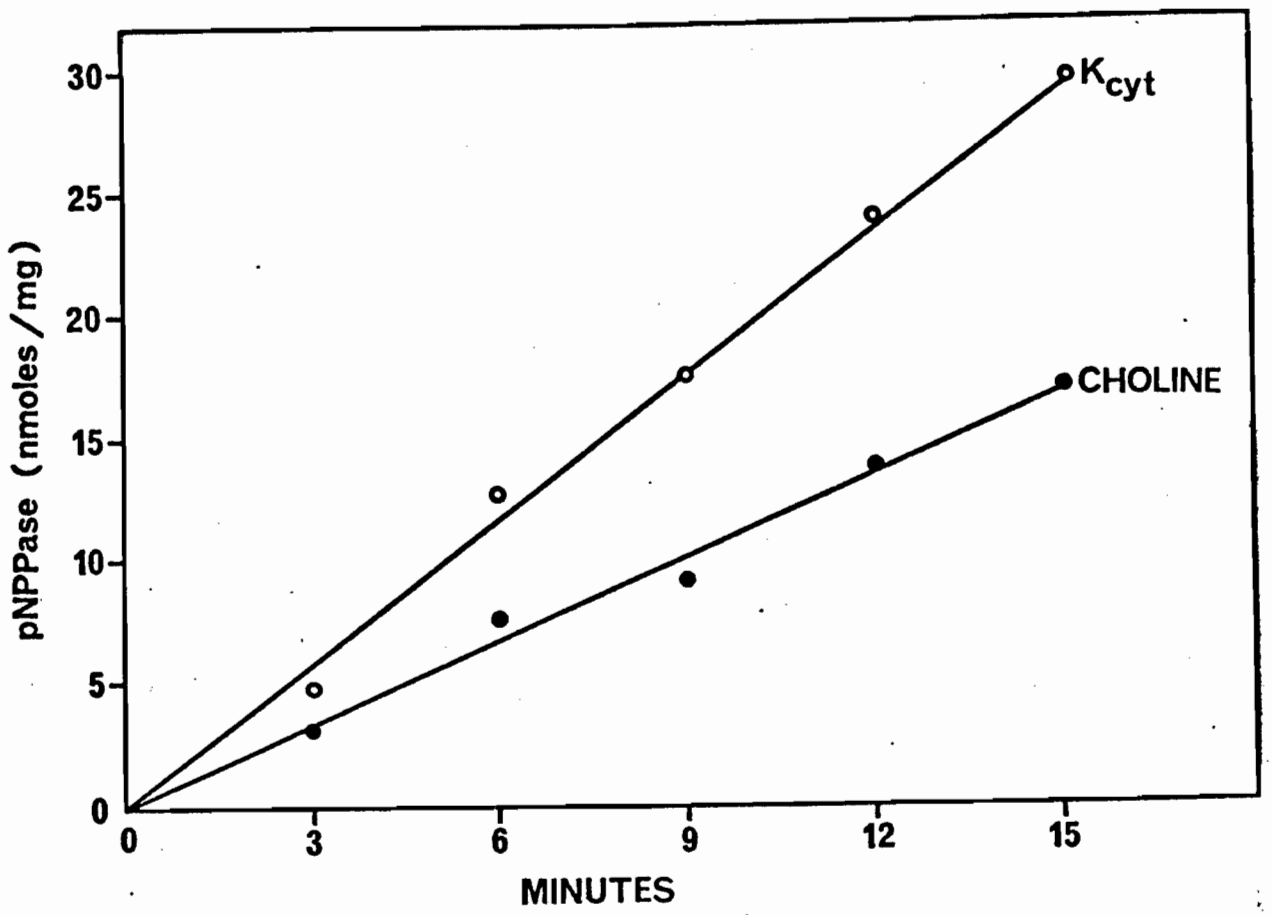

FIGURE 5: TIME-COURSE OF pNPPase

Vesicles are concentrated 10 fold by centrifugation and then added to 10 volumes of reaction medium in a final volume of 0.55 ml containing 3.6 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, and 10 mM choline chloride or KCl, as indicated. The reactions are carried out at 37° and at the times indicated, 0.1 ml samples are assayed by adding them to 0.2 ml of a quench solution (see Section 2.3). The values shown are from single determinations.

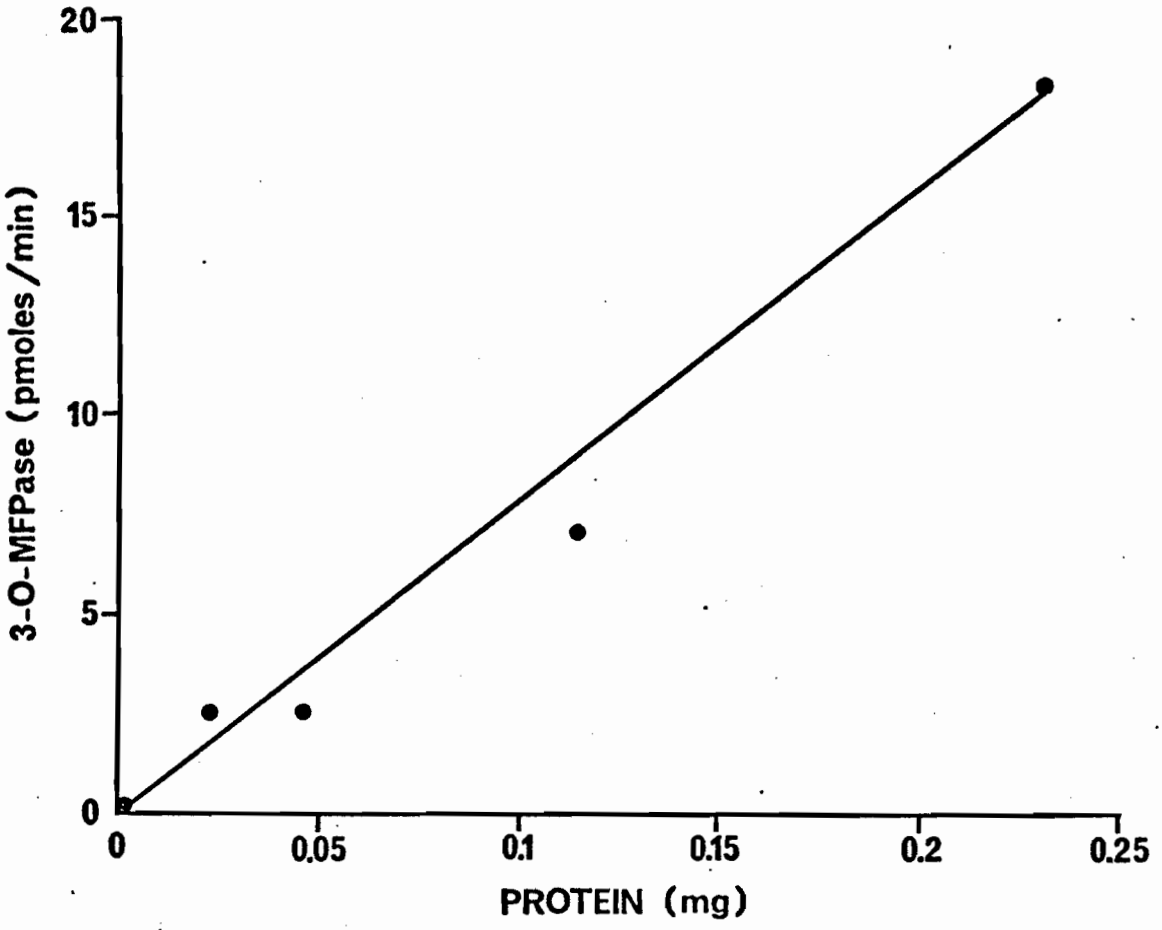

FIGURE 6: EFFECT OF PROTEIN CONCENTRATION ON K^+-STIMULATED 3-O-MFPase

Vesicles are added to 31 volumes of reaction medium in a final volume of 3.1 ml containing 40 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 μM 3-O-methylfluorescein phosphate, and 10 mM choline chloride or KCl. The assays are performed for 10 minutes at 37° as described in Section 2.4. At each protein concentration tested, the baseline activity obtained with choline chloride is subtracted from the value obtained with KCl(7.0, 9.5, 13.5, and 22.1 p moles/min at 0.023, 0.046, 0.115, and 0.230 mg protein, respectively). The values shown are the differences obtained from single determinations.

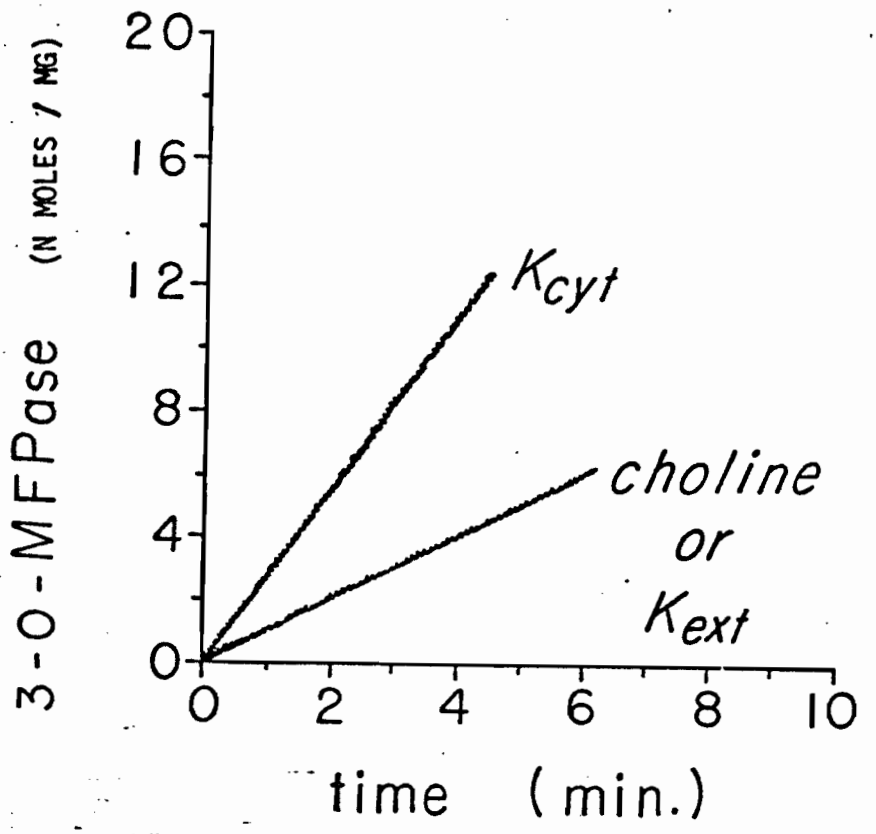

FIGURE 7: TIME-COURSE OF 3-O-MFPase

0.01 volumes of 1 M choline chloride or KCl, as indicated, are added to one volume of vesicles which are then allowed to incubate overnight on ice followed by 5 minutes at 37°. The vesicles are then added to 30 volumes of reaction medium in a final volume of 3.1 ml containing 40 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 50 μM 3-O-methylfluorescein phosphate, and 10 mM choline chloride or KCl, as indicated. The assays are performed at 37° for the time periods indicated (see Section 2.4). Traces of the original recordings are shown.

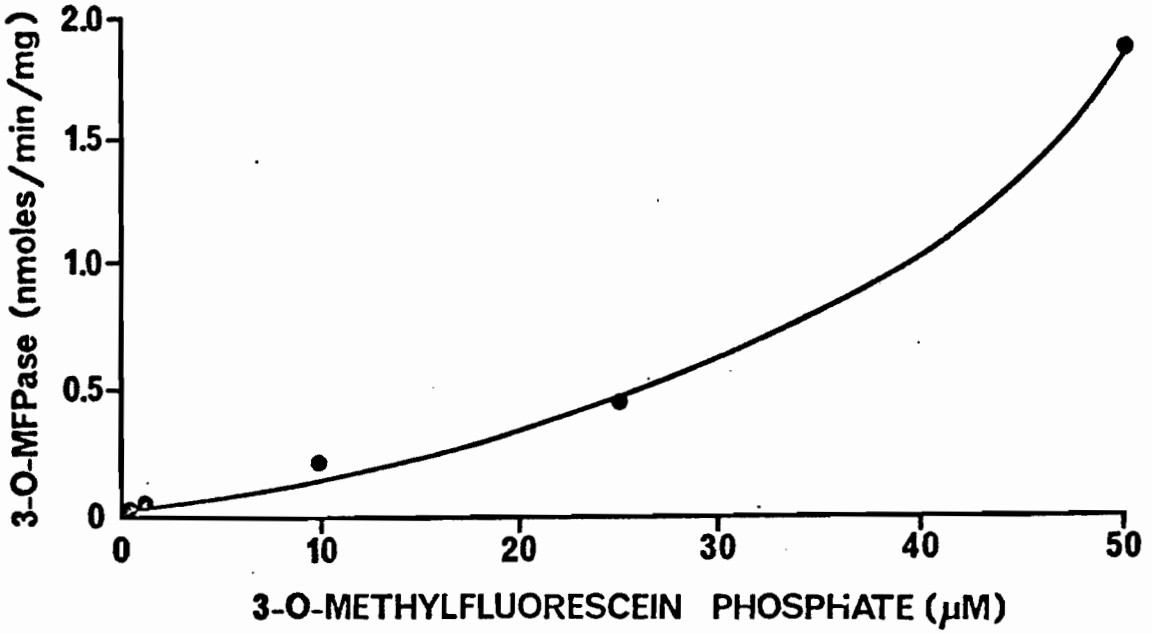

FIGURE 8: EFFECT OF 3-O-METHYLFLUORESCEIN PHOSPHATE CONCENTRATION ON 3-O-MFPase

0.01 volumes of 10 mM choline chloride are added to one volume of vesicles, and are then allowed to equilibrate overnight on ice followed by 5 minutes at 37°. The vesicles are then added to 30 volumes of a reaction medium in a final volume of 3.1 ml containing 40 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 3-O-methylfluorescein phosphate as indicated, and 10 mM choline chloride or KCl. The assays are performed for 10 minutes at 37° (see Section 2.4). The values shown are the K^+-stimulated activities, corrected for the activity observed in the absence of KCl and presence of choline chloride (0.03, 0.13, 0.26, and 1.46 n moles/min/mg at 1, 10, 25, and 50 uM 3-O-methylfluorescein phosphate, respectively). The curve is drawn by eye.

Thus, in a test of permeability of $^{86}Rb^+$ in the presence of 5 mM pNPP (see Figure 15), 10% penetrates the vesicles in 5 minutes and about 20% penetrates in 10 minutes. Penetration is reduced by 50% when pNPP is omitted.

3.2 EFFECTS OF Na^+

Na^+ at either or at both membrane surfaces, i.e. in the presence of gramicidin, does not significantly activate pNPPase activity (Experiment B, Table 2 and Experiment A,Table 3). In the presence of K^+ at either or both membrane surfaces, a significant further stimulation by Na_{cyt} could not be detected (see Experiments B and C of Table 3). In fact, Experiment B of Table 3 shows that a relatively high concentration of Na_{cyt}(50 mM) inhibits the pNPPase observed in the presence of 10 mM K_{cyt}. Although an activation by Na^+ plus K^+ at $K^+ \leqslant 1$ mM has been observed by others (259,262,265), at 1 mM K_{cyt} the K^+ activation, per se, was relatively small, the baseline activity without Na^+ or K^+ being 0.75± 0.03 n moles/min/mg in Experiment B of Table 3 (see Legend). Thus, this effect of Na^+, if present, could not be evaluated with sufficient confidence. Except for the usual stimulation by K_{cyt} and inhibition by Na_{cyt}(50 mM) mentioned above, other differences were not statistically significant at the $p \leqslant 0.05$ level (see Table 3).

3.3 EFFECTS OF ATP

As seen in Figure 9, ATP inhibits pNPPase observed in the presence of K_{cyt} alone with a $K_{0.5}$ for inhibition of about 0.1 mM ATP*. In contrast, ATP stimulates in the presence of Na_{cyt}, K_{ext}, and K_{cyt}, as shown in Figure 10. With Na^+ and K^+, maximal activation is observed at 50 uM ATP, inhibition occuring at higher ATP concentrations.

