CHAPTER II

REVIEW OF THE RELATED LITERATURES

The literatures were reviewed in 2 topics

1. Structure-function studies of the Na-K-ATPase
2. Physiology of exercise
3. Fluid and electrolyte metabolism in exercise

Structure-function studies of the Na-K-ATPase

Introduction

The Na-K-ATPase or sodium pump represents a universal machinery in the membranes of animal cells, which transfers chemical energy of hydrolysis of ATP to potential energy of electrochemical ion gradients for Na^+ and K^+ across the cell membrane. A large number of basic and specialized cellular functions like regulation of cell volume, excitability, cytoplasmic enzyme activity or muscle contraction essentially depends on the intra- and extracellular Na^+ and K^+ concentrations(Fig. 1). The electrochemical gradients for K^+ and Na^+ contribute via ion-selective K^+ and Na^+ channels to the resting potential across cell membranes and the generation of action potentials. A variety of secondary active systems that transport Ca^{2+}, H^+ ions or nutritions across the cell membrane are driven by the inwardly directed electrochemical gradient for Na^+. The maintenance of

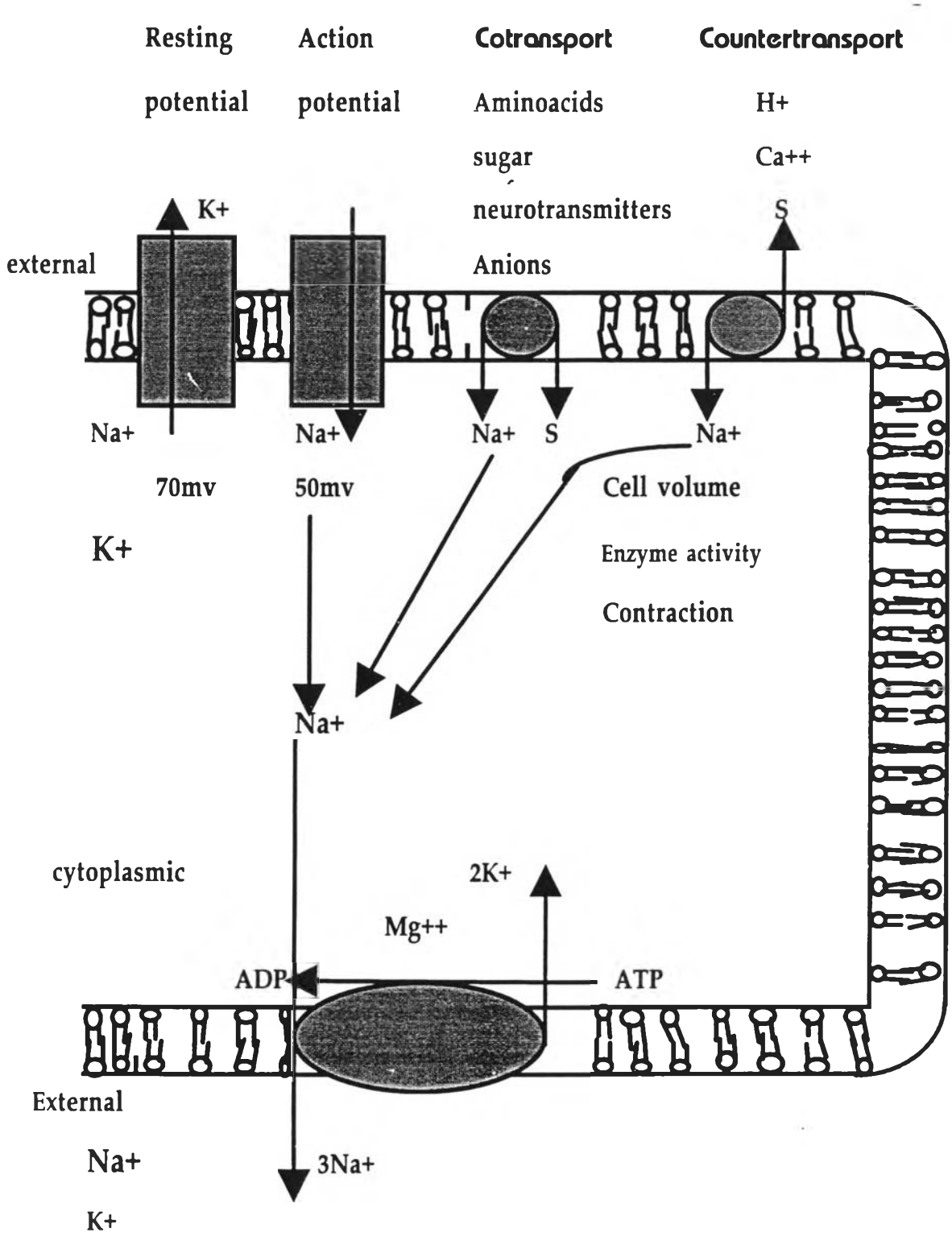


Fig.1. Schematic illustration of membrane transport and cellular functions that depend on the intra-extracellular distribution of [K+] and [Na+] . The intracellularly high [K+] and low [Na+] are maintained by the ATP-consuming 3Na+, 2K+ pump.

the ion gradients with low intracellular [Na^+] and high [K^+] is achieved by the operation of the Na-K-ATPase that transports under physiological conditions three Na^+ ions out of the cell and two K^+ ions into the cell molecule of MgATP hydrolyzed. Changes of pump activity, therfore, will have pronounced effects on cell functions.

The phenomenological description of the transport is based on the hypothesis proposed in the late 60's by Albers(1967) and Post et al(1969) that the enzyme exists in two different conformation, an E_1 and an E_2 conformation. According to the extended Albers-Post reaction scheme(Fig.2), the Na-K-ATPase undergoes a sequence of transitions between the E_1 conformation with inward-facing cation binding sites and high affinity for Na^+ and the E_2 conformation with outward-facing cation binding sites and high affinity for K^+. Transitions between these two conformations are induced by phosphorylation-dephosphorylation reaction(P-type ATPase). In the E_1ATP form, three intracellular ions become bound. Hydrolysis of ATP and phosphorylation of the protein leads to occlusion of the three Na^+ ions followed by a transition to the E_2P form. Na^+ is now released externally, and instead two K^+ ions become bound; this leads to spontaneous dephosphorylation and occlusion of the K^+ ions. Stimulated by ATP, a conformational change back to the E_1 form is induced, K^+ is liberated to the cytoplasm, and the transport cycle is completed.