* Figure 9, estimated by eye

TABLE 3: EFFECTS OF Na^+ ON pNPPase

In Experiment A, vesicles are concentrated 4-fold by centrifugation and are added to an equal volume of a solution containing 0.134 M choline chloride and 0.1 mM $MgCl_2$ to obtain a final concentration of 20 mM Tris glycylglycine, pH 7.4, 67 mM choline chloride, and 0.1 mM $MgCl_2$. The vesicles are allowed to equilibrate overnight on ice followed by 20 minutes at 37^0. 0.01 volumes of absolute ethanol (control) and 0.01 volumes of 0.7 mg gramicidin per ml of absolute ethanol are added to one volume of vesicles and the vesicles are incubated for 10 minutes at 37^0. One volume of vesicles is then added to 4 volumes of reaction medium in a final volume of 0.15 ml containing 14 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 10 mM NaCl, as indicated, with choline chloride present to maintain a final chloride concentration of 64.4 mM, and 0.01 volumes of absolute ethanol or 0.7 mg gramicidin per ml absolute ethanol, as indicated. The reaction is allowed to proceed for 10 minutes at 37^0 and then stopped with 0.15 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.31±0.01 n moles/min/mg).

In Experiment B, vesicles are concentrated 10-fold by centrifugation and are loaded as described above. 0.0022 volumes of absolute ethanol or 0.45 mM valinomycin in absolute ethanol are added as indicated to samples of vesicles and the samples are incubated for 10 minutes at 37^0. One volume of vesicles is added to 9 volumes of a reaction medium in a final volume of 0.15 ml containing 14 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, KCl and NaCl, as indicated, with choline chloride present to maintain a final chloride concentration of 64.4 mM, and 0.0022 ml of absolute ethanol or 0.45 mM valinomycin in absolute ethanol, as indicated. The assays are performed as described above and the choline chloride baseline was 0.75±0.03 n moles/min/mg.

In Experiment C, vesicles are concentrated 5-fold by centrifugation and are added to an equal volume of a solution containing 40 mM Tris glycylglycine, pH 7.4, 8 mM $MgCl_2$, and 20 mM KCl to obtain a final concentration of 40 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 10 mM KCl. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37^0 and are washed by diluting them 19-fold with a solution of similar composition, except that K^+ was replaced by choline. After centrifugation, the supernatant is removed and the pellet is washed without resuspension as follows: 1 ml of the same solution is added and after 2 minutes on ice, removed, and the washing is repeated three times; the vesicles are then resuspended to their original volume (after loading).

One volume of vesicles is added to 4 volumes of reaction medium in a final volume of 0.1 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, NaCl, as indicated, with choline chloride present to maintain a final chloride concentration of 10 mM. The reactions are allowed to proceed for 15 minutes at 37^0 and then stopped with 0.2 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.66±0.03 n moles/min/mg).

a, b, c } Differences between these activities (total activity, choline baseline not subtracted) were not statistically significant ($p>0.05$ as determined by Student's t-test).

TABLE 3

EXPERIMENT	ADDITION (mM)					Na^+ or K^+-STIMULATED
	Na_{cyt}	K_{cyt}	K_{ext}	Val.	Gram.	ACTIVITY (nmoles/min/mg)
A	10	0	0	-	+	0.10± 0.05
	10	0	0	-	+	0.09± 0.06
B	0	1	0	-	-	0.34± 0.07
	10	1	0	-	-	0.24± 0.10[a]
	50	1	0	-	-	0.31± 0.07[b]
	0	1	(1)	+	-	0.40± 0.08
	10	1	(1)	+	-	0.52± 0.10[a]
	50	1	(1)	+	-	0.34± 0.08[b]
	0	10	0	-	-	0.72± 0.07
	10	10	0	-	-	0.69± 0.08
	50	10	0	-	-	0.23± 0.10
	10	0	0	-	-	0.17± 0.10[c]
	50	0	0	-	-	0.00 [c]
C	0	0	10	-	-	0.15± 0.06
	10	0	10	-	-	0.18± 0.06

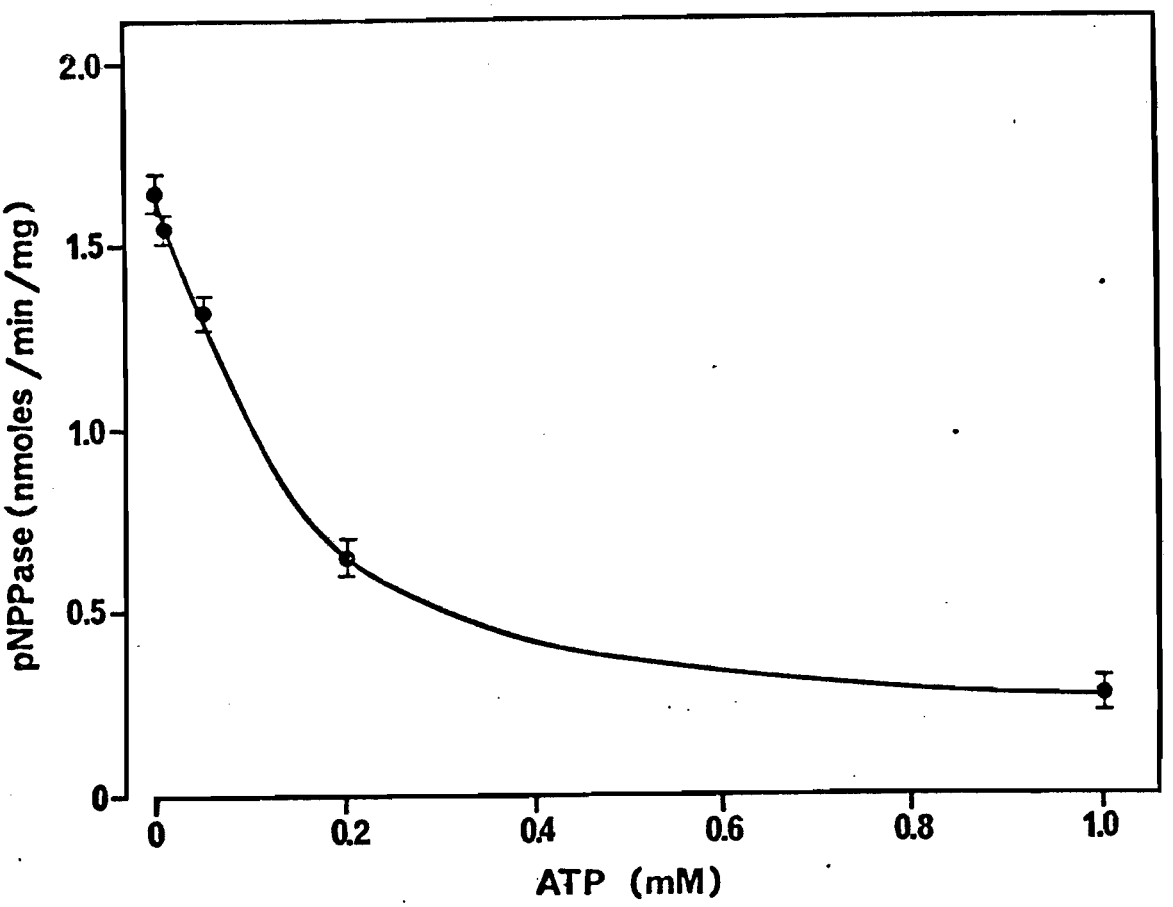

FIGURE 9: EFFECT OF ATP ON K_{cyt}-ACTIVATED pNPPase

Vesicles are concentrated 7.5-fold by centrifugation and are added to an equal volume of a solution containing 8 mM $MgCl_2$ and 20 mM choline chloride to obtain final concentrations of 20 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 10 mM choline chloride. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. One volume of vesicles is then added to 4 volumes of reaction medium in a final volume of 0.1 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 10 mM KCl, and ATP as indicated. The reactions are allowed to proceed for 15 minutes at 37° and then stopped with 0.2 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of KCl and presence of choline chloride (0.63±0.02 n moles/min/mg). The curve is drawn by eye. Preliminary experiments (not shown) ascertained that at all concentrations tested, ATP did not change the choline baseline activity.

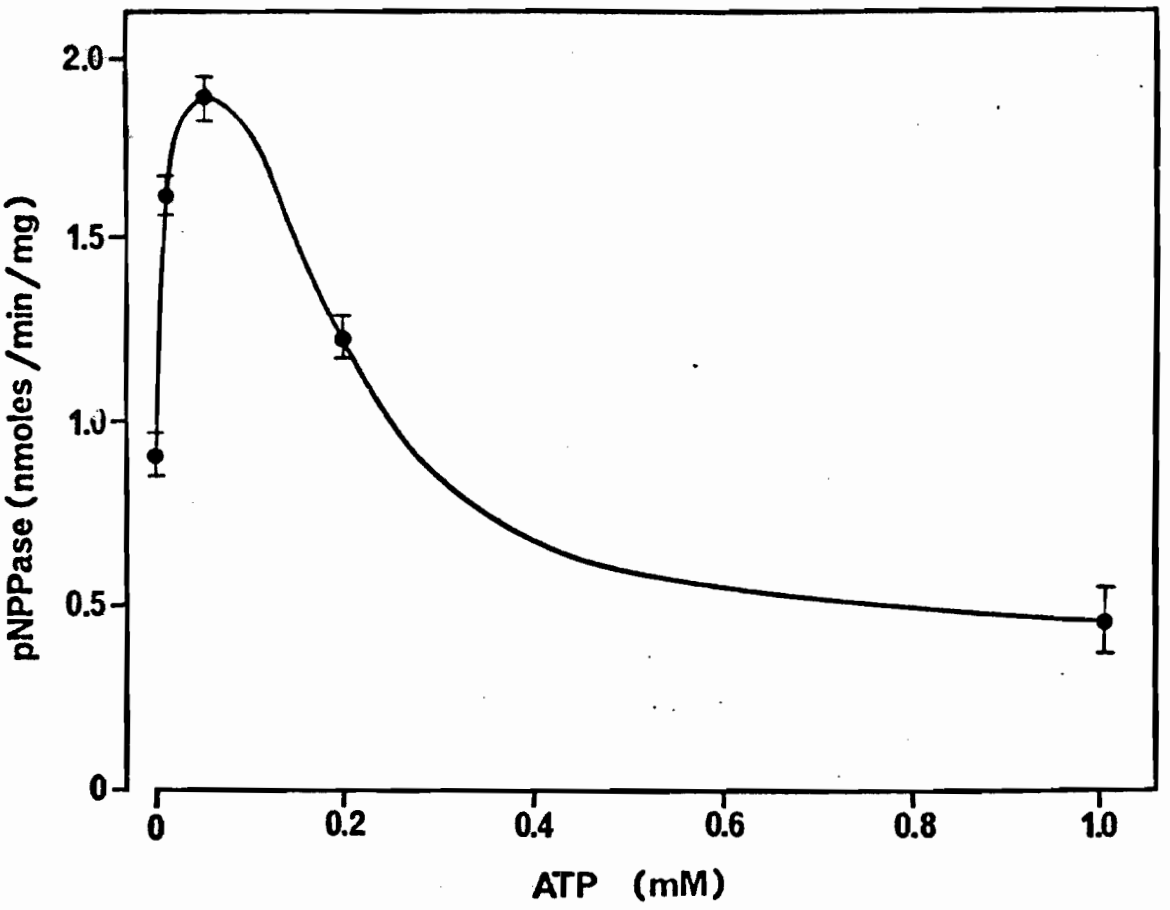

FIGURE 10: EFFECTS OF ATP ON K^+-ACTIVATED pNPPase WITH Na_{cyt}

Vesicles are concentrated 7.5-fold by centrifugation and are then added to an equal volume of a solution containing 8 mM $MgCl_2$ and 20 mM KCl to obtain a final concentration of 20 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 10 mM KCl. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. One volume of vesicles is added to 4 volumes of reaction medium in a final volume of 0.1 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 10 mM NaCl, 2 mM KCl, and ATP as indicated. The reactions were allowed to proceed for 15 minutes at 37° and stopped with 0.2 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.67±0.02 n moles/min/mg). Preliminary experiments (not shown) ascertained that at all concentrations tested, ATP did not change the choline baseline activity. The curve is drawn by eye.

In the experiment shown in Figure 10, the presence of 2 mM K_{cyt} in the presence of Na_{cyt} and K_{ext} is due to the 5-fold dilution of the vesicles equilibrated with 10 mM K^+. The question then re mained whether K_{cyt} or K_{ext} or both were required for the ATP plus Na_{cyt} activation.

With K^+-loaded, washed vesicles (Experiment A, Table 4) and Na_{cyt}, ATP was without effect. A small degree of activation with ATP, K_{cyt} and Na_{cyt} above that observed with K_{cyt} alone was apparent in some experiments, e.g. Figure 11 and Experiment C, Table 4, and is presumably due to K_{ext} i.e. K^+ which had penetrated the vesicles since, in other experiments (Experiment B, Table 4), activation could not be detected. Howerver, with K^+ at both surfaces, even at low concentrations a marked stimulation with ATP plus Na_{cyt} was detected (Experiments A and C, Table 4). The effects of varying the K_{ext} concentration in the presence of Na_{cyt} and K_{cyt} is illustrated in Figure 11 and shows that K_{ext} is ineffective unless 10 uM ATP is added, in which case almost maximal activity is observed with 0.2 mM K_{ext}. Similarly, Figure 12 shows that Na_{cyt} has no effect in the presence of K_{cyt} and K_{ext} unless 10 uM ATP is added, in which case as much as a doubling in activity occurs and almost maximal activity is observed with 1 mM Na_{cyt}.

It is of particular interest to note that whereas valinomycin addition fails to increase K^+-activation with 0.2 mM K_{cyt} added alone, marked stimulation is observed when ATP and Na_{cyt} are added as well (Experiment C, Table 4).

3.4 EFFECT OF Pi

The effect of increasing Pi concentrations on the pNPPase in the presence of 10 mM K_{cyt} is shown in Figure 13.

EXPERIMENTS	ADDITIONS (mM)					Na^+ and/or K^+-STIMULATED ACTIVITY (nmoles/min/mg)
	ATP	Na_{cyt}	K_{cyt}	K_{ext}	Val.	
A	0	10	0	0.5	-	0.00
	0	10	0.5	0.5	-	0.20 ± 0.06
	0.01	10	0	0.5	-	0.07 ± 0.05
	0.01	10	0.5	0.5	-	0.50 ± 0.06
B	-	10	1.0	0	-	0.24 ± 0.10
	0.01	10	1.0	0	-	0.32 ± 0.07
C	0	10	0.2	0	-	0.46 ± 0.07
	0	10	0.2	(0.2)	+	0.56 ± 0.07
	0.01	10	0.2	0	-	0.74 ± 0.07
	0.01	10	0.2	(0.2)	+	1.53 ± 0.10

TABLE 4: EFFECTS OF K^+ ON pNPPase WITH Na_{cyt} AND ATP

In Experiment A, vesicles are concentrated 5-fold by centrifugation and are then added to an equal volume of a solution containing 40 mM Tris glycylglycine, pH 7.4, 8 mM $MgCl_2$, 1 mM KCl, and 19 mM choline chloride to obtain a final concentration of 40 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 0.5 mM KCl, and 9.5 mM choline chloride. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37° and are then diluted 19-fold with a solution of similar composition, except that K^+ was replaced by choline, and centrifuged. The supernatant is removed and the pellet is washed without resuspension as follows: 1 ml of the same solution is added and after 2 minutes on ice, removed, and the washing is repeated three times; the vesicles are then resuspended to their original volume (after loading). One volume of vesicles is then added to 4 volumes of a reaction medium in a final volume of 0.1 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP(Tris form), pH 7.4, 10 mM NaCl, 0.5 mM KCL, as indicated, and 10 μM ATP, as indicated. The reactions are allowed to proceed for 15 minutes at 37° and then stopped with 0.2 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.66±0.03 n moles/min/mg).

In Experiment B, the vesicles are concentrated 10-fold by centrifugation and are added to an equal volume of a solution containing 20 mM choline chloride and 0.1 mM $MgCl_2$ to obtain a final concentration of 20 mM Tris glycylglycine, pH 7.4, 10 mM choline chloride, and 0.1 mM $MgCl_2$. The vesicles are allowed to equilibrate overnight on ice followed by 30 minutes at 37°. One volume of vesicles is then addded to 4 volumes of reaction medium in a final volume of 0.15 ml containing 14 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 1 mM KCl, 10 mM NaCl or choline chloride, as indicated, and 10 μM ATP, as indicated. The assays are performed as described above. The values are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.75±0.03 n moles/min/mg).

In Experiment C, vesicles are concentrated 4-fold and 0.0022 volumes of absolute ethanol or 0.45 mM valinomycin in absolute ethanol are added to samples of vesicles and the samples are incubated for 10 minutes at 37°. One volume of vesicles is added to 9 volumes of reaction medium in a final volume of 0.15 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 10 mM NaCl, 0.2 mM KCl, 10 μM ATP, as indicated, and 0.0022 volumes of absolute ethanol or 0.45 mM valinomycin in absolute ethanol, as indicated. The reactions were allowed to proceed for 15 minutes at 37° and stopped with 0.15 ml of a quench solution (see Section 2.3). The values are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.82±0.04 n moles/min/mg). ATP (10 μM) alone had no effect on the choline baseline activity (preliminary experiments, not shown).

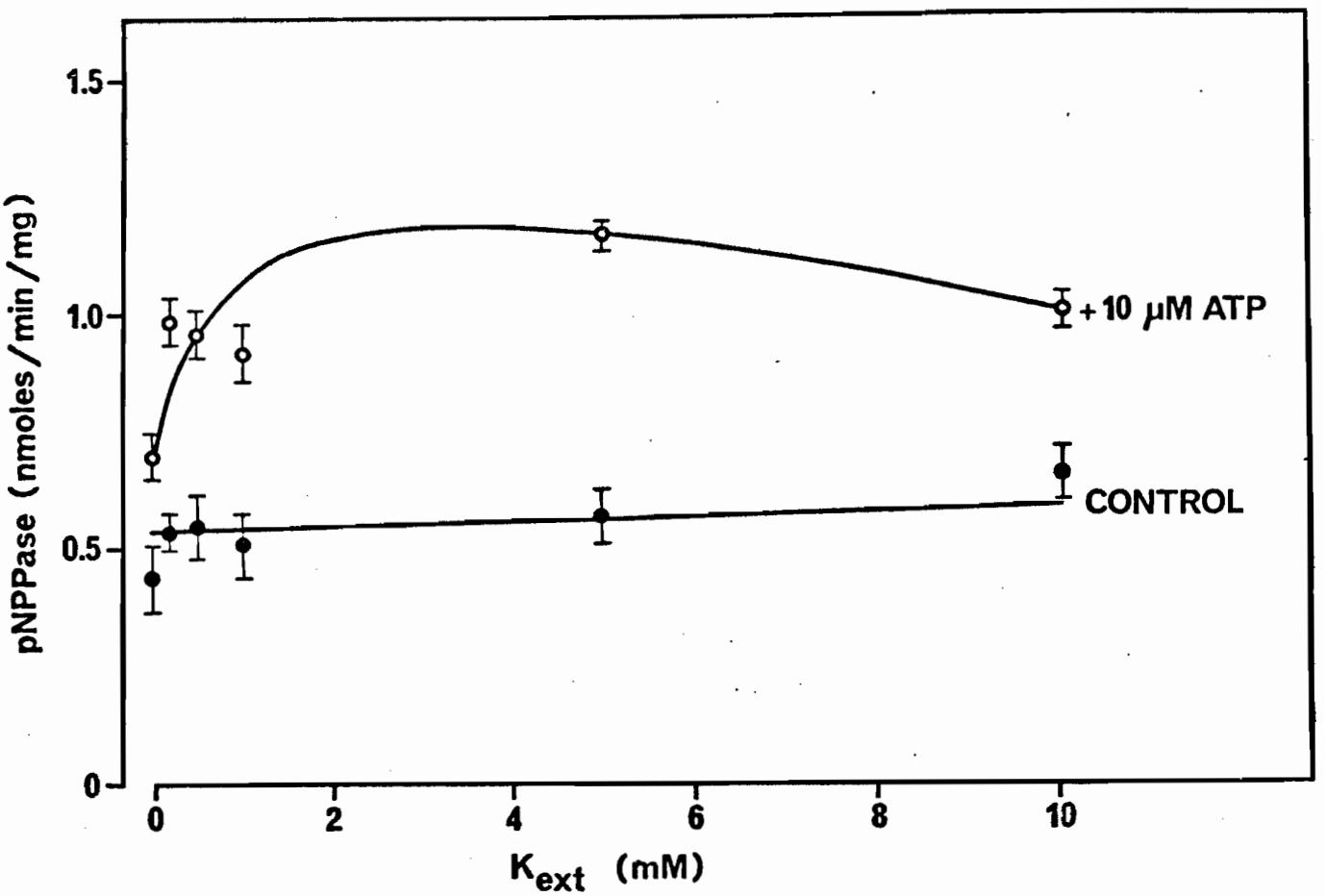

FIGURE 11: EFFECT OF VARYING K_{ext} ON pNPPase WITH K_{cyt}, Na_{cyt} AND ATP

Vesicles are concentrated 5-fold by centrifugation and are added to an equal volume of a solution containing 8 mM $MgCl_2$ and KCl with choline chloride present to maintain the total chloride concentration at 20 mM so that final concentrations of 20 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and KCl, as indicated, are obtained with choline chloride present to maintain the total chloride concentration at 10 mM. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of reaction medium in a final volume of 0.15 ml containing 18 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 1mM KCl, 10 mM NaCl, and 10 μM ATP as indicated. The reactions are allowed to proceed for 15 minutes at 37° and then stopped with 0.15 ml of a quench solution (see Section 2.3). The values are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.67±0.02 n moles/min/mg). ATP (10 μM) alone had no effect on the choline baseline activity (preliminary experiments, not shown). The curves are drawn by eye.

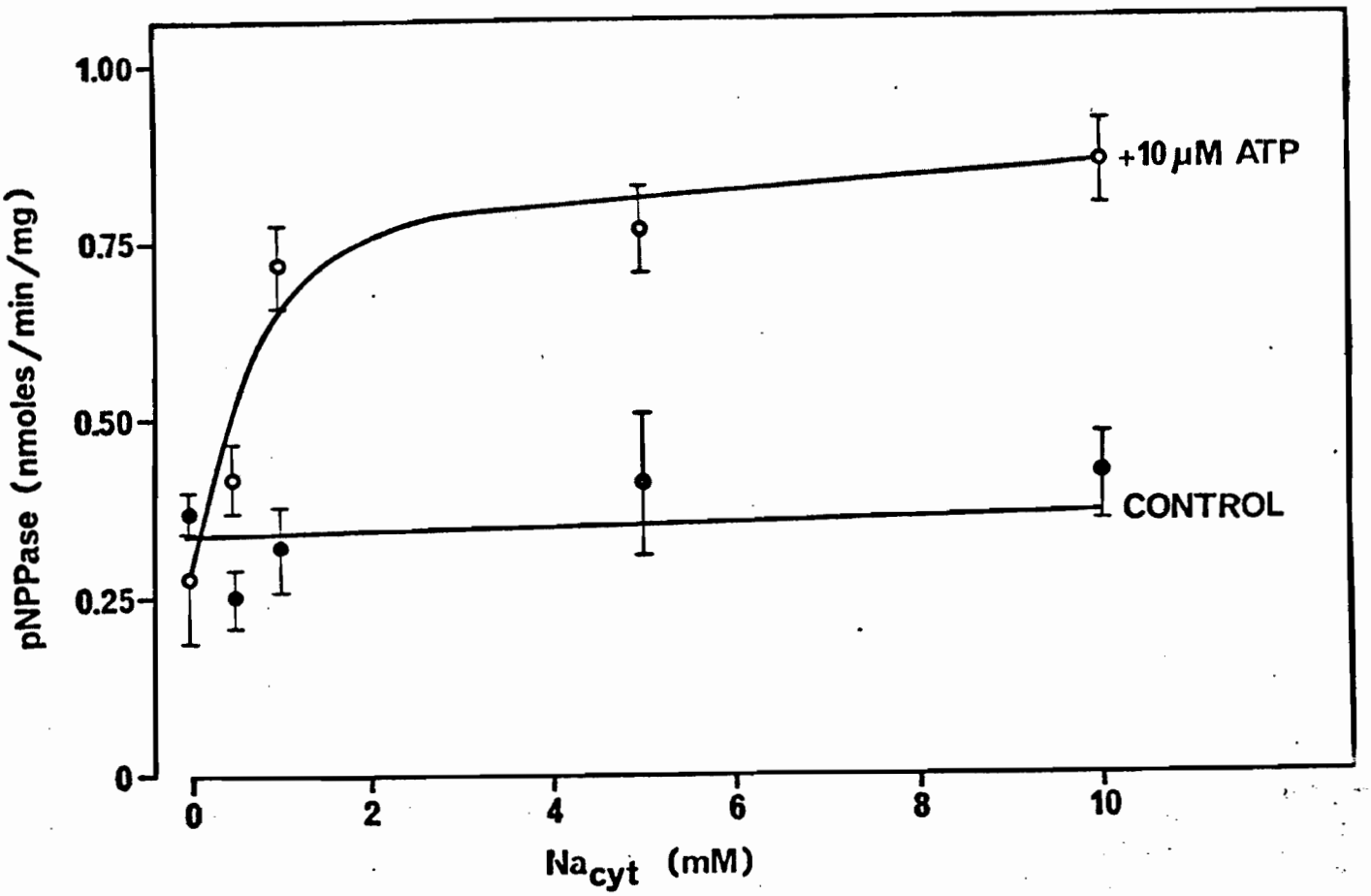

FIGURE 12: EFFECT OF VARYING Na_{cyt} ON pNPPase WITH K^+ AND ATP

Vesicles are concentrated 5-fold by centrifugation and added to 0.1 volumes of a solution containing 44 mM $MgCl_2$ and 110 mM KCl to obtain a final concentration of 36 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 10 mM KCl. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of reaction medium in a final volume of 0.1 ml containing 36 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 1 mM KCl, NaCl as indicated, with choline chloride present to maintain the final chloride concentration at 10 mM, and 10 μM ATP as indicated. The reactions are allowed to proceed for 15 minutes at 37° and then stopped with 0.2 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of NaCl or KCl and presence of choline chloride (0.63±0.02 n moles/min/mg). ATP (10 μM) alone had no effect on the choline baseline activity (preliminary experiments, not shown). The curves are drawn by eye.

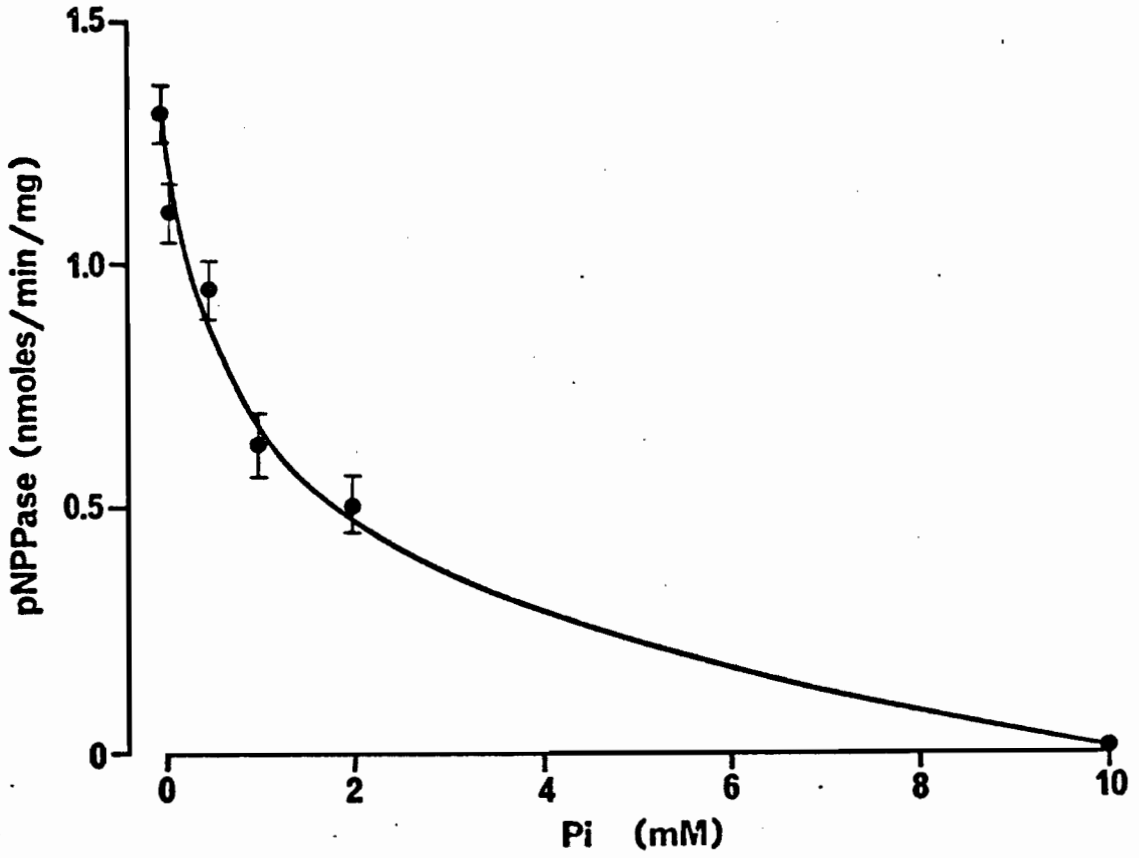

FIGURE 13: EFFECT OF Pi ON K_{cyt}-ACTIVATED pNPPase

Vesicles are concentrated 10-fold by centrifugation and are added to an equal volume of a solution containing 40 mM Tris glycylglycine, pH 7.4, 14 mM choline chloride, and 0.1 mM $MgCl_2$ to obtain final concentrations of 40 mM Tris glycylglycine, pH 7.4, 7 mM choline chloride, and 0.1 mM $MgCl_2$. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of reaction medium in a final volume of 0.15 ml containing 4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP (Tris form), pH 7.4, 10 mM KCl, and Tris PO_4, pH 7.4, as indicated, to maintain a final Tris concentration of 10 mM. The reactions are allowed to proceed for 10 minutes at 37° and then stopped with 0.15 ml of a quench solution (see Section 2.3). The values shown are corrected for the activity observed in the absence of KCl and presence of choline chloride (1.32±0.03 n moles/min/mg). In other experiments, 10 mM Pi did not change the choline baseline activity (not shown). The curve is drawn by eye.

A marked inhibition with a $K_{0.5}$ of about 1 mM* is observed, similar to the competitive inhibition of the pNPPase by Pi described previously (262, 314).

3.5 EFFECT OF pNPP ON $^{86}Rb^+$ UPTAKE

Although no effect of pNPP on ouabain-sensitive $^{86}Rb^+$ uptake and $^{22}Na^+$ efflux in ATP-depleted resealed ghosts was observed by Garrahan and Rega (315), my observation that K_{cyt} is crucial to the pNPPase activity in inside-out vesicles under these conditions would suggest that a pNPP-dependent K^+ (Rb^+) uptake (equivalent to K^+ efflux from ghosts) might be present.

Since $^{86}Rb^+$ is a more stable isotope than $^{42}K^+$, I examined the effects of Rb^+ on the pNPPase in order to determine whether it would be a suitable congener for K^+ influx studies. Figure 14 shows the effects of increasing Rb_{cyt} concentrations on the pNPPase and indicates a $K_{0.5}$ of about 2 mM with a maximal velocity at 10 mM that is only 20% lower than that observed with 10 mM K_{cyt} and thus demonstrates that Rb^+ is indeed a suitable congener of K^+.

Figure 15 shows the effects of pNPP in the presence and absence of 25 μM strophanthidin on $^{86}Rb^+$ uptake into the vesicles. In the absence of pNPP, a strophanthidin-insensitive uptake of $^{86}Rb^+$ is observed and, presumably, represents the passive leak of $^{86}Rb^+$ into the vesicles. Addition of pNPP increases this $^{86}Rb^+$ uptake, consistent with the previous observation of a pNPP-induced increase in passive cation permeability (315).