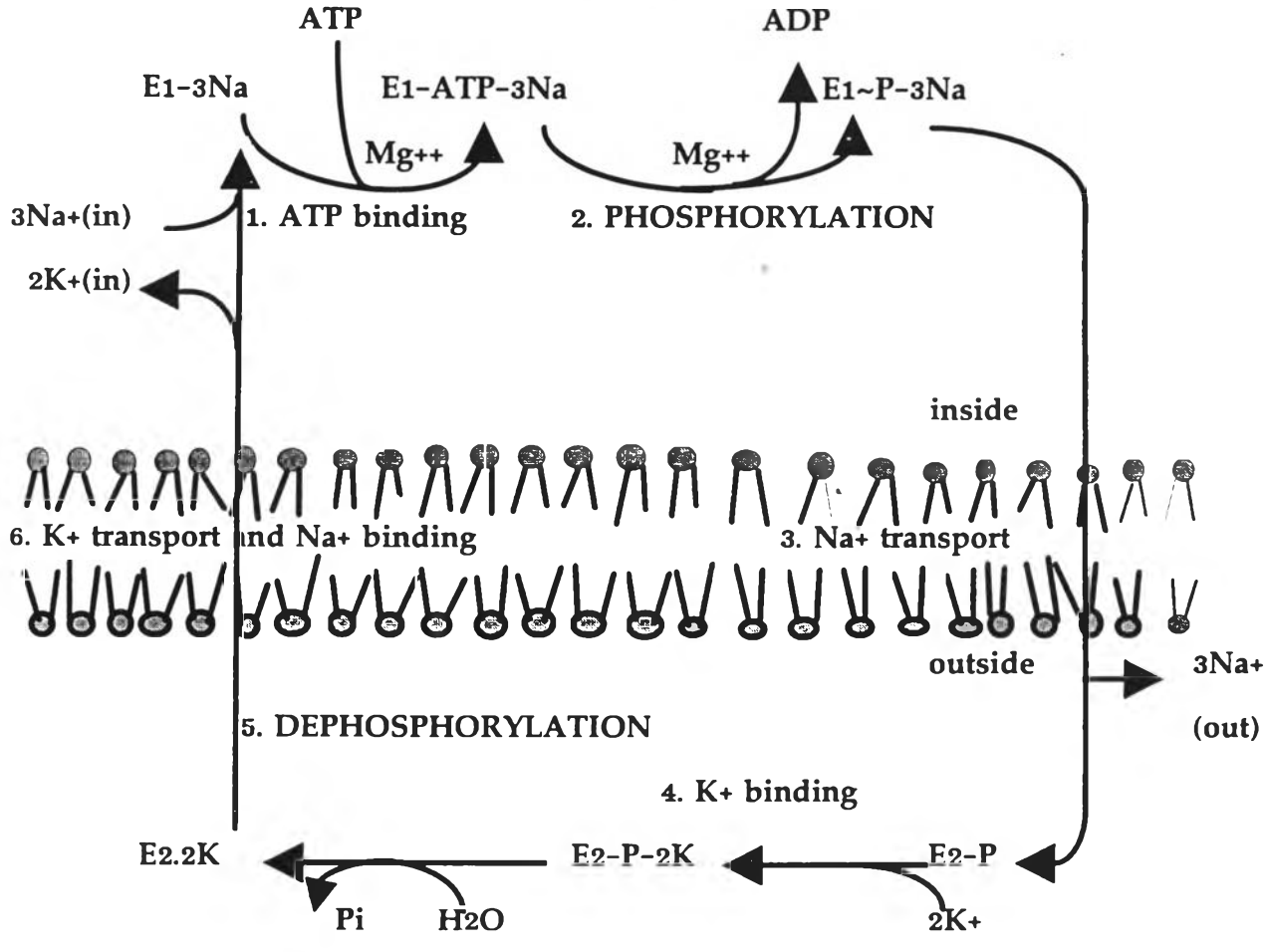


Figure 2 Simplified Albers-Post scheme of the Na,K-pump cycle. E1 and E2 are the conformations of the enzyme with the cation-binding sites facing the cytoplasm and the extracellular space., respectively.

Structural features of the Na-K-ATPase

A. Amino acid sequence and transmembrane topology

Na-K-ATPase is composed of two subunits, a larger catalytic α subunit with a molecular mass of about 112000 Da and a smaller glycosylated β subunit with a molecular mass of about 55000 Da(Fig. 3). The α subunit comprises an acceptor for the γ-phosphate of ATP ; phosphorylation of the aspartic residue Asp-369 leads to formation of the phosphorylated intermediate during the reaction cycle. In addition to the cation binding sites, also the receptor for the cardiac glycosides is located on the α subunit(Price and Lingrel, 1988). The β subunit was for a long time believed not to participate directly in the catalytic cycle or the binding of cardiac glycosides. Recently, it was demonstrated that the assembly of an (αβ) heterodimer is necessary for a stable and functionally competent configuration of the pump; in particular, the β subunit is needed for the α subunit to exit from the endoplasmic reticulum and to the correct configuration(Geering, et al., 1989). A third subunit with a molecular mass of about 12000 Da has been detected as a part of the native enzyme assembly(Collins and Leszyk, 1987., Haarris and Stahl, 1988). However, the importance of this so-called γ subunit for neither ATPase or phosphatase activity nor ion transport characteristics has been demonstrated so far. In addition, there are multiple isoforms of both subunits. In case of the α chain, three isoforms exist(Sweadner, 1989). The α_1 isoform is found in all cells while α_2 is the major isoform in skeletal muscle and is also present in neuronal tissue and heart. The α_3 isoform is limited to the neuronal system and the heart. At least two isoforms exist for the β subunit in mammalian cells with β_1 being found in all tissues, while

β_2 is predominantly in the nervous system(Martin-Vasallo, et al., 1989). Two distinct explanations have been proposed to account for the existence of several Na-K-ATPase isoforms differentially expressed in various tissues (Takeyasu, Lemas and Fambrough, 1990). One possible explanation for the existence of a multigene family is that it represents a selective advantage in evolution because it simplifies the problem of regulating gene expression to suit the needs of each developmental stage or each cell type of the organism. Alternatively, each isoform could be distinct from each other by specific functional characteristics.

In the mid 80's a molecular genetic approach combined with sequencing of proteolytic fragments of the Na-K-ATPase allowed to elucidate the entire amino acid sequences for α subunits first for sheep kidney(Shull, Schwartz and Lingrel., 1985) and pig kidney(Ovchinnikov, et al., 1986). At present, the primary structure of a α subunits of human is known(Kawakami, et al., 1986).

Six to 10 transmembrane segments were derived from hydropathy plots and were suggested for the model of two-dimensional transmembrane topology of the Na-K-ATPase(Jorgensen and Anderson,1988., Modyanov, et al., 1992). The transmembrane domains form 40-50% of the total protein mass that are protected from intensive tryptic digestion by membrane lipids (Jorgensen, 1992). In early examinations of the transmembrane organization, a cytoplasmic orientation of the N-terminus of the α subunit has been demonstrated and became generally accepted. (Giotta, 1975., Jorgensen,1975.) Previous uncertainty concerning the orientation of the C-terminus seems to be settled now in favour of a cytoplasmic location(Antolovic, et al., 1991., Modyanov, et al., 1992). On the basis of the close similarity of hydropathy plots of the Na-K-ATPase to the Ca^{2+} and H^{+}-K^{+}-ATPase a model of 10 transmembrane domains may be favoured also for the Na-K-ATPase. Ten

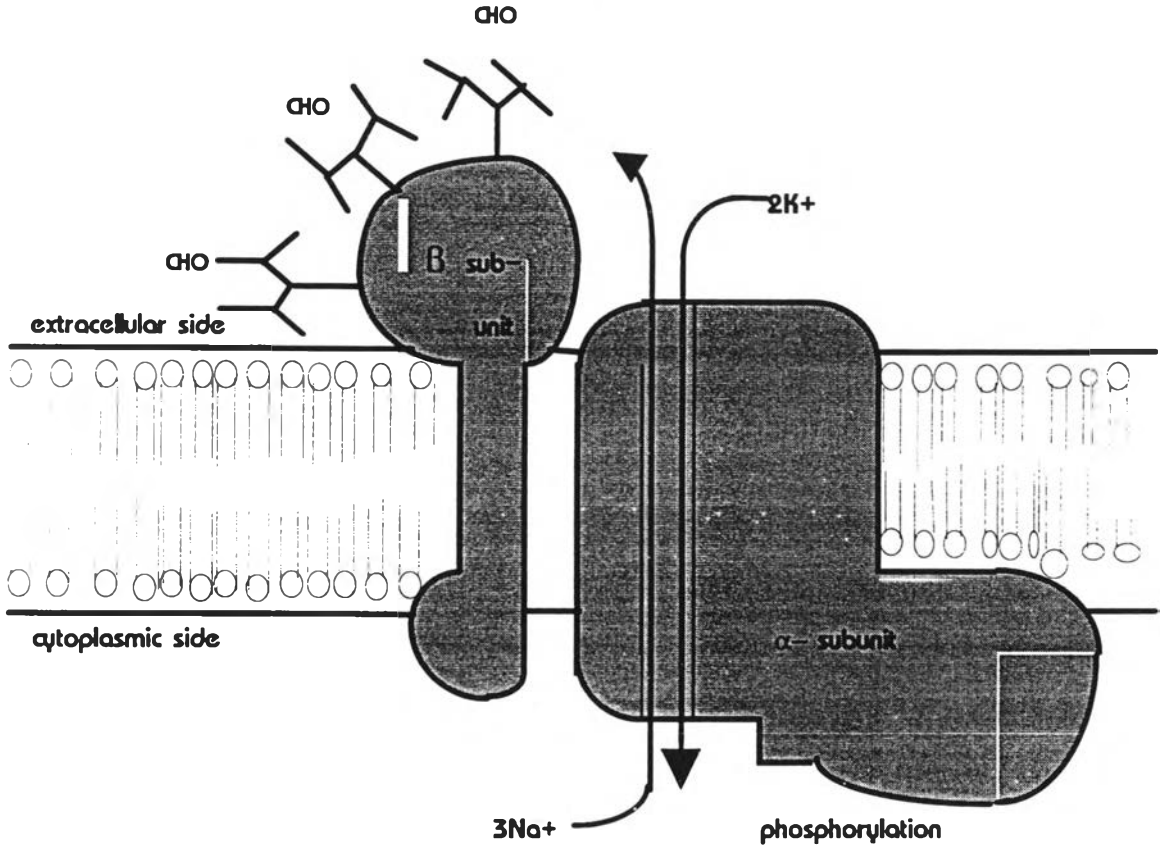


Fig. 3 Model of Na-K-ATPase showing the α and β subunits and the movement of Na+ and K+ across its membrane.

transmembrane segments seem also reasonable from observations on a 19 kDa tryptic fragment with an N-terminal asparagin(Asn-837) that has a cytoplasmic location(Karlish, Goldshleger and Jorgensen., 1993).

B. Structure and assembly of subunits

The three-dimensional structure of crystallized Na-K-ATPase has been analyzed by electron microscopy and image processing(Skriver, et al., 1992). The best resolution obtained in these crystals so far in the range of 20-25 °A. To identify amino acids residues in membrane proteins, a resolution better than 3 °A is required. However, even at the above level of resolution some gross information on intramolecular association has been worked out. The enzyme seems to consist of $(\alpha\beta)_2$ dimers under the conditions of pump-enriched membrane crystals(Skriver, et al., 1992., 1989) They protude about 40°A to the cytoplasmic side of bilayer where according to labeling and sequence studies most of the molecular mass of the α subunit is localized(Kawakami, et al., 1985., Shull, Schwartz and Lingrel., 1985). Much less of the molecules, about 20°A, extends to the extracellular side(Skriver, et al., 1992). An opening between adjacent $(\alpha\beta)$-protomers was observed on the intracellular side and an opening of somewhat smaller depth on the extracellular side(Skriver, et al., 1992). The two rod-shaped protomers are connected by a bridge that is differently placed in the lipid bilayer depending on whether crystals were induced by vanadate or magnesium favouring the E_2 conformation, or by cobalt-tetraamine-ATP that binds to the low affinity ATP-binding site and produces two different populations of the crystals(Hebert, et al., 1990).

The oligomeric structure of the Na-K-ATPase has also been investigated in radiation inactivation studies combined with measurements of enzymatic activity and ligand binding in purified enzyme preparation. In recent experiments by Norby and Jensen(1991) tried to determine the minimal structural unit at which the enzyme still possesses catalytic activity of Na^+-and K^+-activated hydrolysis of ATP. Target size analysis suggests that membrane bound Na-K-ATPase is structurally organized in $(\alpha\beta)_2$ diprotomers. Structural contact between two $(\alpha\beta)$ units seems to be required for complete catalytic activity of the enzyme. However, findings about the number of cation and substrate binding sites per functional catalytic subunit have led to controversy with respect to the number of subunits that form the fully active unit. The single $(\alpha\beta)$ unit can exhibit ATPase activity and cation binding. Even when when α subunit the has been partially destroyed, some partial reactions of the enzyme are still observed(Norby and Jensen, 1991) .

C. Isoform and species differences

The history of characterization of the isoforms of the Na-K-ATPase can be traced back to the early experiments of Skou(1962) who reported that brain contained Na-K-ATPase with higher cardiac glycoside sensitivity than kidney or heart. The observation of two components for [^{3}H]ouabain binding in brain indicates heterogeneity within the population of Na-K-ATPase molecules. On the molecular level, heterogeneity of the α subunit was initially demonstrated by Peterson et al., 1978 and Sweadner(1979). Two electrophoretically separable forms of the α subunit of rat brain designated as α and α + isoforms (Sweadner,1979) had different affinities to ouabain-insensitive form was found in all tissues and is the only isoform in

mammalian kidney. The more slowly migrating α + form, which is more sensitive to ouabain, was found in brain tissue(Sweadner,1979). Later on, it became clear that the existence of doublets on SDS gels is not sufficient to indicate the presence of more than one isoform. In fact, heating conditions of SDS treated isoform preparations may lead to formation of subunits migrating as a doublet(Sweadner, 1990), or even produce multiple bands corresponding to conformational "pseudoisozymes". Nevertheless, clear evidence for the existence of isoforms of the Na-K-ATPase was provided by the finding of distinct genes encoding three rat α subunit , which according to modern nomenclature are designated as α_1, α_2, and α_3(Shull, Schwartz and Lingrel., 1985). The α_1 subunit is encoded as the largest polypeptide with 1021-1023 amino acids and corresponds to the ouabain-insensitive kidney form earilier marked as α. The polypeptides of 1017-1020 amino acids correspond to the α_2 or α+ isoform with higher affinity for ouabain though a larger apparent molecular mass of this isoform has been determined on the basis of its electrophoretical mobility(Sweadner, 1990). The amount of α_2 isoform is high in skeletal and heart muscle, adipose tissue and stomach. The α_3 isoform consisting of 1010-1013 amino acids is predominantly expressed in brain(Specht, 1988), but was also found in other vertebrate and human tissue(Lingrel,1992). Among the same isoforms of different species homology is very high(more than 90% of identity) while the degree of identity between different isoforms of the same specious is only about 87% (Sweadner, 1989).

Sensitivity towards cardiac glycosides for a given tissue is higher for α_2 and α_3 isoforms compared to the α_1 isoforms. However, pronounced variation of ouabain sensitivity have been demonstrated for the same isoforms of different tissues or species(Skou and Esmman., 1992). Therefore, this parameter can hardly be used for an absolute definition of the isoforms.

Present identification of isoform is based predominantly on the comparison of the amino avid sequence of the N-terminus. The degree of variability in this range is higher between isoforms of the same species than for the same isoforms of different species that are evolutionary distant(Shull, Greeb and Lingrel., 1986., Sweadner, 1989). For example in the chicken Na-K-ATPase the amino acid residues that are believed to be isoform-specific are located predominantly at the N-terminal end and mostly include charged residues (Takeyasu, Lemas and Fambrough., 1990). The boundary amino acids of the first ectodomain implicated in ouabain binding are also considered to be isoform-specific attributes.

In contrast to the highly glycosylated β subunit, the α subunit is not recognized as a glycoprotein. Indeed, it has been demonstrated that the α subunit can be glycosylated with carbohydrate chains facing the cytoplasm(Pedemonte, Sachs and Kaplan., 1990). In a more recent report (Pedemonte and Kaplan., 1992), it has been shown that only a single N-acetylglucosamine is the N-linked carbohydrate moiety attached to the α subunit of the sodium pump.