* Figure 13, estimated by eye

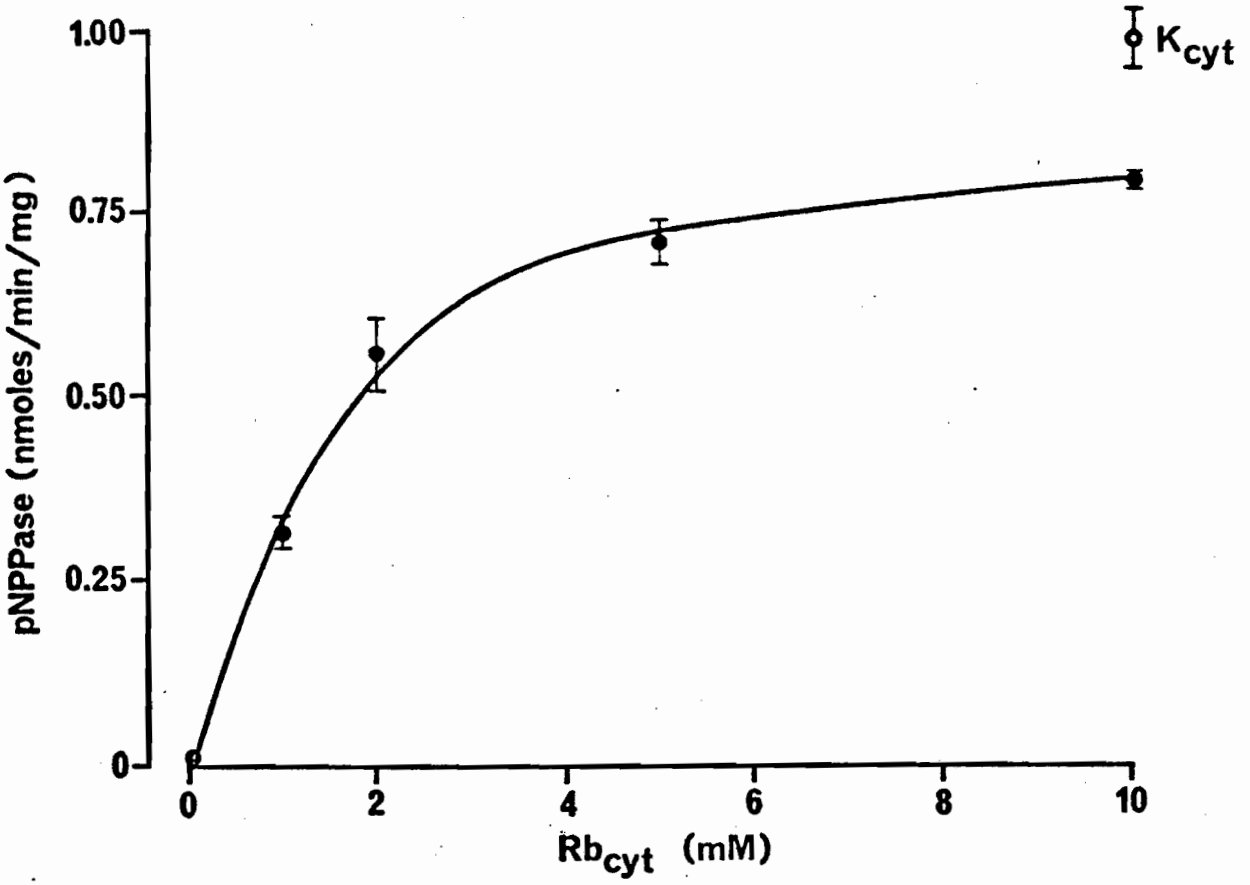

FIGURE 14: EFFECT OF VARYING Rb_{cyt} ON pNPPase

Vesicles are concentrated 5-fold by centrifugation and are added to 0.1 volumes of a solution containing 44 mM $MgCl_2$ and 110 mM choline chloride to obtain final concentrations of 36 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, and 10 mM choline chloride. The vesicles are equilibrated overnight on ice followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing 21.4 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 5 mM pNPP, pH 7.4, and RbCl or KCl as indicated, with choline chloride present to maintain the final chloride concentration at 10 mM. The reactions are allowed to proceed for 10 minutes at 37° and then stopped with 0.2 ml of a quench solution (see Section 2.3). The values are corrected for the activity observed in the absence of RbCl or KCl and presence of choline chloride (1.18±0.01 n moles/min/mg). The curve is drawn by eye. Linear regression analysis of double reciprocal plots of these data were carried out and the value for the $K_{0.5}$ obtained was 2.2±0.2 mM (r= 0.9651).

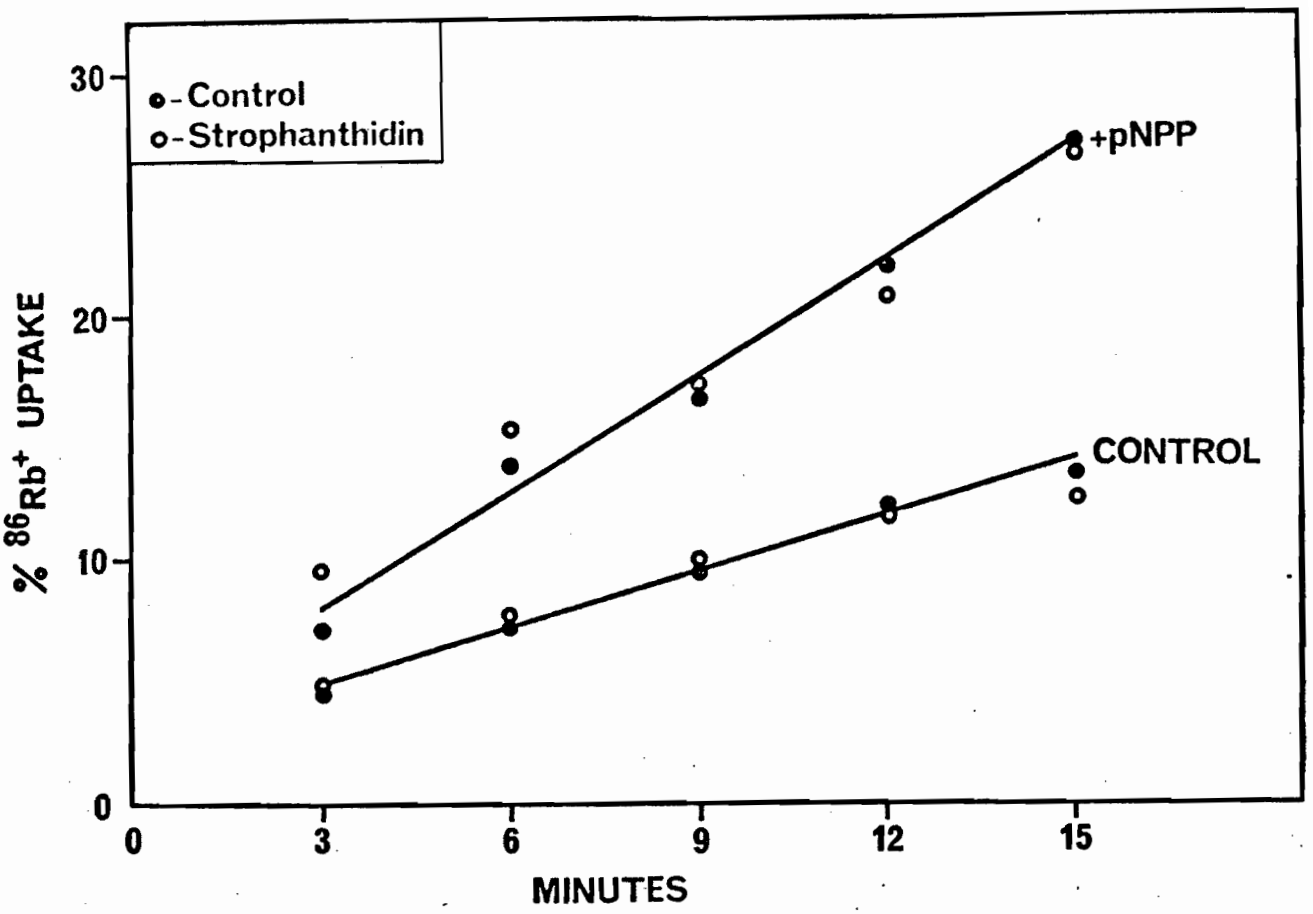

FIGURE 15: EFFECT OF pNPP ON $^{86}Rb^+$ UPTAKE

Vesicles are concentrated 5-fold by centrifugation and are added to an equal volume of a solution containing 36 mM choline chloride and 8 mM $MgCl_2$ to obtain final concentrations of 20 mM Tris glycylglycine, pH 7.4, 18 mM choline chloride, and 4 mM $MgCl_2$. The vesicles are equilibrated overnight on ice followed by 20 minutes at 27°. 0.005 volumes of absolute ethanol or 5 mM strophanthidin in absolute ethanol are added to samples of vesicles, as indicated, and the vesicles are incubated for 10 minutes at 37°. One volume of vesicles is then added to 4 volumes of a reaction medium in a final volume of 0.35 ml containing 11.8 mM Tris glycylglycine, pH 7.4, 4 mM $MgCl_2$, 1 mM $^{86}RbCl$ (3 μCi/ml), 21.4 mM choline chloride, 5 mM pNPP (Tris form), pH 7.4, as indicated, and 0.005 volumes of absolute ethanol or 5 mM strophanthidin in absolute ethanol as indicated, at 37°. Single 0.035 ml samples are taken at the times indicated and the $^{86}Rb^+$ uptake is measured as described in Section 2.7. After 15 minutes, 0.0022 volumes of 0.45 mM valinomycin are added, the $^{86}Rb^+$ space is measured as above, and the results are presented as a percentage of this space (≃ 15 μl/mg).

A strophanthidin-sensitive, pNPP-dependent $^{86}Rb^{+}$ transport could not be detected. The magnitude of the pNPP-dependent $^{86}Rb^{+}$ uptake that could have been observed, if it were present, can be predicted from the pNPPase activity under these conditions if one assumes the stoichiometry for $^{86}Rb^{+}$ transport is at least one Rb^{+} transported per pNPP molecule hydrolyzed. Thus, with a pNPPase of 0.3 n mole min^{-1} mg^{-1} at 1 mM Rb_{cyt} (see Figure 14), 0.3 n mole $^{86}Rb^{+}$ min^{-1} mg^{-1} would have been pumped into the vesicles and would have been readily detected.

4. DISCUSSION

The results of this study show that a cardiac glycoside-sensitive K^+ activated phosphatase activity is observed in the presence of K_{cyt} but not in the presence of K_{ext} alone. A requirement for K_{ext} in the presence of K_{cyt} could not be demonstrated under a variety of conditions, i.e. by i) adding valinomycin to equilibrate K^+ on both sides of the membrane; ii) by pre-equilibrating vesicles with 50 mM Na^+ to allow Na_{ext} competition with K^+ that may penetrate the vesicles when assayed with 1 mM K_{cyt}; iii) by measuring the time-course of pNPPase, or iv) the time-course of 3-O-MFPase. In these latter two cases, it was assumed that a lag would be observed until enough K^+ penetrates the vesicles to activate the phosphatase. A requirement for K_{ext} less than about 0.05 mM in addition to K_{cyt} cannot be excluded. This is the amount of K_{ext} estimated to penetrate during the 5 minute assays at 0.5 mM added K_{cyt} (see page 50), the lowest amount of K_{cyt} causing a measurable stimulation of pNPPase. Since a significant further increase in activity in the presence of valinomycin was not detected, it is concluded, that an amount of K_{ext} greater than about 0.05 mM is not required.

The conclusion that K_{cyt} alone activates pNPPase in red cells conflicts with an earlier study in which the authors concluded that K^+ activates pNPPase at the external surface of resealed red cell ghosts (286).

These ghosts contained some Na^+ and probably low levels of ATP as well; the consequences of the presence of these ligands on the sidedness of K^+ interactions with the enzyme system will be discussed below.

The K_{cyt} activation has a $K_{0.5}$ of about 2 mM which is similar to that observed in studies with other tissues (222, 267, 276, 317). K^+ sites with a similar "moderate" apparent affinity have been observed during beryllium and fluoride inactivation of the (Na^+, K^+)-ATPase in the absence of enzyme phosphorylation (222, 223) and by measuring the dissociation constant for Rb^+ binding (318). These sites are different from the high affinity K^+ sites involved in activation of the (Na^+, K^+)-ATPase and the sodium pump, which are presumably on the external surface. This lends support to a role of "moderate" affinity K^+ sites distinct from the high affinity external catalytic sites, These cytoplasmic sites do not appear to be the K^+ discharge sites of the sodium pump, which have a $K_{0.5}$ of 0.3 M (141) or 0.14 M (223). They are similar, though not identical, to the Na_{cyt} activation sites for the sodium pump, which have a Km for Na_{cyt} of 0.19 mM (128, 129) and a $K_{0.5}$ for K_{cyt} inhibition in the presence of Na^+ of 9 mM (128, 130). Although the $K_{0.5}$ for K_{cyt} for K^+-K^+ exchange is higher (Km = 10 mM) (155), it should be noted that in the exchange studies ATP is present since it is required in a non-phosphorylating role (157). Thus, it may be that ATP decreased K_{cyt} binding under this condition. The final identification of the cytoplasmic sites for the phosphatase with a class of cytoplasmic sites for the (Na^+, K^+)-ATPase depends on further examination of the effects of the various ligands on cation binding at each membrane surface.

Na^+ at either or both membrane surfaces does not significantly activate pNPPase. Albers and Koval (265) have shown that about 3% of the maximal K^+-activated pNPPase in electric organ preparations is observed in the presence of Na^+ alone. Such a low level of activity is beyond the sensitivity of the assays used here in which case a significant increase in activity by Na_{cyt} (10 mM) in the absence or presence of K^+ at either or both membrane surfaces is not observed. Na_{cyt} (50 mM) inhibits the pNPPase in the presence of 10 mM K_{cyt} and is similar to observations by others (273, 318) of competitive Na^+ inhibition at high K^+ concentrations. Na^+ has been reported to activate pNPPase in the presence of $K^+ \leq 1$ mM (259, 263, 265). The level of activation expected is, however, too low to be evaluated with sufficient confidence in the present study.

ATP inhibits the K_{cyt}-activated pNPPase with a $K_{0.5}$ for ATP inhibition of about 0.1 mM. Both competitive (266, 268, 270) and non-competitive (260, 270) inhibition by ATP have been reported and suggest that ATP binding to a low affinity site(s) is competitive with K^+ and non-competitive with pNPP. In the presence of Na_{cyt} and K^+ at both surfaces, low ATP concentrations (up to 50 μM) stimulate pNPPase, with a high apparent affinity for Na_{cyt} and an even higher apparent affinity for K_{ext}, whereas higher concentrations of ATP inhibit pNPPase. Similar biphasic effects of ATP have been reported by others (270,276). As mentioned above, a K_{ext} requirement for pNPPase previously observed in red cell ghosts (286) may be due to the presence of contaminating Na_{cyt} and probably micromolar concentrations of ATP.

These data can be accounted for, in part, by the model shown in Figure 16. Accordingly, K_{cyt} binds to the enzyme and forms an E·K complex that hydrolyzes pNPP. K^+ binding to the enzyme has been described previously and is believed to involve the steps shown in equation 5 (204):

$$E + K^+ \rightleftharpoons E_1 \cdot K \rightleftharpoons E_2 \cdot K \qquad (5)$$

where $E_2 \cdot K$ is an occluded complex as demonstrated by Post et al (202) and which they postulated to be responsible for pNPP hydrolysis. ATP would inhibit pNPPase by binding to its low affinity site on the (Na^+, K^+)-ATPase (18, 162, 215-217), consistent with the $K_{0.5}$ for ATP inhibition of 0.1 mM, by displacing K^+ from the enzyme ($E_2 \cdot K$) as it does with the (Na^+, K^+)-ATPase (202, 204-207). An E·ATP complex would be formed and is consistent with competitive inhibition of K^+ by ATP (266, 268, 270). Observations of non-competitive inhibition by ATP (260, 270) suggest that ATP may also combine with a different enzyme form than does pNPP, and the two forms are reversibly connected.

At micromolar concentrations of ATP, Na_{cyt} can phosphorylate the enzyme and thus expose the high apparent affinity K_{ext} sites (168,170); K_{ext} binding to E_2-P would produce the $E_2 \cdot K$ complex (202). Thus, pNPPase would be sequential to ATPase and would allow $E_2 \cdot K$ formation via the forward sequence of steps in the (Na^+, K^+)-ATPase reaction cycle (see Figure 2, p. 26). Higher concentrations of ATP would inhibit the pNPPase by promoting K^+ release from $E_2 \cdot K$ by acting at its low apparent affinity site(s) and/or by competing with pNPP, as described above.