Functional domains of the Na-K-ATPase

A. Receptor for cardiac glycosides

The Na-K-ATPase is the receptor for the cardiac glycoside class of compounds which are used in the treatment of congestive heart failure and certain arrhythmias. The enzyme, of course, is inhibited by this class of drugs in all tissues and, while an understanding of the ligand receptor interaction may be helpful in developing better drugs, it is also likely to

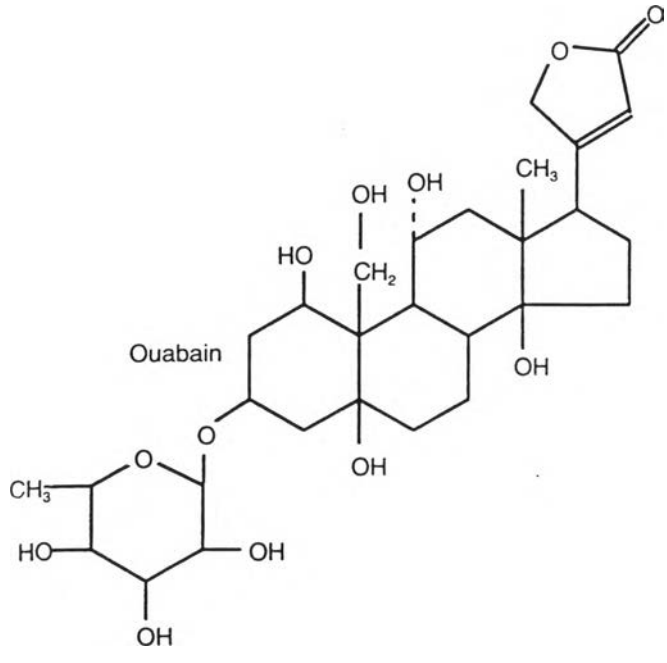


Fig. 4 Structure of ouabain

provide an important approach for exploring structure-function relationships. the cardiac glycoside most often used in the laboratory is ouabain, a powerful inhibitor of the enzyme which is also readily solubilized in water. Ouabain, as well as other naturally occurring cardiac glycosides, is composed of a lactone ring, a hydrophobic steroid moiety, and a sugar. The structure of ouabain is shown in Figure 4. Because cells require Na-K-ATPase for survival, selection procedures can be used to identify alternations in the enzyme which prevent its interaction with ouabain or other inhibitors of this class of drugs. This approach was used initially to determine the structural differences responsible for the differential ouabain sensitivity exhibited by naturally occurring resistant and sensitive isoforms(Price, 1988). The rat and the mouse express both high and low affinity isoforms of the subunit. The I_{50} for the low affinity α_1 isoform is approximately 1000-fold greater than the I_{50} for the high affinity α_2 and α_3 isoforms. When the α_1 subunit of rat, that is, the low affinity form, is introduced into an expression of the isoform confers resistance to 1 μM ouabain. HeLa cells are of human origin and therefore express a sensitive α_1 isoform. Without the expression of the transfected cDNA, HeLa cells are killed by this concentration of inhibitor. The identity of the amino acids responsible for the differential sensitivity between the sensitive and insensitive isoforms was determined by preparing chimeras between the insensitive rat α_1 isoform and the sensitive sheep α isoform. The chimera containing the N-terminal half of the resistant rat α_1 isoform, and the C-terminal half of the sensitive isoform did not confer the resistance when expressed in sensitive cells. Based on this findings it was concluded that the determinants of differential sensitivity reside in the N-terminal half of the molecule. This half contains the first two extracellular regions and these are considered targets for ouabain bindings, as it is known that this class of drugs binds to the outside of the cell. Interestingly, the

amino acid sequence of the second extracellular domain is identical between the sensitive and insensitive isoforms, and therefore cannot be responsible for the differential sensitivity. The first extracellular region, however, contains four amino acid differences. When these residues in the sensitive sheep α_1 isoform were changed to those found in the resistant rat α_1 isoform, the resulting enzyme is able to confer ouabain resistance to HeLa cells and exhibits an affinity for ouabain identical to that of the rat α_1 isoform(that is, the affinity for ouabain was reduced 1000-fold). These four amino acids were then substituted singly and in various combinations to determine whether all of a subset of residues is responsible. The double substitution Gln111 to Arg and Asn122 to Asp was necessary and sufficient to lower the sensitivity of the ship α_1 subunit to that of the resistant rat α_1 subunit. These two amino acids are located at the border of the H1-H2 extracellular domain and are depicted by bold arrows at amino acid positions 111 and 122. The *Bufo marinus* toad and the monarch butterfly(*Danaus plexippus*) also express a ouabain insensitive Na-K-ATPase. Interestingly, the enzymes from these organisms contain a charged residue at only one of the border positions, not both, as in the resistant rat α_1 isoform(Jaisser, Canessa, Horisberger and Rossier., 1992., Holzinger, Frick and Wink., 1992). Utilizing the same transfection assay, specific mutations were introduced into other regions of the α subunit and the alterted enzymes were tested for their ability to confer resistance when expressed in otherwise sensitive HeLa cells. Initial studies examined additional amino acids in the first extracellular region. Substitution of one amino acid in this region, aspartic acid121, yield an enzyme able to confer resistance, although, not to the same degree as the rat α_1 isoform. Mutations in the second extracellular region, however, failed to yield resistant colonies. These and other substitutions which failed to confer resistance are shown in Figure 4 and are depicted by small arrows.

Transmembrane amino acids containing side chains capable of participating in hydrogen bonding were also substituted. Enzyme containing alanine at cysteine 104(Schultheis, 1993) and tyrosine108 in the first transmembrane region are able to confer resistance to ouabain. While it is unknown at this time whether amino acids in other parts of the molecule contribute to ouabain. While it is unknown at this time whether amino acids in other parts of the molecule contribute to ouabain sensitivity, it is clear that residues in the first extracellular region and the first transmembrane region are involved. This finding suggests that this region may represent a binding site for ouabain. It is also possible that the binding site is located elsewhere in the molecule and that substitutions in the first transmembrane and extracellular region influence ouabain binding through indirect effects or changes in conformation.

B. Cation binding sites

The amino acid residues involved in the binding and transport of cations have not been identified. The overall enzymatic mechanism of Na-K-ATPase is depicted in simplified form in Figure 2. It is envisioned that Na^+ binding sites exposed to the cytoplasmic surface bind three Na^+ ions, permitting the phosphorylation of the enzyme. Phosphorylation triggers a conformational transition from an E1 form of the enzyme to an E2 intermediate exposing Na^+ binding sites to the exterior surface where they are released. Potassium ions then bind to their respective sites on the outside of the cell. The sites for potassium may or may not be distinct from those binding sodium. The binding of K^+ stimulates the dephosphorylation of the enzyme, and following dephosphorylation, another major conformational shift occur (E2 to E1), exposing K^+ sites to the inside of the cell, where the K^+

ions are liberated. It has been proposed that negatively charged amino acids may be an integral part of the cation binding sites, as these residues would be expected to neutralize the positive charge of the cation. Furthermore, these negatively charged residues are thought to be located within membrane-spanning regions of the enzyme. In theory this should lower the activation energy required to move cations across the hydrophobic environment of the plasma membrane.

Chemical modification/protease digestion studies(Karlish, Goldshleger and Stein., 1990., Goldshleger, et al., 1992., Capasso, et al., 1992) along with the high degree of conservation of E955 and E956 among isoforms and species, suggest the involvement of glutamic acids at positions 955 and 956 in cation binding(Lingrel, et al., 1990). In the current studies, each of these residues was converted to aspartic acid and glutamine(Van Huysse, Jewell and Lingrel., 1993). These substitutions were introduced into a cDNA encoding the rat α_1 isoform, which is relatively resistant to ouabain (this isoform is approximately 1000-fold more than the human α_1 enzyme, and thus has the ability to confer ouabain resistance when expressed in sensitive cells). If the amino acid in question is required for cation transport, the subunit carrying a substitution at the same position should not be active and should therefore lack the ability to confer ouabain resistance to sensitive cells. On the other hand if the substitution does not affect activity of the subunit, it will confer resistance and colonies should be observed in the presence of ouabain. Interestingly, substitutions at either of these positions (E955 or E956) confer resistance as colonies are obtained in the presence of the drug. The number of colonies obtained in transfections with all mutant cDNA was similar to the number obtained with the unaltered rat α_1 cDNA indicating that the encoded substitutions have little or no effect on the enzymatic activity of the expressed enzyme(Fig. 6). When the expressed

enzyme was isolated and analyzed for Na^+ and K^+ dependence of ATPase activity, only a modest alternation in apparent Na^+ and K^+ affinities was observed. On this basis, it is reasoned that these two amino acids are not absolutely required for function and are unlikely to be integral components of the cation binding site. The other negatively charged transmembrane amino acid residues still represent potential cation binding sites and are presently being tested for their role in maintaining overall Na-K-ATPase.

C. ATP binding pocket

The main strategy for elucidation of the ATP binding pocket was based on using spectroscopic probes and chemical modification. The fact that ATP prevents inactivation of enzymatic activity of fluorescent labeled Na-K-ATPase was interpreted as an indication that the modified residues form the ATP-binding site. The labeled residues were identified by sequencing of the peptide fragments containing the label. In *Torpedo* electroplax, seven amino acid residues Asp-376, Lys-487, Lys-507, Cys-663, Asp-716, Asp-720 and Lys-725 are suggested to participate in the formation of the ATP binding pocket(Pedemonte and Kaplan., 1990). Asp-716 and Asp-720 are the residues selectively labeled with the ATP derivative γ-(4-*N*-2-chloroethyl-N-methylamino)benzylamide-ATP (C1R-ATP), Cys-663 and Lys-725 by an affinity analog of adenosine p-(fluorosulfonyl benzoyladenosine) (FSBA) (Ohta, Nagano and Yoshida., 1986), Lys-507 is the binding site labeled by fluorescein-isothiocyanate (FITC)(Farley, et al., 1984) and also by N-(2-nitro-4-isothiophenyl)-imidazole (NIPI)(Ellis-Davies and Kaplan., 1992) and by 4-acetamido-4'-isothiocyanatostilbene-2, 2'-disulfonic acid SITS) (Pedemonte, et al., 1992). Two other residues Lys-487 and Lys-773 were also reported to be labeled by FITC in an ATP-protectable way(Xu, 1989)

suggesting that three Lys residues are clustered around the binding site of FITC. Recent investigation, however, demonstrated that 5'-diphospho-5'-adenosine AP_2PL and pyridoxal phosphate(Hinz and Kirley, 1990) labeled only Lys-487. Therefore, it was suggested that this lysine participates in the formation of the putative ATP-binding domain, and possibly is in close proximity to the phosphorylation site Asp-376 and also to the generally accepted FITC-binding site Lys-507.

Short-term regulation of Na-K-ATPase activity

Short-term regulation of activity has been traditionally viewed mainly as secondary to changes in intracellular sodium, which alone among its primary reactants is rate-limiting for pump in intact cells(Jorgensen, 1986). This rate of pumping is exactly balanced by the passive entry of Na^+ and the loss of K^+. When the Na-K-ATPase were partially inhibited by reducing the concentration of external K^+, the rate of pumping no longer balanced the passive leak, and the cells began to rise, however, it stimulated the pumps, resulting in a slight increase in the rate of transport. This stimulatory effect of Na^+on the pump rate partially counterbalanced the accumulation of Na^+ via the passive leak and would eventually establish a new steady state. Consideration of the short-term response illustrates a key factor in the moment-to-moment control of the Na-K-ATPase. The rate of Na-K-ATPase by the pump depends on the intracellular Na^+ concentration, which, in turn, is a complex function of the number of pumps and the passive permeability of the membrane. Although the number of functional pumps may be reduced slightly, say by partial inhibition with ouabain, the total rate of pump-mediated transport will not change appreciably. Because of the increase in intracellular Na^+ concentration, each of the remaining

pumps will operate at a faster rate. Indeed, the binding of the first to its internal site on the pump facilitates the binding of the second and the third. With this cooperativity, a slight increase in intracellular Na^+ concentration can produce a significant increase in pumping rate. As a consequence, any increase in the permeability of the membrane to will stimulate pump-mediated transport with a minimal increase in the intracellular concentration of that ion. Considerable information has now emerged, however, suggesting that, in addition to fluctuations in intracellular sodium concentration, and structural intracellular networks and that this regulation is cell specific and has physiological relevance.

Transporting epithelia, particularly the renal tubule, are rich in Na-K-ATPase, and its regulation is critically important both to the countertransport of sodium and potassium at the basolateral membrane and to the translocation of several solutes cotransported with sodium across the apical membrane of tubule cells(Doucet, 1988). The mammalian nephron is highly heterogeneous, and so is the distribution of Na-K-ATPase and its regulation along its longitudinal axis(Katz, 1982). Control of Na-K-ATPase activity varies according to the specific function of each nephron segment and the localization of specific hormone receptors therein, as illustrated in the following selected examples:

Dopamine Endogenous dopamine (of renal origin) is an important intrarenal natriuretic factor that acts in paracrine and/or autocrine fashion to modulate tubular sodium transport, thereby contributing to the regulation of final sodium excretion and body sodium homeostasis(Felder, et al., 1989.). That its action is on the tubule is suggested by observations that enhanced sodium excretion can occur without detectable changes in renal hemodynamics. The cellular mechanisms involved in the action of dopamine at the tubular level

include inhibition of Na-K-ATPase(Aperia, Bertorello and Seri., 1987) and Na/H exchanger activity(Gesek and Schoolwerth., 1990).

Norepinephrine Norepinephrine and other α-adrenergic agonists have an effect opposite to that of dopamine, stimulate Na-K-ATPase activity(Aperia, et al., 1992), especially in the proximal convoluted tubule. It is likely that the final sodium excretion (as far as catecholamine are concerned) represents a balance between the dopaminergic and α-adrenergic on transporter function in renal tubules(IBarra, et al., 1993).

Endothelin Endothelin, a peptide synthesized by vascular endothelial cells, is a potent vasoconstrictor agent. Its action on the renal tubule, which can result in natriuresis, appears to be located both in the proximal tubule and in the medullary collecting duct(IMCD) and to involve, at least in part, inhibition of Na-K-ATPase activity(Garvin and Sundus, 1991). Although several intracellular mechanisms following membrane receptor activation by endothelin have been described in various tissues, its effect on renal Na-K-ATPase has been attributed to stimulation of prostaglandin E_2 (PGE_2 synthesis).

Parathyroid hormone Parathyroid hormone (PTH) inhibit sodium transport in the proximal tubule. Although this effect could be secondary to inhibition of the apical sodium-proton exchanger, it has been recently reported that PTH also decreases Na-K-ATPase activity(Garvin and Sundus, 1991).

Cytokines Produced by the immune system and other tissues, cytokines regulate response in lymphocytes (e.g. activation of chemotaxis), as well as diverse functions in other tissues, including PLA2 activation and increased transcription of cyclooxygenase mRNA in endothelial and smooth muscle cells(Hajjar and Pomerantz, 1992). In the kidney interleukin-1 is natriuretic when given intravenously, and it modulates sodium excretion without changes

in renal hemodynamics(Beasley, Diarello and Cannon, 1988), largely by inhibiting its reabsorptionin the collecting duct(Kohan, Merli and Simon, 1989). Inhibition of Na-K-ATPase activity by interleukin-1 has been demonstrated in IMCD cells, and this effect has been implicated in part in the natriuretic action of interleukin-1 in the collecting duct.