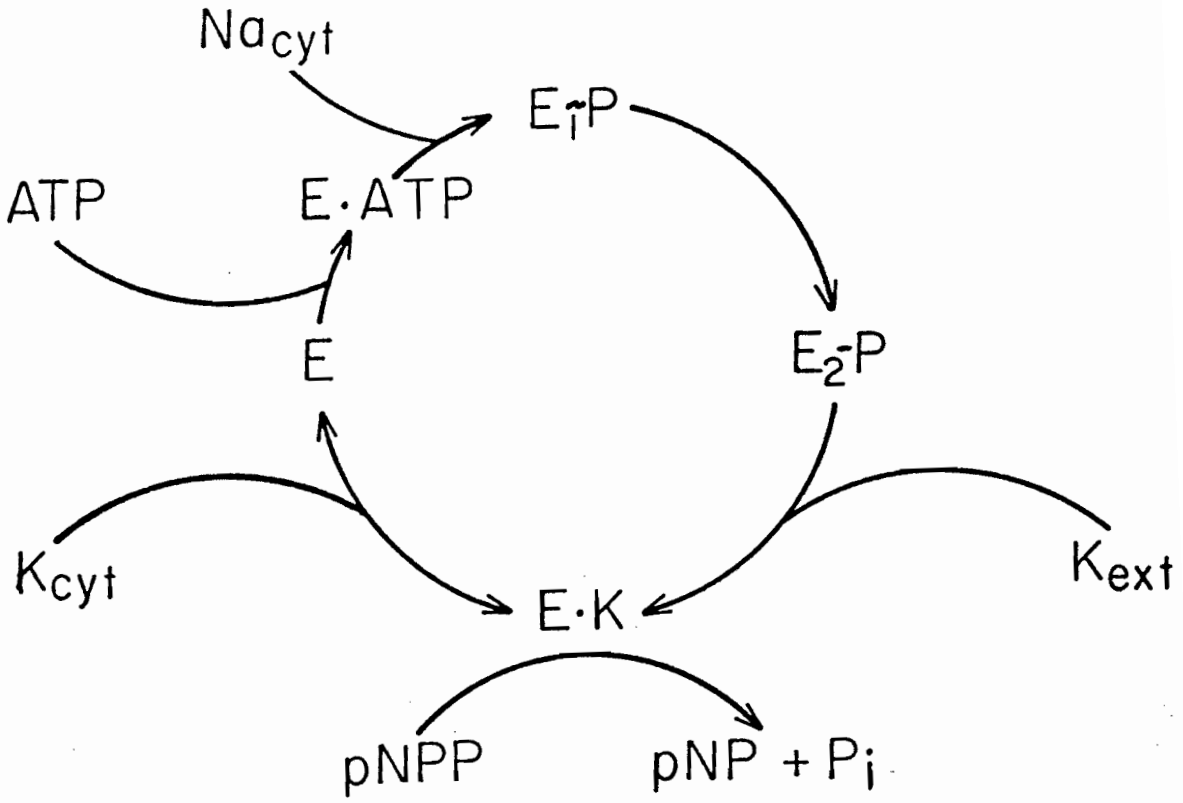

FIGURE 16: MODEL FOR THE pNPPase

(See text for explanation)

This model is similar, though more detailed, to one proposed by Skou (259) in which he postulated K^+ and Na^+-plus-K^+ forms of the phosphatase. Albers and Koval (265), who used dimethylsulfoxide to increase the Na^+-activated pNPPase activity, suggested that the phosphatase had high affinity catalytic sites for K^+ but required Na^+ or K^+ occupation of separate low affinity regulatory sites as well. Occupancy of the regulatory sites "unmasks" the catalytic sites, particularly in the presence of Na^+ and ATP. Their low affinity regulatory sites may be related to the sites at which K_{cyt} or Na_{cyt} (plus ATP) are observed to activate pNPPase in the present study.

Assuming that $K_{0.5}$ is a measure of the affinity of the enzyme for K^+, the high affinity K^+ catalytic sites described by Albers and Koval (265) and by Robinson (221,223) may be related to the high apparent affinity for K_{ext} observed in the presence of Na_{cyt} and ATP, although such high apparent affinity K_{ext} sites, if present with K_{cyt} alone, must be of much higher apparent affinity than those observed in the presence of Na_{cyt} plus ATP, i.e. maximal activity by Na_{cyt} plus ATP, with K_{cyt} present, was not observed unless the vesicles contained K_{ext} (see Experiment C , Table 4). Thus, with Na_{cyt} and ATP, the K_{ext} present due to penetration of K_{cyt} was not sufficient to give maximal activation, which is in contrast to the results obtained with K_{cyt} alone. It should be noted that dimethylsulfoxide decreases the $K_{0.5}$ for K^+ (319) and its importance to the interpretation of the Albers and Koval model is unknown.

If $E_2 \cdot K$ can also be formed by K_{cyt} binding via the sequence $E_1 + K^+ \leftrightarrows E_1 \cdot K \leftrightarrows E_2 \cdot K$, then K^+ efflux from cells (or K^+ uptake by vesicles) might be expected. Since a strophanthidin-sensitive pNPP-dependent uptake of the congener $^{86}Rb^+$ into vesicles is not observed, then either $E_2 \cdot K$ is not formed upon K_{cyt} binding, which is difficult to reconcile with other results in the present study as well as the study of Karlish et al (204,205), or K^+ remains occluded i.e. is not readily released at the external surface. The high apparent affinity for K^+ at catalytic sites as observed in some kinetic studies of others (as described above) may be due to K_{ext} required during pNPP hydrolysis in a manner ($E_2 + K^+ \longleftrightarrow E_2 \cdot K$) that differs from its requirement during ATP hydrolysis ($E_2\text{-}P + K^+ \leftrightarrows E_2 \cdot K + Pi$). Thus, K_{ext} may be required, under certain circumstances, to regenerate $E_2 \cdot K$ from E_2.

The K_{cyt} requirement for pNPPase activation in the presence of Na_{cyt}, K_{ext} and ATP may be related to the observation that low levels of K_{cyt} stimulate the sodium pump (113,122,130,148) and Na^+-Na^+ exchange (122), although the nature of this effect remains to be elucidated.

Robinson has suggested that phosphorylation (to form E_2-P) is required to expose the high affinity K^+ sites on the (Na^+,K^+)-ATPase since these are only observed during beryllium or fluoride inactivation of the enzyme in the presence of Na_{cyt} plus ATP or phosphatase substrates (221,223).

pNPP phosphorylates a serine at high acidity (pH 5.0) after long periods of exposure (281) or in the presence of ouabain (282), but its specificity is questionable. Furthermore, lack of a p-nitrophenol-ATP or pNPP-ADP exchange (279, 282, 284) and a lower water oxygen incorporation during pNPP hydrolysis than during the hydrolysis of acetyl phosphate and ATP (283) suggest that phosphorylation is not occurring or that the phosphoenzyme intermediate during pNPP hydrolysis differs significantly from E-P formed with other substrates. A different form of E-P that is unable to translocate K^+ might account for the lack of pNPP-dependent $^{86}Rb^+$ transport described here and by others (315) (and might also account for the low reactivity in the presence of Na^+ alone (265)). It must be stressed, however, that further studies of the relationships between the mechanisms of pNPP and of ATP hydrolysis are required before a new form of phosphoenzyme becomes plausible.

In the presence of only K^+, radiation inactivation results suggest that only half of the (Na^+, K^+)-ATPase system is required for the phosphatase activity (64). Since studies of the structure of the (Na^+, K^+)-ATPase suggest a dimeric enzyme (52, 67, 68 and ref. 25), Na_{cyt} and/or ATP may be responsible for coupling the dimers e.g. ATP binding to its regulatory site(s). This coupling action may be related to the suggestion of half-of-the-sites reactivity (251-253) according to which each half of the dimer proceeds through the entire reaction cycle chemically, mechanically, and energetically coupled in a "flip-flop" mechanism (254), 180° out of phase with the other half.

Accordingly, uncoupled dimers might express only phosphatase activity and the coupled enzyme (in the presence of Na_{cyt} and/or ATP) would express ATPase activity. An uncoupling of subunits during SDS treatment has also been suggested to increase the phosphatase/ATPase activity ratio, although this observation may not be related to a physiological effect of subunit interactions (54).

5. Na^+-ATPase OF HK AND LK SHEEP RED CELL MEMBRANE INSIDE-OUT VESICLES

Studies of ion transport in HK and LK sheep red cells have shown differences not only in the rate of pumping (288) and number of pump sites between these two types of sheep (290-292), but also a difference in apparent affinity for K^+ at the external activation sites (296) and most noticeably at the cytoplasmic surface, where K^+ is much more inhibitory in LK than in HK (131, 295). These differences have been partly related to differences in the kinetics of the (Na^+, K^+)-ATPase activity in broken membranes (see ref. 303), particularly with regards to the inhibitory effects of K^+ in LK membranes at very low ($\lesssim 0.2$ μM) ATP concentrations. Under this condition, the Na^+-ATPase of HK sheep is activated by $K^+ \lesssim 5$ mM but the LK Na^+-ATPase is strongly inhibited by K^+ (294, 302, 303).

Although one study of the (Na^+, K^+)-ATPase of fragmented goat red cell membranes included a correction for incomplete occupation of external K^+ sites and the authors concluded that K_{cyt} inhibition is much greater in LK than in HK membranes (296), information regarding the sidedness of the interactions of K^+ and the effects of ATP on the enzyme is lacking. Since direct observations of these interactions should yield useful information regarding not only the interactions of K^+ with the enzyme but also the molecular basis of the sheep dimorphism, inside-out vesicles were prepared from HK and LK red cells by a modification of the method described for human red cells (306).

With this system, the concentrations of ligands can be controlled at both membrane surfaces (see ref. 168 and 170) and the vesicles are relatively free of contaminating cytoplasmic enzymes which could interfere with the low (micromolar) ATP levels required for these studies.

5.1 GENERAL PROPERTIES OF THE VESICLES

The sealing and orientation of the vesicles are assessed as follows: The sensitivity of the Na^+-ATPase activity to ouabain inhibition is assayed since sealed inside-out vesicles should have their ouabain binding sites, originally at the external surface, inaccessible to ouabain binding and inhibition (11). As can be seen from the data in Table 5, both HK and LK Na^+-ATPase are about 90% insensitive to ouabain (2×10^{-5} M). In contrast, ouabain added prior to vesiculation completely inhibited the Na^+-ATPase and LK ghosts are almost completely inhibited by ouabain present in the reaction medium. These data suggest that about 90% of the external ouabain binding sites are at the inner surface of the vesicles and thus inaccessible to ouabain in the reaction medium, at least under the conditions of relatively low ouabain concentrations and short reaction periods.

The susceptibility of sialic acid groups, present only at the originally external membrane surface (316), to cleavage by neuraminidase is assayed and the data are presented in Table 6.

SHEEP TYPE	Na^+-ATPase (p moles min^{-1} mg^{-1}) CONTROL	+0.02 mM OUABAIN	% CONTROL
HK	45.8	36.6	82
	40.6	34.8	86
	40.5	37.2 *0.35	92
MEAN ±S.E.M.:	42.3±1.7	36.5±0.9	87±3
LK	2.8	2.5	89
	3.9(2.4)	4.1(0)	105(0)
	3.3	3.0	91
	1.4(0.79)	1.0(0.07)	72(9)
	2.4	2.5	104
	1.4	1.2	86
MEAN ±S.E.M.:	2.5±0.4	2.4±0.5	91±5

TABLE 5: EFFECT OF OUABAIN ON HK AND LK Na^+-ATPase

Vesicles (approximaly 1 mg protein/ml) are concentrated to one-tenth or one-fifth their original volume and then added to 9 or 19 volumes of reaction medium, respectively, in a final volume of 0.1 ml containing 20 mM NaCl, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 0.2 uM [γ-^{32}P] ATP. 0.02 mM ouabain is added as indicated. Assays of Na^+-ATPase are carried out for 2 minutes at 37°. The reaction is terminated and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6.and the values are corrected for the activity observed in the absence of NaCl and presence of KCl. These values were (from top to bottom) 6.5,8.6 and 3.9 p moles/min/mg for HK and 0.4,1.1,1.5,0.6, 0.8, and 0.2 p moles/min/mg for LK; the values for the ghosts (parentheses) were 1.9 and 0.7 p moles/min/mg (see below).

In the HK assay indicated by an asterisk, the vesicles are exposed to 0.02 mM ouabain present both in the assay medium and during the vesiculation step, i.e. ouabain is present at the originally external membrane surface, and an activity of 0.35 p moles/min/mg is observed.

Values in parentheses are for ghosts (taken before vesiculation) from the same preparation as the vesicles and assayed as described above. The data in each row are from separate

SIALIC ACID CONTENT (n moles mg^{-1})

SHEEP TYPE		TOTAL	NEURAMINIDASE-SENSITIVE	%INSENSITIVE
HK	Vesicles:	90.7	9.1	90
		49.5	10.1	80
	Ghosts:	79.0	76.6	3
LK	Vesicles:	125.3	8.0	94
		135.9	27.1	80
		46.5	5.9	87
	Ghosts:	102.6	99.7	3

TABLE 6: SIALIC ACID DISTRIBUTION IN HK AND LK INSIDE-OUT VESICLES

For the measurement of total sialic acid, ghosts and vesicles (approximately 1 mg protein/ml) are diluted 2.5-fold and are then heated at 80° for 1 hour in a final volume of 0.25 ml containing 0.2N H_2SO_4. Neuraminidase-sensitive sialic acid is measured following incubation at 37° for 1 hour in a final volume of 0.25 ml containing 0.2M Tris-acetate (pH 5.7) and 5 µg neuraminidase. The measurement of sialic acid released by these treatments is carried out as described in Section 2.5. The data in each row are from separate experiments.

Only 10% of the total sialic acid content of both HK and LK vesicles is removed by neuraminidase in the reaction medium, whereas 97% is removed from ghosts, suggesting that the vesicles are about 90% inside-out.

The suitability of the vesicles for studies of Na^+-ATPase activity was then tested. As seen in Figure 17, the HK Na^+-ATPase activity, which is the more active of the two types of sheep red cells, is linear over the range of protein concentration tested and used for these studies (up to 0.5 mg). Figure 18 shows that ATP hydrolysis by the HK Na^+-ATPase is also linear for up to 10 minutes at 37°, the longest incubation used in these assays.

The time course of $^{86}Rb^+$ uptake into the vesicles is shown in Figure 19. Uptake is expressed as μl $^{86}Rb^+$ space per mg membrane protein. Thus, the total intravesicular space is that estimated at 100% equilibration; this was measured by allowing $^{86}Rb^+$ to equilibrate in the presence of valinomycin (10^{-6} M). The results indicate that the $^{86}Rb^+$ content of the vesicles after overnight incubation on ice is about 55% of the equilibrated level indicated in the legend to Figure 19. A somewhat greater extent of $^{86}Rb^+$ loading (70%-90%, not shown) was observed in subsequent studies when the overnight incubation on ice was followed by 30 minutes at 37°. The time-course of $^{22}Na^+$ uptake into the vesicles in the presence of K^+ is shown in Figure 20.

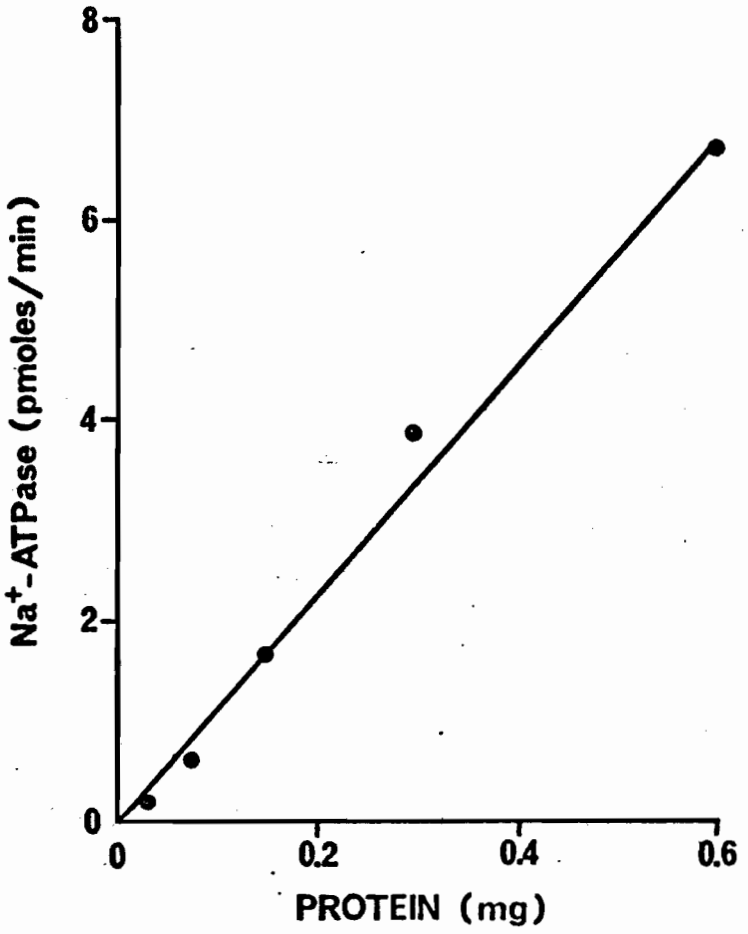

FIGURE 17: EFFECT OF VARYING THE PROTEIN CONCENTRATION ON HK Na^+-ATPase

Vesicles are concentrated 10-fold by centrifugation. Samples diluted 2-, 4-, 8- and 20-fold with a solution containing 40 mM Tris glycylglycine, pH 7.4, and 0.1 mM $MgCl_2$ and an undiluted sample of the same composition as the solution described above are then added to 1.5 volumes of reaction medium containing 16 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, 20 mM NaCl, and 0.2 μM [γ-^{32}P] ATP. The protein concentration of each sample is indicated. The reactions are allowed to proceed for 2 minutes or 4 minutes (for the 20-fold diluted vesicles) at 37°. The reaction is terminated and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for the activity measured in the absence of NaCl and presence of KCl. These values are 0.0,1.9,0.6,0.2,and 0.4 p moles/min at 0.03, 0.07, 0.15, 0.30, and 0.60 mg, respectively.