Long term regulation of Na-K-ATPase.

As effective as short-term regulation might be in limiting the accumulation of Na^+ in response to low-K^+ treatment, it could not account for the partial recovery of the intracellular concentration of Na^+. It was recognized that this recovery was mediated by a long-term regulatory response: an increase in the number of functional Na-K-ATPase. If the total number of pumps was increased, the overall rate of pumping would be sufficient to reverse the accumulation of Na^+, despite the continued partial inhibition of the pumps by the low external concentration of K^+. If that inhibition was suddenly releived by the addition of K^+ to the medium, the rate of pumping might be expected to greater than normal. Although several attempts were made to test this prediction, the rates of Na^+ extrusion were much too fast to measure reliably. Of course, the rapid exit of Na+ could only be sustained for the few moments required to reduce the intracellular Na^+ concentration.

Recent evidence suggests that regulators shown to have short-term effects on Na-K-ATPase activity may also influence the total pool size of Na-K-ATPase molecules. For example, Baines *et al.*(1992) established that inhibition of dopamine production in animals on a high-salt significantly increased proximal tubule Na-K-ATPase activity and $\alpha 1$ subunits abundance

to the same extent. They concluded that chronic dopamine production reducesd Na-K-ATPase activity by decreasing the number of Na-K-ATPase α_1 subunits.

Factors that influence the number of pumps in the membrane are listed in the following topics:

Steroids

Glucocorticoids administration to adrenalectomized rats have been shown to restore Na-K-ATPase activity in the liver(Miner *et al.*,1980) the submandibular glands(Batolomei *et al.*, 1983), and the cardiac muscle(Klein *et al.*, 1984). Klein *et al.*(1984) demonstrated that corticosterone increased cardiac Na-K-ATPase activity when administered in multiple doses, a single dose, and different dosages. The possibility that enhancement of myocardial Na-K-ATPase activity by corticosterone is mediated by early changes in intracellular Na^+ and K^+ was investigated. The results demonstrated that the increase in Na-K-ATPase activity preceed the changes in intracellular Na^+ and K^+. In addition, corticosterone did not change the transition temperature and activation energy of the reaction in myocardial Na-K-ATPase. This implies that corticosterone does not act by altering the lipid matrix in the microenvironment of the Na-K-ATPase system. Corticosterone also increases renal Na-K-ATPase (Rodriguez, Sinha and Starling., 1981), renal blood flow, and glomerular filtration rate (Kleeman, Levi and Better., 1975). The elevated Na-K-ATPase activity in the kidney could be due to the enhanced glomerular filtration rate and filtered sodium load. One possdibility was that a change in net Na^+ reabsorption could mediate the change in Na-K-ATPase activity on treatment with glucocorticoids. The results indicated that the corticosterone restores renal Na-K-ATPase activity in adrenalectomized rats

prior to any enhanced sodium delivery (Klein and Lo, 1992). Using the monospecific polyclonal antibodies of the rat α and β renal Na-K-ATPase subunits, it was demonstrated that after adrenalectomy, Na-K-ATPase α and β subunit levels decreased 48% and 52%($p < 0.05$), respectively, below those found in control animals. After adrenalectomy, mRNA α and β subunit levels were reduced 61% and 64%, respectively, below control. Within 1 hour of corticosterone administration, mRNA returned to control values(Klein and Lo, 1992). It was concluded that corticosterone restores Na-K-ATPase activity in adrenalectomized rats by acting at the level of renal tubular epithelial cells, prior to any enhanced sodium delivery, to increase the levels of the Na-K-ATPase subunits, at least in part, though increased mRNA content(Klein and Lo, 1992).

Thyroid hormones

Thyroid hormones increase the activity of Na-K-ATPase in various tissues from hypothyroid and euthyroid rats, respectively, including small intestine(Liberman, Asano, and Lo., 1979), skeletal muscle(Asano, Liberman, and Edelman., 1976), cardiac muscle(Philipson and Edelman., 1977) and kidney(Lo, August and Liberman., 1976). Because hypothyroidism results in a decrease in glomerular filtration rate(GFR) and in renal plasma flow(RPF) in the rats, Katz and Lindheimer(1973) suggested that decreased tubular sodium transport is a major determinant of the reduction in Na-K-ATPase activity in the thyroid-deficient rat. A change in net Na^+ reabsorption may mediate the change in Na-K-ATPase activity on treatment with T_3. To test this possibility, experiments were carried out to compare the time course of the changes in GFR, filtered Na^+ load, and cortical Na-K-ATPase after administration of a single dose of T_3 (50 μg/100 g body weight). The cortex

showed an increase in at 24 hours and progressive increases to a peak of 62% at 48 hours. GFR and filtered Na^+ load showed no changes at 24 and 48 hours. At 72 hours, however, significant increases of 62% and 63%(per rat) were observed in GFR and filtered Na^+ load, respectively(Lo,1979). In addition, clearance of PAH(C_{PAH}) and sodium reabsorption(R_{Na}) showed no changes at 24 and 48 hours. At 72 hours, however, significant increases of 41% and 42% (per g kidney wet wt) were observed in C_{PAH} and R_{Na}, respectively(Lo,1981). The results show that the early increase in Na-K-ATPase activity upon T_3 treatment precedes the increase in GFR, filtered Na^+ load, C_{PAH} and R_{Na} suggesting a direct effect of T_3 on the regulating of Na-K-ATPase activity in the hypothyroid rat kidney cortex, rather than a secondary response to a primary increase in filtered Na^+ load(Lo,1979).

Because Na-K-ATPase activity is increased significantly in the transition from the hypothyroid to the euthyroid and hyperthyroid states in a variety of tissues, including rat submandibular gland(Eng and Lo., 1987). The enhancement of Na-K-ATPase activity and energy consumption coupled to active Na, K transport both correlate well with the level of T_3 occupaaaancy of specific nuclear binding sites.T_3 -induced enhancement of Na-K-ATPase results from an increase in the number of enzyme sites rather than a stimulation of pre-existing units(Kim and Smith., 1984). Moreover, the stimulation of Na-K-ATPase activity occurs in the absence of a change in either the affinity of the enzyme for ATP, Na^+, and K^+, or in the energy of activation of Na-K-ATPase(Asano, Liberman, and Edelman., 1976., Philipson and Edelman., 1977). It has also been shown that T_3 increases the abundance of Na-K-ATPase in the renal cortex by stimulating the rate of synthesis of both α and β subunits while their degradation rates remained unaltered(Lo and Edelman., 1976). The enhanced biosynthesis of Na-K-ATPase in the renal cortex was found to be associated with increased in vitro

translational activity of mRNA directing the synthesis of the α-subunits of the pump(Edelman, Pressley and Hiatt., 1984). These data suggest that thyroid hormone stimulation of Na-K-ATPase activity results from an increase in the number of Na, K-pump sites-mediated at the pretranslational level rather than a modification of preexisting sites.

Insulin

Insulin enhances pump activity in adipose tissue and skeletal muscle. The mechanism appears to involve either an unmasking of latent pump sites or an increase in the turnover rate of the pump rather than the synthesis of new pump units. This action of insulin may underlie the ability of this hormone to shift K from the intracellular compartment, an important component of nonrenal K homeostasis.

Circulating inhibitors

Circulating factors that may affect electrolyte balance by inhibiting the Na, K pump have been postulated to play a role in hypertension. Such inhibitors have been referred to as one of potential factors in regulating Na excretion by the kidney.

Erythrocytes have been widely used as a cell model for demonstrating defect of the enzyme in human because the cells are easily obtained and the methods for analysis are simple and not invasive. The number of Na,K pump can be determined by evaluating specific binding of [^{3}H] ouabain to the cells and activity of Na-K-ATPase in erythrocyte membranes as can be by measuring ouabain-sensitive K^+-dependent

generation of inorganic phosphorus(Pi) after hydrolysis of ATP or by measuring the rate of Na^+ or K^+ transport cell membranes. Schmalzing et al, 1981 and Deluise et al, 1985 have demonstrated a constancy of ouabain-binding capacity of erythrocyte membranes in an individual. Decreased number of ouabain-binding site(OBS) indicates reduction of the Na,K pump unit and, consequently, correlates with decreased Na-K-ATPase activity or increased intracellular sodium concentration [Cheng et al., 1984]. Abnormalities in number and activity of Na,K pump on erythrocyte have been reported in various diseases [table 1]

Table 1 The change of erythrocyte Na-K-ATPase status with various diseases

Diseases	Erythrocyte Activity	Na-K-ATPase number	RNa	References
1. Hypertension	D	D	I	Rashman et al, 1986 Quintanilla et al,1988.
2. Hyperthyroidism	D	D	I	Deluise, Flier, 1983, Dasmahapatra et al,1989
3. Chronic renal failure	D	D	I	Cheng et al, 1984, Kaji, Thomas, 1987.
4. IDDM and NIDDM	D	D	-	Finotti, Palatini,1986, Baldini et al, 1989.
5. Hypothyroidism	I	I	D	Dasmahapatra et al,1989 Deluise, Flier, 1983.
6. Kwarshiorkor	I	I	-	Narayanareddy, Kaplay, 1982
7. Myotonic dystrophy	I	I	-	Mishra et al, 1980.
8. Sickle cell anemia	I	I	I	Luthra, Secirs, 1982, Izumo et al, 1987.
9. Hypokalemia	I	I	D	Rubyton, Morgan, 1983, Cumberbatch, Morgan, 1983

D = decreased, I = increased, - = no data available

Physiology of exercise

Metabolism-Production of energy for work

Energy is defined as the ability or capacity to perform work, whereas *work* is defined as the application of a force through a distance. For example, lifting the book from the desk to the bookshelf will constitute a certain amount of work requring a certain amount of energy. Energy and work are inseparable.

The immediate energy source- ATP

The human eat food for energy. However, the immediate energy needed at rest or to perform work does not come directly from the food we eat, but rather, from a chemical compound called *adenosine triphosphate*, or more simply, ATP. ATP is stored in various quantities in most living cells, particularly in muscle cells. As its name implies, the structure of ATP consists of a complex component called adenosine and three simpler component called phosphate groups. When ATP is chemically broken down, between 7 and 12 kilocalories(kcal) of energy are liberated. Chemically, this can be represented as:

$$ATP \longleftrightarrow ADP + Energy$$

This energy becomes immediately available to support any work required by the cell, such as muscular contraction. In other words, the body's immediately useful supply of energy is that which is released when ATP is chemically broken down.

The principle of coupled reactions

The arrow pointing to the left in the above chemical equation indicates that energy from another source is required in order to resynthesize ATP. The energy for resynthesizing ATP comes from three different series of chemical reactions that also take place within the body. The energy released from any one of these series of reactions, such as from the breakdown of the food we eat, is coupled with the energy needs of the reaction that resynthesizes ATP. In other words, the two series of reactions are functionally linked together such that the energy released by the one is always used by the other. Biochemically, this is referred to as coupled reactions and is the fundamental principle involved in the metabolic production of ATP.

Metabolic production of ATP

As just mentioned, the energy needed for ATP resynthesis comes from three different series of reactions. Although all three series of reactions take place within the cell, two of them do not require the presence of oxygen and are thus *anaerobic*. The third series of reactions can operate only under *aerobic* conditions, i.e., in the presence of oxygen. During exercise, both anaerobic and aerobic series of reactions are important sources of ATP energy.

One of the anaerobic series of reactions is referred to the *phosphagen system*, and the other is termed *anaerobic glycolysis*. The aerobic source is referred to as *oxidative phosphorylation*.

The Phosphagen system

Phosphocreatine(PC) is a chemical substance closely related to ATP. Like ATP, PC is also stored in muscle cells, and when it is chemically broken down, a large amount of energy is released(at least 12 kcal). The end products of this breakdown are creatine(C) and inorganic phosphate(P_i). Chemically:

$$PC \longrightarrow P_i + C + \text{Energy}$$

The released energy, of course, is coupled with the energy requirement necessary for the resynthesis of ATP. In other words, as rapidly as ATP is broken down during muscular contraction, it is continuously resynthesized from ADP and P_i by the energy liberated during the breakdown of the stored PC. Again on chemical terms:

$$PC \longrightarrow P_i + C + \text{Energy}$$
$$ATP \longleftarrow ADP + P_i$$

The muscular stores of ATP and Pc (collectively referred to as phosphagens) are very small, only about 20 mM per kg of muscle. For an average-size male, this would be a total body store of about 0.6 mole, and for an average size female, about 0.3 mole. Thus, the amount of energy available from this system is quite limited. In fact, in a 100-meter dash, the phosphagen stores in the working muscles would probably be empty by the end of the run. However, the usefulness of the stored phosphagens lies in their rapid availability (power) rather than in their quantity. Although this is not important at rest, it is extremely important with respect to the kinds of physical

activities performed. Activities such as sprinting, jumping, swinging, kicking, and other similar movements that require only a few seconds to complete, are all dependent upon the stored phosphagens for their primary energy source. For example, in sprinting 100 meters, it can be estimated from energy-cost data that a total of only 0.43 mole of ATP is required, but at an average rate of utilization of 2.6 moles per minute. As will be pointed out later, such a power requirement can be met only by the phosphagen system.

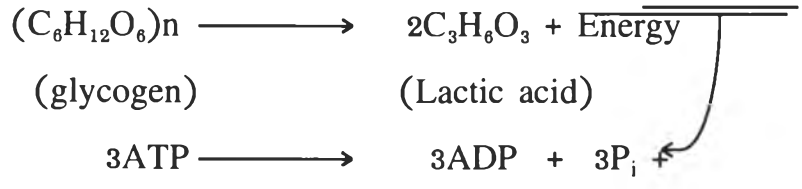
Anaerobic glycolysis

The term *glycolysis* means to break down glycogen, and, as mentioned earlier, anaerobic means without oxygen. Thus, as the name implies, in anaerobic glycolysis, glycogen or glucose is broken down without oxygen, and energy is released for ATP resynthesis. However, since oxygen is not required, glycogen can be broken down only partially, with the end product being *lactic acid.* When lactic acid(LA) accumulates in the muscles and blood to very high levels, temporary muscular fatigue results. This, of course, is a very definite limitation during exercise.

Another limitation that also stems from oxygen not being required is that only a few moles of ATP can be resynthesized from glycogen during anaerobic glycolysis, compared with the yield possible when oxygen is present, for example, during anaerobic glycolysis only 3 moles of ATP can be resynthesized from breaking down 180 grams of glycogen. As will soon see, in the sufficient

oxygen, the complete breakdown of the same amount of glycogen yields 39 moles of ATP.

The summary equations for ATP resynthesis from anaerobic glycolysis are:



During exercise, the userful ATP production from anaerobic glycolysis is actually less than 3 moles of ATP shown in the above equation. The reasdon for this is that during heavy exercise, the muscles and blood can tolerate the accumulation of only about 60 to 70 grams of lactic acid before fatigue sets in. If all 180 grams of glycogen were broken down anaerobically during exercise, 180 grams of lactic acid would be formed. Therefore, from a practical viewpoint, only about 1 to 1.2 moles of ATP can be manufactured from anaerobic glycolysis during heavy exercise before lactic acid in blood and muscle reaches exhausting levels.