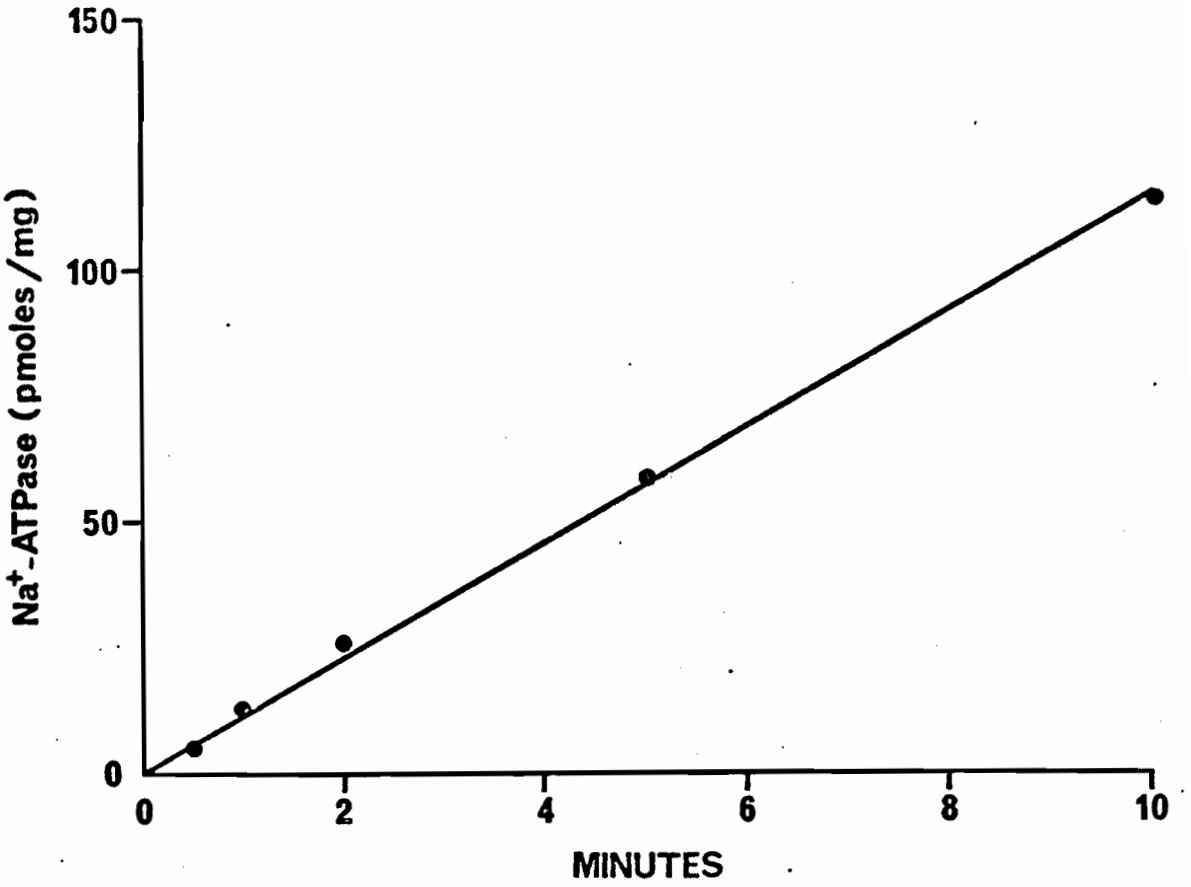

FIGURE 18: TIME-COURSE OF ATP HYDROLYSIS BY HK Na^+-ATPase

Vesicles are concentrated 2.5-fold by centrifugation and are then added to 1.5 volumes of reaction medium containing 16 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, 20 mM NaCl, and 0.2 uM [γ-^{32}P] ATP. The reactions are allowed to proceed at 37^0 for the time periods indicated. The reactions are terminated and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for the activity observed in the absence of NaCl and presence of KCl. These values are 0.0,0.0,0.0,1.5, and 4.7 p moles/mg at 0.5,1,2.5, and 10 minutes, respectively.

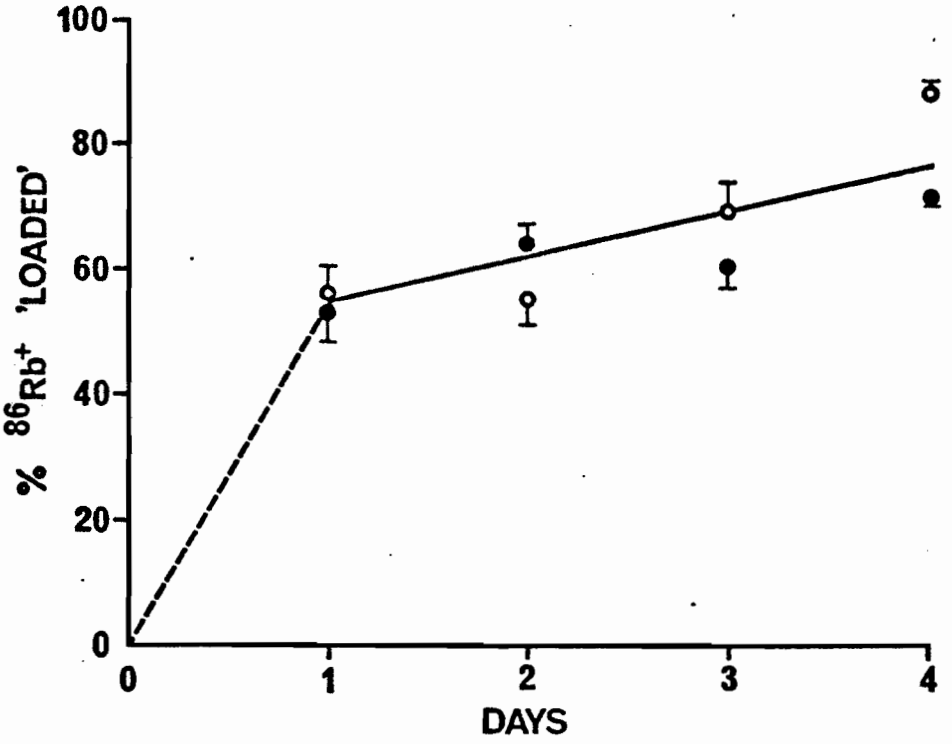

FIGURE 19: TIME-COURSE OF $^{86}Rb^+$ LOADING OF HK AND LK VESICLES

HK or LK vesicles are added to an equal volume of a solution containing 78 mM NaCl, 2.2 mM $MgCl_2$, and 1 mM $^{86}RbCl$ (5.5 μCi/ml) to obtain a final concentration of 20 mM Tris glycylglycine, pH 7.4, 39 mM NaCl, 1.1 mM $MgCl_2$, and 0.5 mM RbCl. The vesicles are incubated on ice and triplicate 0.05 ml samples are then taken at the times indicated and $^{86}Rb^+$ uptake is measured as described in Section 2.7. After the last samples are taken, 0.0022 volumes of 0.45 mM valinomycin in absolute ethanol are added and after 2 minutes at 37°, duplicate samples are assayed (intravesicular space = 100% $^{86}Rb^+$ equilibrated). The values are expressed as percentages of the total space, which is 12.4 μl/mg for HK (open circles) and 14.6 μl/mg for LK (closed circles) vesicles.

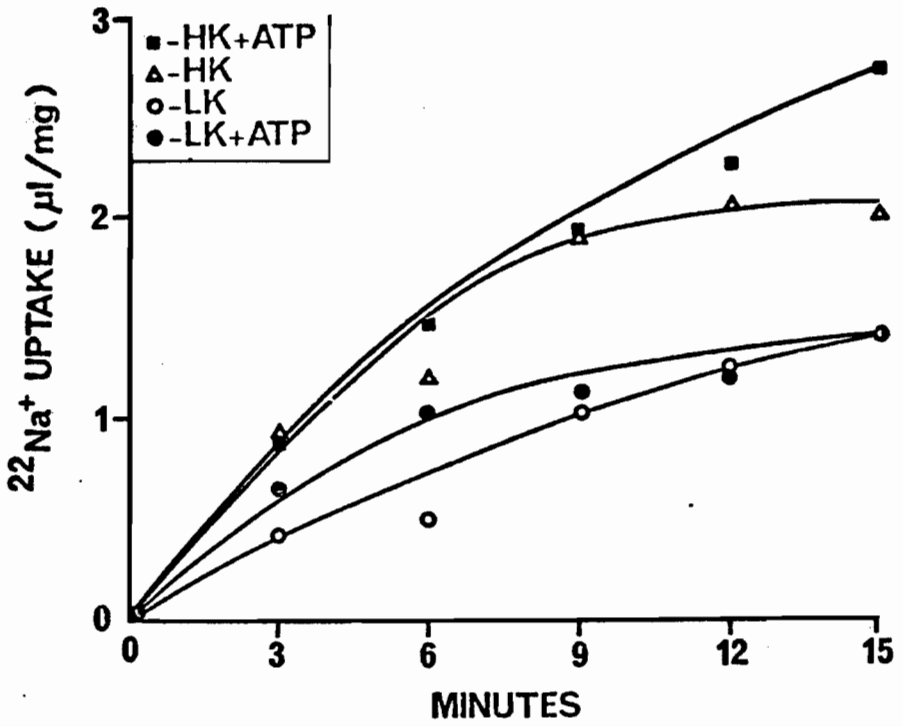

FIGURE 20: $^{22}Na^+$ UPTAKE INTO HK AND LK VESICLES IN THE PRESENCE OF K^+

0.04 volumes of 0.1M choline chloride and 0.002 volumes of 1M $MgCl_2$ are added to one volume of HK or LK vesicles to obtain a final concentration of 40 mM Tris glycylglycine, pH 7.4, 4 mM choline chloride, and 2 mM $MgCl_2$. The vesicles are allowed to equilibrate overnight on ice followed by 30 minutes at 37°. The vesicles are then concentrated 10-fold by centrifugation and then added to 9 volumes of reaction medium containing 40 mM Tris glycylglycine, pH 7.4, 2 mM $MgCl_2$, 1 mM KCl, 5 mM $^{22}NaCl$ (6.4 µCi/ml), and 1 mM ATP as indicated. The vesicles are incubated at 37° and single 0.05 ml samples are taken at the times indicated and $^{22}Na^+$ uptake is measured as described in Section 2.7. The values are corrected for the $^{22}Na^+$ uptake measured after 10 seconds (about 0.6 µl/mg). The curves are drawn by eye. To convert ul/mg to n moles/mg, multiply by 5 (1 ul= 5 n moles).

Since the total intravesicular volume, taken as the $^{86}Rb^+$ space measured in the presence of valinomycin, is about 15 ul/mg (see Figure 19), up to about 10% penetration of cations ($^{22}Na^+$) is observed during the first 10 minutes at 37°. Addition of 1 mM ATP had no significant effect on $^{22}Na^+$ uptake into LK vesicles, probably because the ATP-driven sodium pump activity in these vesicles is too low to be observed. Addition of 1 mM ATP increased the $^{22}Na^+$ uptake into HK vesicles to a small extent in most experiments if valinomycin was present, as has been observed in studies with human red cell membrane inside-out vesicles (170). In that study, it was concluded that valinomycin is probably required to maintain sufficient intravesicular K^+ (K_{ext}).

5.2 EFFECTS OF Na^+ ON Na^+-ATPase

In Figure 21, Na^+-ATPase activity is assayed for short periods at 37° at 0.02 and 0.2 μM ATP using varying amounts of Na_{cyt} (up to 40 mM). The "control" values in Figure 21 are the activities observed at the highest Na_{cyt} concentration tested. Baseline activities are measured without Na^+ and with K^+ added to counteract effects of any residual and/or tightly bound Na^+; these baseline values are subtracted. At these low ATP concentrations, Na_{cyt} alone, even at 1mM, significantly stimulates ATPase activity.

The response of the LK enzyme to Na_{cyt} is different from that of HK over the range of Na_{cyt} concentrations tested, the apparent affinity being clearly lower for LK.

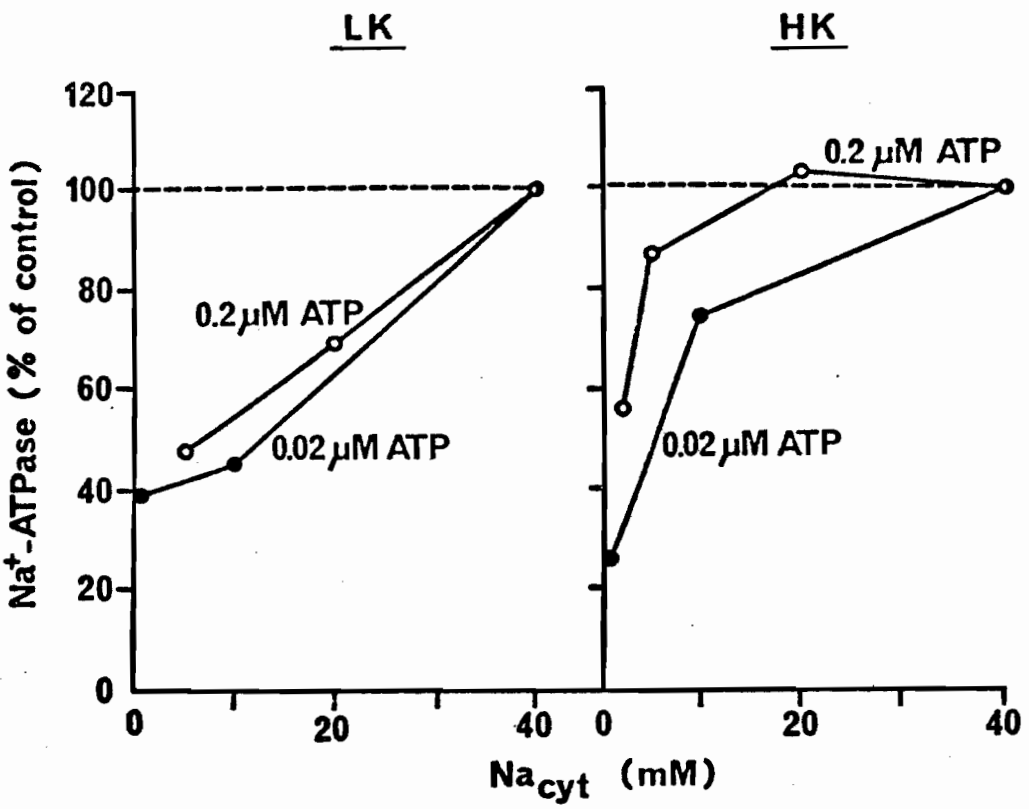

FIGURE 21: EFFECT OF Na_{cyt} ON HK AND LK Na^+-ATPase

Vesicles are concentrated to one-tenth their original volume by centrifugation and are added to 9 volumes of a solution containing 45 mM choline chloride, and 0.1 mM $MgCl_2$ to obtain a final concentration of 20 mM Tris glycylglycine, pH 7.4, 40 mM choline chloride, and 0.1 mM $MgCl_2$. The vesicles are centrifuged again and then allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing varying amounts of NaCl, as indicated, plus choline chloride so that the final chloride concentration is 40 mM, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$ and 0.02 or 0.2 μM [γ-^{32}P] ATP. The reactions are carried out at 37° for 2-5 minutes. The reactions are terminated and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for thebaseline activityin the absence of NaCl and presence of 40 mM KCl. These activities are (p moles/min/mg) 1.93 (100% control) and 0.29 (KCl baseline) for LK at 0.02 μM ATP, 11.3 (100% control) and 2.8 (KCl baseline) for LK at 0.2 μM ATP, 7.8 (100% control) and 0.5 (KCl baseline) for HK at 0.02 μM ATP, and 68.3 (100% control) and 6.8 (KCl baseline) for HK at 0.2 μM ATP. The values are expressed as the % control activity observed at 40 mM Na_{cyt} (control).

This is apparent at both ATP concentrations tested. The considerable level of activity observed in the absence of added Na^+ (40% of the activity observed at 40 mM Na_{cyt}) is likely due to traces of tightly bound Na^+.

Na^+-ATPase activity of HK vesicles tends to reach maximal activity above about 5 mM Na_{cyt}, particularly at 0.2 μM ATP. Increasing the ATP concentration decreases the level of Na_{cyt} required for maximal activation. With 40 mM Na_{cyt}, intravesicular Na^+ (Na^+ at the originally external surface, Na_{ext}) further increases the activity (Table 7). This is more apparent in LK compared to HK vesicles.

5.3 EFFECTS OF K^+ ON Na^+-ATPase

Na^+-ATPase activity is assayed as a function of KCl in the media (K^+ at the originally cytoplasmic surface, K_{cyt}) or as a function of intravesicular K^+ (K_{ext}). Activities are assayed at 0.02, 0.2, and 2.0 μM ATP since earlier studies showed that the K^+ response profile, at least in HK, is a function of ATP concentration (302). As shown in Figures 22-24, the activity of LK vesicles is inhibited by K^+ at either surface. This inhibition is apparent at all ATP concentrations tested, although K_{cyt} inhibition is always greater than that observed with K_{ext}. In one set of tubes in the experiment at 0.2 μM ATP (Figure 23), 1 μM valinomycin is included with the KCl in the reaction medium, so that K^+ has access to both surfaces of the membrane.

% ACTIVITY

SHEEP TYPE	[ATP]	MEAN ±S.D.	n	p
HK	0.02 μM	105 ± 5	6	$0.1 < p < 0.05$
	0.2 μM	124 ±21	6	$0.05 < p < 0.01$
LK	0.02 μM	124 ±12	10	$p < 0.001$
	0.2 μM	165 ±40	11	$p < 0.001$

TABLE 7: EFFECT OF Na_{ext} ON HK AND LK Na^+-ATPase

Vesicles are concentrated to one-tenth their original volume by centrifugation and then diluted 19-fold with 52.5 mM choline chloride or NaCl containing 0.1 mM $MgCl_2$ to obtain a final concentration of 2 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and either 50 mM choline chloride or 50 mM NaCl, as indicated. They are then centrifuged again and allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing 40 mM NaCl, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 0.02 or 0.2 uM [γ-^{32}P] ATP, as indicated. The reaction are carried out at 37° for 2-5 minutes. The reactions are terminated and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for the baseline activity in the absence of NaCl and presence of 40 mM KCl. These activities (p moles/min/mg) were 12.3± 5.9 (+ Na_{ext}) and 0.9± 0.4 (KCl baseline) for HK at 0.02 uM ATP, 57± 30 (+ Na_{ext}) and 6± 2 (KCl baseline) for HK at 0.2 uM ATP, 1.72± 1.1 (+ Na_{ext}) and 0.55± 0.19 (KCl baseline for LK at 0.02 uM ATP, and 8.5± 4.3 (+ Na_{ext}) and 4.4± 0.5 (KCl baseline) for LK at 0.2 uM ATP.

The values shown are the percentage of activity measured in Na^+-loaded vesicles relative to choline-loaded vesicles. S.D. is the standard deviation, n is the number of experiments performed, and p is the level of significance of Student's t-test performed on paired data i.e. with and without Na_{ext}.

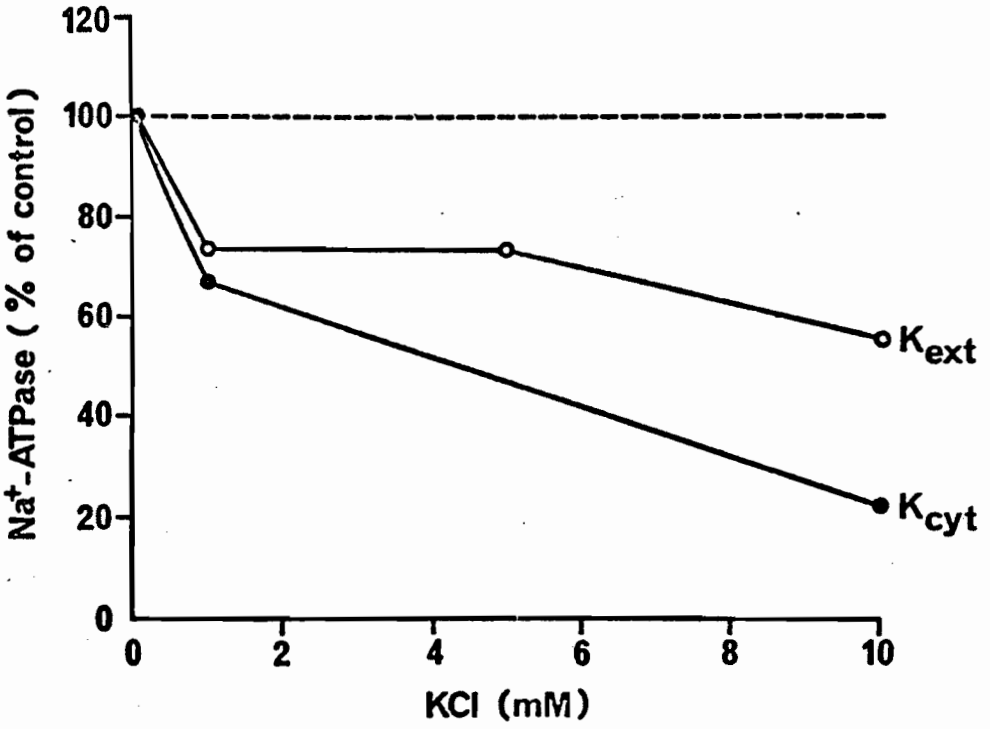

FIGURE 22: EFFECT OF K_{cyt} AND K_{ext} ON LK Na^+-ATPase AT 0.02 μM ATP

Vesicles are concentrated to one tenth their original volume by centrifugation and are added to 9 volumes of a choline chloride - KCl solution so that the final KCl varied as indicated. The final chloride concentration is 40 mM and 0.1 mM $MgCl_2$ is present throughout. The vesicles are recentrifuged and allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing varying amounts of KCl as indicated, 40 mM NaCl, and choline chloride to maintain the final chloride concentration at 50 mM, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 0.02 μM [γ-^{32}P] ATP. The reactions are terminated after five minutes at 37° and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for thebaseline activityin the absence of NaCl and presence of 40 mM KCl,The activities (p moles/min/mg) are 3.0 (100% control) and 1.0 (KCl baseline). The results of an experiment typical of at least 5 others are shown. The values are expressed as the % control activity observed in the presence of NaCl and absence of KCl.

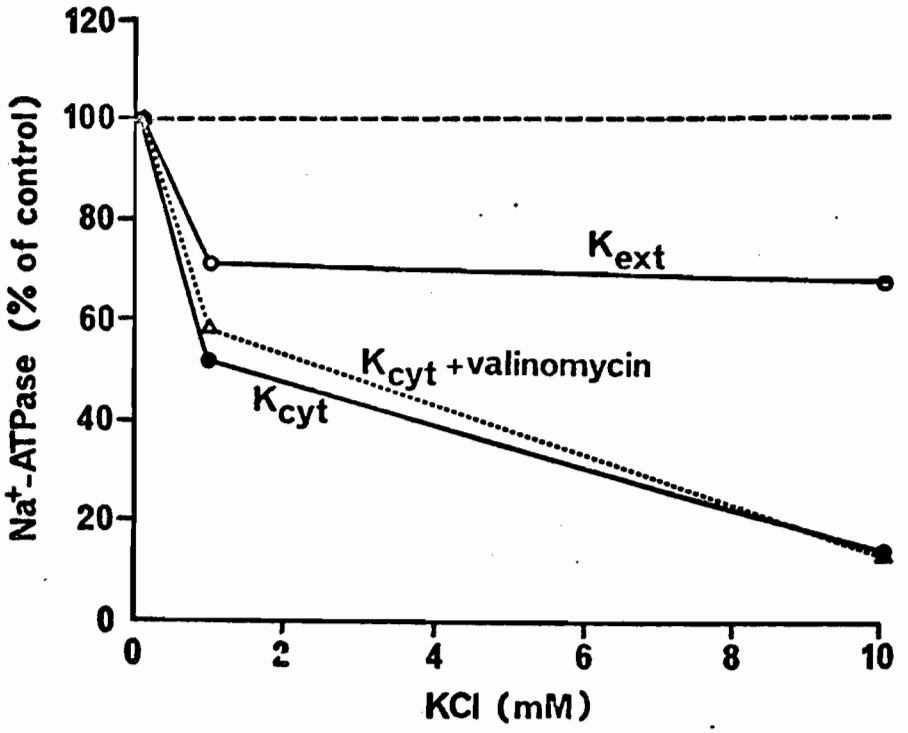

FIGURE 23: EFFECT OF K_{cyt} AND K_{ext} ON LK Na^+-ATPase AT 0.2 μM ATP

Vesicles are concentrated to one tenth their original volume by centrifugation and are added to 9 volumes of a choline chloride - KCl solution so that the final KCl varied as indicated. The final chloride concentration is 40 mM and 0.1 mM $MgCl_2$ is present throughout. The vesicles are recentrifuged and allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing varying amounts of KCl as indicated, 40 mM NaCl, and choline chloride to maintain the final chloride concentration at 50 mM, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 0.2 μM [γ-^{32}P] ATP. The reactions are terminated after five minutes at 37° and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for thebaseline activityin the absence of NaCl and presence of 40 mM KCl. The activities (p moles/min/mg) are 4.9 (100% control) and 2.0 (KCl baseline). The results of an experiment typical of at least 5 others are shown. The values are expressed as the % control activity observed in the presence of NaCl and absence of KCl. As indicated, 0.0022 volumes of 0.45 mM valinomycin dissolved in absolute ethanol are added

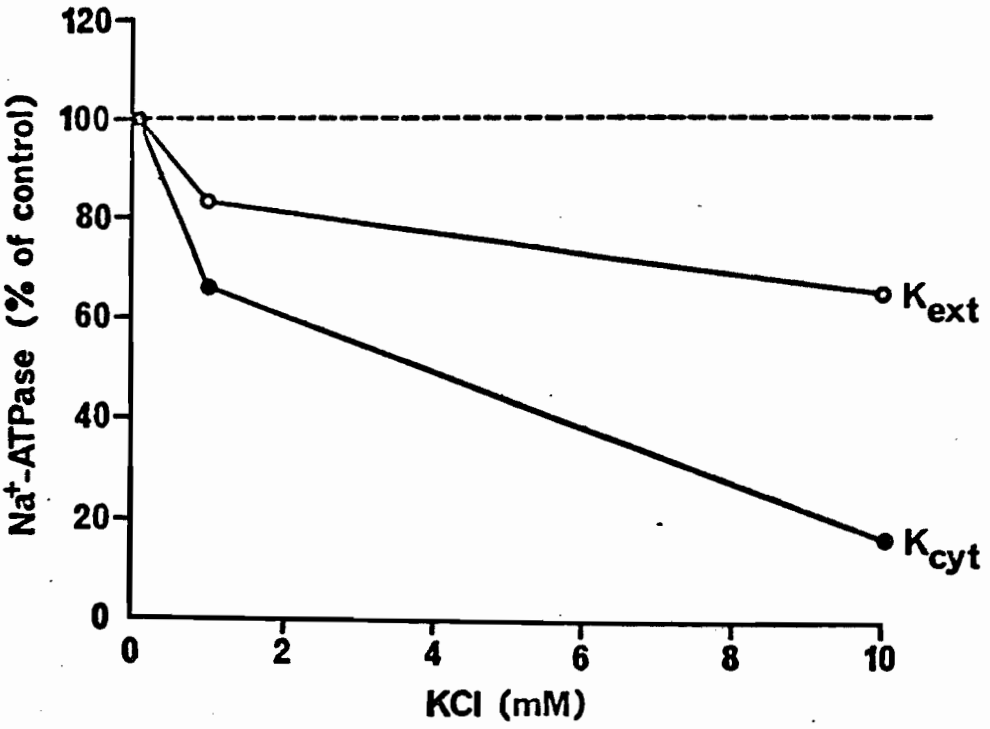

FIGURE 24: EFFECT OF K_{cyt} AND K_{ext} ON LK Na^+-ATPase AT 2.0 μM ATP

Vesicles are concentrated to one tenth their original volume by centrifugation and are added to 9 volumes of a choline chloride - KCl solution so that the final KCl varied as indicated. The final chloride concentration is 40 mM and 0.1 mM $MgCl_2$ is present throughout. The vesicles are recentrifuged and allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing varying amounts of KCl as indicated, 40 mM NaCl, and choline chloride to maintain the final chloride concentration at 50 mM, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 2.0 μM [γ-^{32}P] ATP. The reactions are terminated after five minutes at 37° and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.5 and the values are corrected for thebaseline activityin the absence of NaCl and presence of 40 mM KCl. The activities (p moles/min/mg) are 9.4 (100% control) and 4.7 (KCl baseline). The results of an experiment typical of at least 5 others are shown. The values are expressed as the % control activity observed in the presence of NaCl and absence of KCl.

Addition of valinomycin does not increase the extent of inhibition of K^+ beyond that observed with K_{cyt} alone. Thus K_{cyt} alone appears to be responsible for inhibition of the enzyme. The data suggest also that the apparent K_{ext} inhibition is due to the presence of K_{cyt} since the 10-fold dilution of K^+-equilibrated vesicles results in K_{cyt} present at one-tenth the K_{ext} concentration. The observation that valinomycin addition does not decrease K_{cyt} inhibition and that K_{ext} never stimulates LK Na^+-ATPase, even at 2 μM ATP, is evidence that K_{ext} does not stimulate overall Na^+-ATPase activity in these membranes.

With 0.02 μM ATP, Na^+-ATPase of HK vesicles shows relatively little response to addition of K^+ at either surface (Figure 25). However, in contrast to LK, at 0.2 μM ATP, K_{ext} markedly activates the Na^+-ATPase and K_{cyt} effects only a slight activation (Figure 26), probably because of slight K^+ penetration into the vesicles. At 2.0 μM ATP (Figure 27), K_{ext} increased activity several fold, characteristic of the Na^+-plus K^+-dependent activation of the ATPase observed at high ATP concentrations.

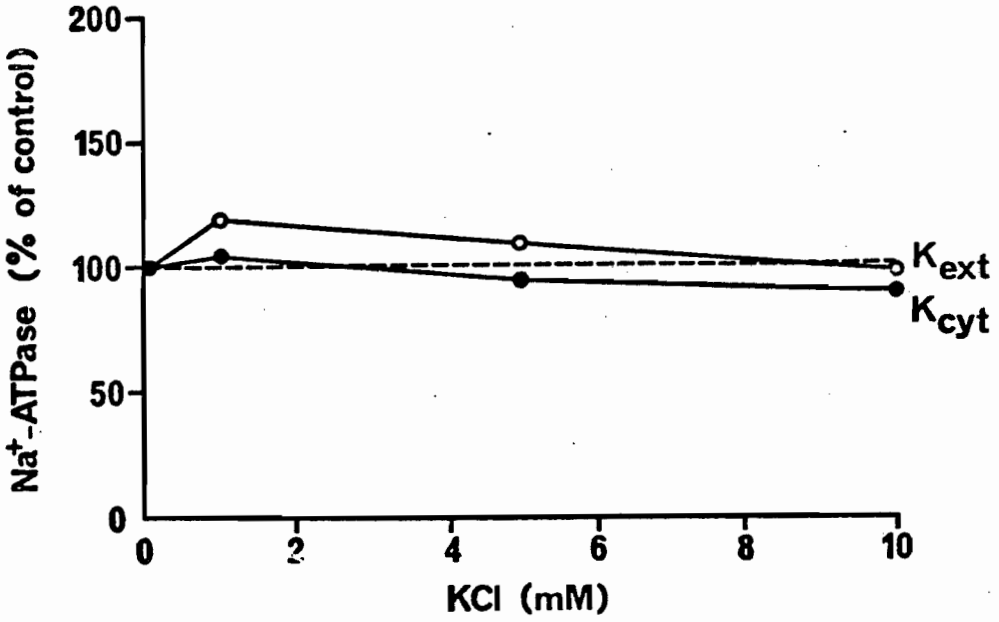

FIGURE 25: EFFECT OF K_{cyt} AND K_{ext} ON HK Na^+-ATPase AT 0.02 μM ATP

Vesicles are concentrated to one tenth their original volume by centrifugation and are added to 9 volumes of a choline chloride - KCl solution so that the final KCl varied as indicated. The final chloride concentration is 40 mM and 0.1 mM $MgCl_2$ is present throughout. The vesicles are recentrifuged and allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing varying amounts of KCl as indicated, 40 mM NaCl, and choline chloride to maintain the final chloride concentration at 50 mM, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 0.02 μM [γ-^{32}P] ATP. The reactions are terminated after five minutes at 37° and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for thebaseline activityin the absence of NaCl and presence of 40 mM KCl. The activities (p moles/min/mg) are 12.6(100% control) and 0.6 (KCl baseline). The results of an experiment typical of at least 5 others are shown. The values are expressed as the % control activity observed in the presence of NaCl and absence of KCl.