Fluid and electrolyte metabolism in exercise

In exercise, a series of reactions are initiated that serve to convert chemically-bound energy into mechanical work. In this process, two-thirds of this chemically-bound energy will be lost as heat, while no more than one-third can be transformed into mechanical work. Thus, an oxygen uptake of 3 liter/min will be accompanied by a heat production rate of about 14.5 kal/min (875 kal/hr). During exercise, maximal sweat rates may be as high as 20 to 25 ml/min(1 to 1.5 liter/hr), thereby providing for a maximal loss of heat by

evaporation of 12 to 15 kal/min (900 kal/hr). Retention of no more than 70 kal may increase body temperature by 1°C. Therefore, this evidence indicates an important role of the thermal exchange system in exercise: in its absence, exercise willbe limited to very short periods(Nadel,1979).

During exercise, the heat produced will flow down a temperature gradient from muscle to body core and further to the skin: from there, heat will be dissipatedinto the environment. The start of muscular exercise is followed quickly by a rise in body core temperature, brought about by a high rate of blood flow from muscle to body core induced by the increased requirements of muscle for oxygen. The rise of body core temperature is primarily the result of convective heat transfer by blood flow, rather than by passsive conductance across body tissues. The subsequent dissipation of heat from the skin to the environment is achieved by conduction, convection, radiation, or evaporation. However, an increasing environmental temperature will reduce the temperature gradient between skin and environment. Consequently, heat loss by conduction, convection, or radiation will also decrease, and dissipation of metabolic heat then hinges on evaporation of sweat.

The ability to sweat is influenced by both physical training(Gisolfi and Robinson, 1969., Piwonka and Robinson, 1967) and acclimatization to heat(Wyndham, 1967). Within ten days of rigorous training an increase in the sweat rate is noted, and the sweat produced will have low concentration of sodium and chloride. If moderate exercise training is then continued to for ten more days in a hot environment, the effects of acclimatization can be observed. Acclimatization causes a decrease in the threshold of sweating, but there is minimal effect on the gain of the system(Nadel, et al., 1974). Thus, an individual who is both trained and acclimatized starts to produce sweat rate than before. He will therfore store less heat during exercise.

Total water loss

It follows, then, that an increasing intensity of mechanical work will be paralleled by an increasing water loss from the body. This is primarily the result of increased sweat production. When trained and acclimatized athletes ran for one hour at room temperature and at 37-74% of their aerobic capacity, more than 90% of the fluid loss from the body was attributable to sweat. At the same time, urine production was decreased. Metabolic water production rose, but it replenished no more than 6-10% of water losses(Pivarnik, Leeds and Wilkerson., 1984).

The electrolyte concentrations in sweat vary: they depend on the state of the state of hydration(Horstman and Horvath., 1972), physical training, and acclimatization to heat (table 2). The sweat of a trained athlete is usually hypotonic, with low concentrations of sodium and chloride; however, the concentrations of potassium, magnesium and calcium essentially remain unchanged.

Table 2. Electrolyte concentration in sweat during exercise (mmol/liter)

Sodium	25-80
Chloride	15-70
Potassium	4-8
Calcium	0.4-1.3
Magnesium	0.2-0.5

Plasma volume in exercise

There is no clearcut correlation of body water loss and subsequent reduction of plasma volume, and conflicting data have been reported on the effects of exercise on plasma volume. These discrepancies may be explained by differences in one of the following: state of hydration, mode of exercise, duration and intensity of exercise, ambient temperature and heat acclimatization, and physical training.

Mode of exercise

There are many ways by which different modes of exercise could be involved: for instance, the changes in vascular volume following treadmill vs a bicycle exercise differ. In this respect, it has been found that bicycle exercise will induce anaerobic metabolism at a lower level of maximal oxygen consumption than will treadmill exercise(Koyal, et al., 1976). On the other hand, endurance athletes such as cross-country skiers(Astrand and saltin., 1964) show little or no reduction of blood volume at the end of the contest. This occurs despite a water loss corressponding to 3-5% of body weight. In contrast, bicycle exercise at 50% of maximal O_2 uptake will be associated with a reduction of plasma volume by 6-12%; plasma volume will return to baseline within 10 to 20 minutes after cessation of exercise.

However, at maximal work intensity on a cycle ergometer, a plasma volume reduction as high as 17.2 $\pm$ 1.2% has been observed(Mohsenin and Gonzalez., 1984); this was accompanied by increased concentration of plasma sodium, chloride, lactate and protein, as well as by a rise of plasma osmolality

and colloid osmotic pressure (table 3). It is of interest that the plasma potassium concentration increased regularly by 1-2 mM/liter during exercise (Affrime, Lowenthal and Falkner., 1981., Carlsson, et al ., 1978). This change has been attributed to β-adrenergic stimulation(Carlsson, et al., 1978) or to a H^+/K^+ exchange during the acidosis of exercise(Kilburn, 1966).

The reduction of plasma volume followed the onset of exercise such as isotonic handgrip exercise almost immediately, and before any production of sweat. It is correlated to the work load; a maximal reduction of around 18% is reached when relative O_2 uptake exceed 65% of maximal oxygen uptake (Greenleaf, et al., 1977, 1979., Lundvall, et al., 1972., Sjogaard and Saltin., 1982). Two factors, both of which are proportional to work intensity(Kjellmer, 1964., Miles, et al., 1983), have been implied in the genesis of the initial reduction in plasma volume. These are an increase in mean capillary pressure

Table 3. Hemoconcentration in exercise with maximal work intensity for 3 minutes on a cycle ergometer (Mohsenin, 1984)

	Before	After
Plasma volume		17.2 ± 1.2%
Sodium mmol/liter	142.6 ± 0.5	148.1 ± 1.0
Chloride mmol/liter	101.8 ± 0.6	104.6 ± 0.9
Lactate mmol/liter	1.4 ± 0.2	14.0 ± 1.5
Osmolality mOsm/kg	283 ± 2	299 ± 3
Protein g/L	7.0 ± 0.2	8.1 ± 0.3
Colloid osmotic pressure *mm. Hg*	25.1± 0.6	30.6 ± 1.4

due to an increased blood flow(Lundvall, 1972) and an increase in cellular osmolality because of glycogen breakdown and of lactic acid production. In

the steady state of exercise, these driving forces appear to reach a new equilibrium within minutes. This is brought about by balancing factors, such as increased interstitial pressure and elevation of intravascular oncotic pressure (Mohsenin and Gonzalez., 1984). As a net result, direct measurement of muscle water content have demonstrated a 10% increase in exercise(Sjogaard and saltin., 1982).

During exercise, plasma potassium increases proportionally more than the plasma level of other electrolytes during exercise chiefly reflecting of potassium from the working skeletal muscles. When exercise is performed at moderate intensity, mixed venous potassium concentration increases by approximately 0.5 meq/L. At higher work levels, the rise in mixed venous potassium concentration may amount to as much as 1 or 2 meq/L. In extreme circumstances, especially when work has been sustained for a long period of time at high intensity, potassium concentration invenous blood have been recorded between 9 and 10 meq/L(Greenleaf, et al., 1979) (fig. 7). Such changes are of sufficient magnitude to produce important electrocardiographic changes of hyperkalemia. Because of such events, it is possible that hyperkalemia during exercise could be a cause of sudden death.

Gisolfi and Robinson, 1969, provided evidence that exercise-induced hyperkalemia canbe reduced in human subjects by moderate training. according to that experimental, venous plasma concentration from 15 conscripts was followed during exercise on a training bicycle before and after 10 weeks of moderate physical training and a putative relationship with skeletal muscle Na-K-ATPase was evaluated. Peak plasma potassium concentration obtained at exhaustion was 6.1 ± 0.2 and 5.6 ± 0.2 mmol/L before and after training, respectively. Neither peak values nor after training was correlated to the 3H-ouabain binding site (Na-K-ATPase) concentration in vastus lateralis muscle. The moderate improvement of capacity to clear intracellular potassium during

exercise may be due to increasded activity of existing Na-K-ATPase in skeletal muscle fibers.