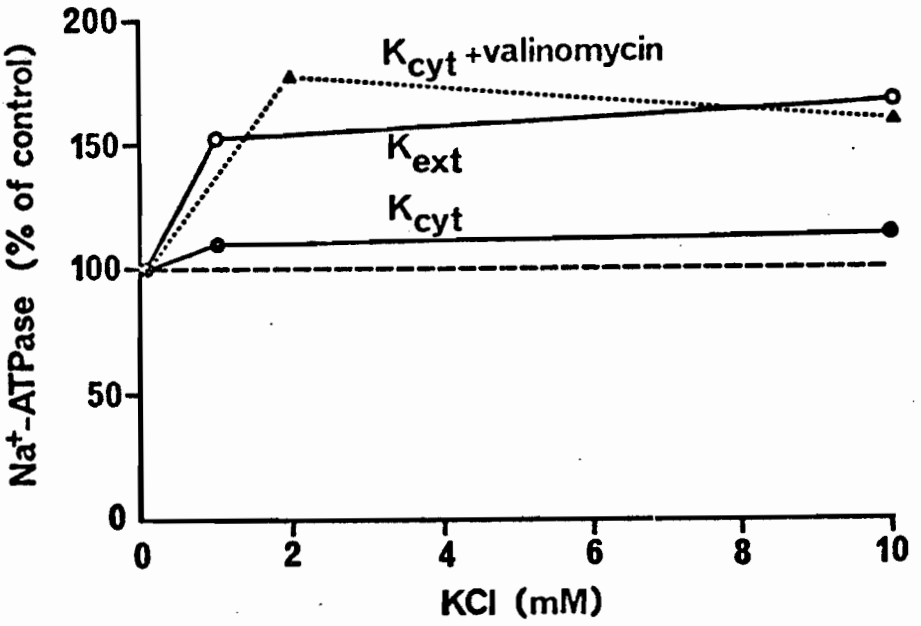

FIGURE 26: EFFECT OF K_{cyt} AND K_{ext} ON HK Na^+-ATPase AT 0.2 μM ATP

Vesicles are concentrated to one tenth their original volume by centrifugation and are added to 9 volumes of a choline chloride - KCl solution so that the final KCl varied as indicated. The final chloride concentration is 40 mM and 0.1 mM $MgCl_2$ is present throughout. The vesicles are recentrifuged and allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing varying amounts of KCl as indicated, 40 mM NaCl, and choline chloride to maintain the final chloride concentration at 50 mM, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 0.2 μM [γ-^{32}P] ATP. The reactions are terminated after five minutes at 37° and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for thebaseline activityin the absence of NaCl and presence of 40 mM KCl. The activities (p moles/min/mg) are 41.8 (100% control) and 4.2 (KCl baseline). The results of an experiment typical of at least 5 others are shown. The values are expressed as the % control activity observed in the presence of NaCl and absence of KCl. As indicated, 0.0022 volumes of 0.45 mM valinomycin dissolved in absolute ethanol are added.

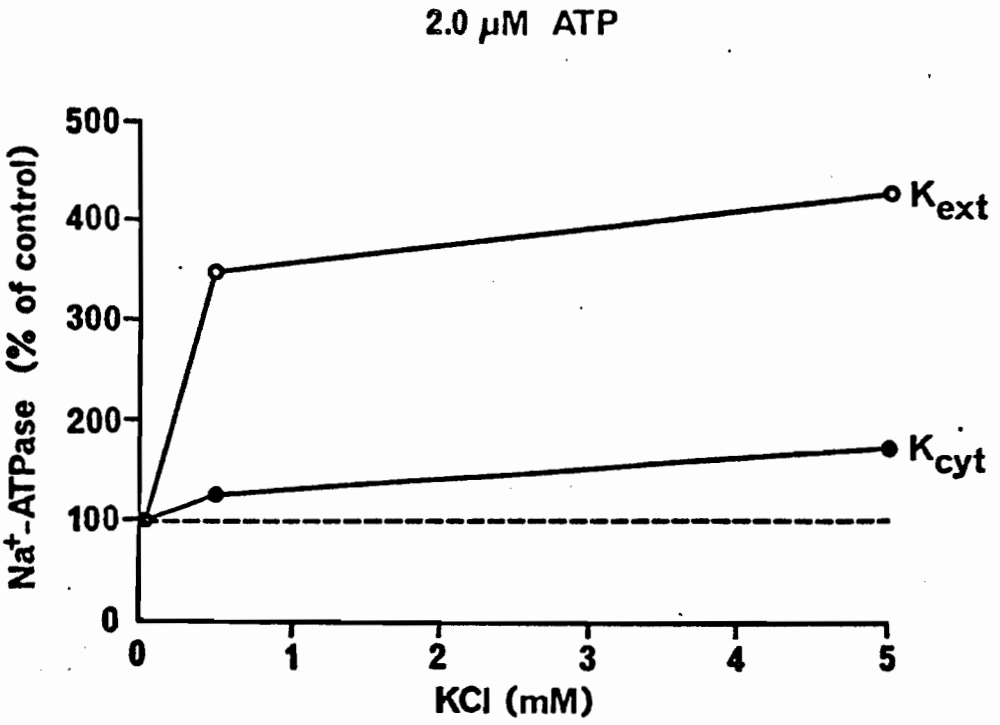

FIGURE 27: EFFECT OF K_{cyt} AND K_{ext} ON HK Na^+-ATPase AT 2.0 μM ATP

Vesicles are concentrated to one tenth their original volume by centrifugation and are added to 9 volumes of a choline chloride - KCl solution so that the final KCl varied as indicated. The final chloride concentration is 40 mM and 0.1 mM $MgCl_2$ is present throughout. The vesicles are recentrifuged and allowed to equilibrate on ice overnight followed by 30 minutes at 37°. One volume of vesicles is then added to 9 volumes of a reaction medium in a final volume of 0.1 ml containing varying amounts of KCl as indicated, 40 mM NaCl, and choline chloride to maintain the final chloride concentration at 50 mM, 10 mM Tris glycylglycine, pH 7.4, 0.1 mM $MgCl_2$, and 2.0 μM [γ-^{32}P] ATP. The reactions are terminated after five minutes at 37° and ^{32}Pi released from [γ-^{32}P] ATP is measured as described in Section 2.6 and the values are corrected for the baseline activity in the absence of NaCl and presence of 40 mM KCl. The activities (p moles/min/mg) are 42 (100% control) and 10 (KCl baseline). The results of an experiment typical of at least 3 others are shown. The values are expressed as the % control of activity observed in the presence of NaCl and absence of KCl.

6. DISCUSSION

Inside-out membrane vesicles prepared from HK and LK sheep red cells by a modification of the method described for human red cells (306) are shown to be about 90% inside-out (Table 5 and 6). The (Na^+, K^+)-ATPase of these vesicles was examined in order to gain insight on the asymmetric interactions of K^+ with the enzyme as well as on the molecular nature of this dimorphism. Previous studies of the sodium pump showed marked differences in K_{cyt} interactions (131, 195) as well as a lower affinity for K_{ext} in LK than in HK red cells (131). Studies of (Na^+, K^+)-ATPase with broken membrane preparations (294, 299-303) were devoid of the asymmetry required to define the surfaces at which the cations were acting in order to precisely correlate the physiological observations with the biochemical measurements performed, although one study included appropriate corrections for the effects of K_{ext} (296). From a comparison of the effects of Na^+ and K^+ on Na^+-ATPase of both types of sheep vesicles the following are evident:

i) K^+ stimulates HK (Figures 25-27) and inhibits LK Na^+-ATPase (Figures 22-24), these effects occurring at the external surface of HK and cytoplasmic surface of LK red cell vesicles. The activation of the HK Na^+-ATPase by K_{ext} is almost maximal with 1 mM K^+ and is, presumably, at the high affinity K^+ loading site (5, 6) which has a Km = 0.6 mM in the absence of Na_{ext} (131). The strong inhibition by K_{cyt} in LK vesicles is likely due to competition for Na^+ at the Na^+ loading sites as in the case of human red cell (Na^+, K^+)-ATPase (168).

These observations agree also with the earlier reports of inhibition of the pump by intracellular K^+ in both sheep (131,295,298) and goat (296) red cells.

ii) Vesicles of HK sheep have a greater apparent affinity for Na_{cyt} and are activated to a greater extent than LK Na^+-ATPase. The response to Na_{cyt} is similar to the observed effects of Na^+ on ATPase of broken membranes from the two types of sheep red cells (302). Whereas increasing the ATP concentration (from 0.02 to 0.2 μM) increased the apparent affinity of HK Na^+-ATPase for Na_{cyt}, this did not affect the response of the LK enzyme to Na_{cyt} (Figure 21). Thus ATP, even at these low concentrations, is observed to modulate the apparent affinity of the HK enzyme for Na_{cyt}, presumably at its loading sites, whereas the LK enzyme is less sensitive to changes in both Na_{cyt} and ATP.

iii) In the absence of K^+, Na_{ext} appears to have a further activating effect on the enzyme, to a greater extent in LK than in HK (see Table 7). With human Na^+-ATPase, this Na_{ext} stimulation observed at very low ATP concentrations has been accounted for in terms of current models for the reaction sequence. Thus, in accordance with the scheme shown in Figure 2 (p. 26), a step in the transition of phosphoenzyme from one form, $E_1 \sim P$, to another, E_2- P, is reversed by Na_{ext} with the result that hydrolysis proceeds mainly by an alternate route: $E_1 \sim P \rightarrow E_1 + Pi$. This alternate route (320) may result in a faster overall rate of ATP hydrolysis if subsequent regeneration of the active E_1 form of the enzyme from E_2 via the normal route is dependent on a modulating effect of ATP.

It is now clear that the higher rates of Na^+ and K^+ transport (288), (Na^+, K^+)-ATPase (289) and Na^+-ATPase (294) activities in HK compared to LK red cells are due not only to a somewhat greater number of pump sites per cell (290-292), but also to kinetic differences. These differences can be considered in terms of the plausible steps involving side-specific interactions of Na^+ and K^+ at the two surfaces. Assuming that K^+ binding at the external surface results in Pi release from phosphoenzyme, i.e. E_2- P + $K_{ext} \longrightarrow$ $E_2 \cdot$ K + Pi (see Figure 2, p. 26), it is possible that the rate of release of K^+ from $E_2 \cdot$ K may determine whether the enzyme is activated by K_{ext}. Several studies suggest that ATP has a role in regulating the release of K^+ from the enzyme and/or the conversion of E_2 back to E_1 (202, 204-207). It is apparent that at low ATP concentrations the enzyme is stimulated maximally by Na^+; at higher concentrations, maximal stimulation is observed with both Na^+ plus K^+. The interaction of ATP with the enzyme at the catalytic (phosphorylation) site and at a regulatory site may be interelated. This would be the case, for example, if (Na^+, K^+)-ATPase were a dimeric enzyme having half-of-the-sites reactivity (251-254).

A basic kinetic difference between HK and LK Na^+-ATPase may be their interaction with ATP which is manifested by different relative apparent affinities of the dephosphoenzyme for Na_{cyt} and K_{cyt} as well as effects of Na_{ext} and K_{ext} on the phosphoenzyme.

Thus it is not surprising that tight binding of K^+ to the dephosphoenzyme is evident in differences between HK and LK Na^+-ATPase with respect to interactions with ligands at both membrane surfaces.

7. SUMMARY

The sidedness of cation interactions with the (Na^+, K^+)-ATPase during pNPP hydrolysis is assayed using human red cell membrane inside-out vesicles. Intravesicular K^+ (K^+ at the originally external surface, K_{ext}) does not significantly activate pNPPase. Extravesicular K^+ (K^+ at the originally cytoplasmic surface, K_{cyt}) causes maximal activation of pNPPase, although a requirement for $K_{ext} \leq 0.05$ mM cannot be excluded. ATP inhibits this K_{cyt}-activated pNPPase at a low affinity site(s) ($K_{0.5}$ for inhibition by ATP = 0.1 mM).

A significant activation of pNPPase by Na^+ at either or both membrane surfaces could not be detected. In the presence of K_{cyt}, K_{ext} and micromolar ATP concentrations, extravesicular Na^+ (Na^+ at the originally cytoplasmic surface, Na_{cyt}) doubles the pNPPase activity. The activation is characterized by a high apparent affinity for Na_{cyt} and an even higher apparent affinity for K_{ext}. Higher ATP concentrations (50 uM) inhibit the pNPPase under these conditions.

These observations are considered in terms of a model for the (Na^+, K^+)-ATPase in which K_{cyt} binds to the enzyme and forms an E·K complex capable of hydrolyzing pNPP. This complex would be similar to an externally oriented form of the enzyme with occluded K^+ ($E_2 \cdot K$) described previously. ATP inhibits pNPPase by promoting K^+ release from E·K by acting at a low affinity regulatory site. In the presence of Na_{cyt}, ATP allows phosphorylation of the enzyme (to yield E_2- P) and exposes high affinity external K^+ sites.

K_{ext} binding then leads to dephosphorylation and formation of the active $E_2 \cdot K$ complex. The inability of pNPP to promote Rb^+ uptake into vesicles, i.e. transport from the "cytoplasmic" to the "external" surface, suggests that K^+ is not released to an appreciable extent at the external membrane surface during pNPP hydrolysis.

Inside-out membrane vesicles of about 90% purity are obtained from HK and LK sheep red cells by a modification of the method described for human red cells. The activation of the Na^+-ATPase of HK sheep by Na_{cyt} is more sensitive to increases in the ATP concentration (from 0.02 to 2.0 μM) than is the Na^+-ATPase of LK sheep. The LK enzyme is shown to be inhibited by K_{cyt} and is insensitive to changes of the ATP concentration in the range mentioned above whereas the HK enzyme is sensitive to K_{ext}-activation in an ATP-sensitive manner. An increase in the Na^+-ATPase activity in both types of sheep vesicles, though more apparent with LK, is seen when intravesicular Na^+ (Na^+ at the originally external membrane surface, Na_{ext}) is included.

These results indicate that the (Na^+, K^+)-ATPase of HK and LK sheep assayed at low ATP concentrations has a similar response to cations at both membrane surfaces as has been described from studies of ion transport. The molecular basis of the sheep dimorphism is accounted for as a difference in the relative effects of ATP (LK being less sensitive) acting at a low affinity regulatory site(s) that increases the sensitivity of the enzyme to both K_{ext} and Na_{cyt}.

8. CLAIMS TO ORIGINALITY

Parts of this investigation have been reported in the publications:

Drapeau, P. and Blostein, R. (1978) "Side-specific interactions of Na^+ and K^+ with Na^+-ATPase of inside-out membrane vesicles of HK and LK sheep red cells". Biophys. J. 21, 72a (Abstract)

Drapeau, P. and Blostein, R. (1979) "Side-specificity of K^+-activated phosphatase of inside-out membrane vesicles from human red cells". Fed. Proc. 38, 4303 (Abstract)

Blostein, R., Pershadsingh, H.A., Drapeau, P. and Chu,L. (1979) "Side-specificity of alkali cation interactions with (Na^+,K^+)-ATPase: studies with inside-out red cell membrane vesicles". In "(Na^+,K^+)-ATPase: Structure and Kinetics", Skou, J.C. and Norby, J., eds., Academic Press, London, pp. 223-235.

Drapeau, P. and Blostein, R. (1980) "Sodium and potassium interactions with Na^+-ATPase of inside-out membrane vesicles from high-K^+ and low-K^+ sheep red cells". Biochim. Biophys. Acta (in press)

Drapeau, P. and Blostein, R. (1980) "Interactions of K^+ with (Na^+,K^+)-ATPase: orientation of K^+-phosphatase sites studied with inside-out red cell membrane vesicles". J. Biol. Chem. (submitted)

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

1. The direct elucidation of the sidedness of K^+ interactions with the (Na^+,K^+)-ATPase during the hydrolysis of the pseudo-substrate p-nitrophenyl phosphate. The studies were carried out with inside-out human red cell membrane vesicles. It was shown that

K^+ stimulates p-nitrophenylphosphatase activity at the cytoplasmic, i.e. extravesicular, membrane surface whereas under conditions of enzyme phosphorylation, extracellular K^+, i.e. intravesicular K^+, stimulates p-nitrophenylphosphatase activity.

2. The direct elucidation of the sidedness of K^+ interactions with the (Na^+,K^+)-ATPase of sheep red cells. Using inside-out vesicles, it was shown that K^+ inhibits LK Na^+-ATPase (assayed at $\leqslant$2.0 uM ATP) at the cytoplasmic membrane surface and has no effect at the extracellular membrane surface. In contrast, HK sheep Na^+-ATPase is stimulated by extracellular K^+ and is insensitive to cytoplasmic K^+.
3. From studies with sheep red cell Na^+-ATPase, evidence was obtained for a regulatory role of ATP on the effectiveness of extracellular K^+ and cytoplasmic Na^+ as activators of Na^+-ATPase.
4. Based on the foregoing results, a mechanism has been proposed whereby K^+ interacts with the dephosphoenzyme at the cytoplasmic membrane surface and interacts with the phosphoenzyme at the extracellular membrane surface. The K^+-enzyme complex hydrolyzes p-nitrophenyl phosphate. ATP modulates the release of K^+ from the dephosphoenzyme.
5. A modification of the method described for the preparation of inside-out vesicles from human red cells was developed for the preparation of inside-out vesicles from sheep red cells. The modification involves the addition of ice-cold water prior to the

dilute phosphate buffers in the steps of the procedure prior to the vesiculation of the membranes.

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