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The Fourth Datta Lecture

The energy coupled exchange of Na^+ for K^+ across the cell membrane. The Na^+,K^+-pump

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1. INTRODUCTION

In a Croonian Lecture in 1946 entitled 'The active and passive exchange of inorganic ions through the surfaces of living cells and through living membranes generally', Krogh concluded: 'The power of active transport of ions is of common occurrence both in the vegetable and the animal kingdom and is possibly a general characteristic of the protoplasmic surface membrane' [1].

The use of isotopes of Na^+ and of K^+ which was introduced in biology in the late 1930s [2], showed that the mammalian cell membrane is not only permeable to K^+ but also to Na^+. Since K^+ as well as Na^+ seemed to be present at least for the major part as freely dissociable ions in the protoplasm, an energy dependent extrusion of Na^+ is necessary in order to explain that Na^+ is at a lower electrochemical potential inside than outside the cell.

Conway, who advocated a low Na^+ permeability of the muscle cell membrane was concerned about the energetic consequences of an active Na^+ transport. In a response to Kroghs criticism in his Croonian Lecture of Conway's view, Conway replied in a paper in Nature in 1946: 'Krogh... considers the apparent impermeability to sodium as due to an active extrusion, sodium ions entering the fibers as fast, if indeed not faster than potassium ions. The following may then be considered: the minimal energy required for extrusion of sodium ions from the normal frog's sartorius, if sodium enters as fast as potassium.' A calculation showed that if sodium is entering as fast as potassium the energy requirement for the sodium extrusion is about twice the resting metabolism of the muscle [3].

Ussing measured the rate of sodium exchange across the muscle membrane with isotopes and came to the same result, that the energy from the metabolism of the muscle was not high enough to account for the sodium flux [4]. The answer to the problem was given by Ussing, namely that besides the active transport of sodium there is a sodium-sodium exchange, an exchange diffusion, which is a one-for-one exchange and which energetically is neutral.

But even if the metabolism can provide for the energy requirement for the active transport, it is pertinent to ask, what is the purpose of having a membrane which is permeable to Na^+ and K^+ and spend energy to keep Na^+ out of and K^+ into the cell? With the present knowledge the answer is that the sodium pump is an energy transducer which converts the chemical energy from the hydrolysis of ATP into a gradient for sodium and potassium, and that these gradients are used as free energy sources for a number of other processes like formation of the membrane potential, for the de- and repolarisation of the membrane potential, for cell volume regulation, for transport of glucose and amino acids into cells against concentration gradients, for co- and countertransport of ions across the cell membrane and thereby of importance for cell homeostasis, and for transepithelial transport in intestine, kidney and secretory glands. The sodium pump thus indirectly makes the energy from the hydrolysis of ATP available for all these processes, i.e., the active transport is not just a compensation for the leak of the cell membrane for sodium and potassium, a waste of energy, but has a key function in the regulation of the exchange of substances between the cell and the surroundings, in transepithelial transport and in transmission of information.

2. ACTIVE TRANSPORT

The concept of active transport developed during the

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forties and fifties from experiments on muscle, frog skin, red blood cells and nerves (for reference see the extensive review by Ussing [5]). It is interesting to see that the development with the different tissues runs parallel but has little cross reference.

Early experiments showed a reciprocal relationship between the exchange of Na^+ and K^+. Frog muscle cells soaked in K^+ free Ringer gain Na^+ and lose K^+. When transferred to a K^+ containing medium Na^+ is excluded and K^+ taken up [6]. Red blood cells lose K^+ and gain Na^+ by cooling and this is reversed by return to 37°C [7,8]. It was uncertain whether this meant an active transport of Na^+ or of K^+. In his Croonian Lecture Krogh was of the opinion that in the red blood cells it is K^+ which is actively transported, while in the muscle it is Na^+ [1]. By introducing the technique of short-circuiting the frog skin, Ussing and Zerahn in 1951 showed that the current through the skin is carried by an active transport of Na^+ [9]. However, following experiments on giant axons [10] and on red blood cells [11–13] showed that the efflux of Na^+ decreases by removal of extracellular K^+ and $K_{0.5}$ for K^+ for the saturable component of K^+ influx and for Na^+ efflux is the same [13] suggesting a linkage between an active K^+ influx and an active Na^+ efflux, i.e. both ions are actively transported [13]. In 1957, Post and Jolly measured the coupling ratio in red blood cells and found it to be close to 3 Na^+ transported out of, for 2 K^+ transported into the cell [14]. This has been confirmed for red blood cells [15] and found in nerve [16], for purified kidney enzyme reconstituted into liposomes [17–19], in the frog skin [20] and in a number of other tissues (for references see [19]). It is, however, an unsettled question whether this stoichiometry is fixed under all conditions and in all tissues, but it is generally agreed that more sodium is pumped out of than potassium into the cell in each pump cycle.

3. SUBSTRATE FOR THE ACTIVE TRANSPORT

In 1951, Maizels showed that glucose supports the reuptake of K^+ and excretion of Na^+ of red blood cells when rewarmed after cold storage, suggesting that glycolysis supports the active transport [21]. The effect of metabolic inhibitors on the active transport in red blood cells pointed to energy-rich triphospate esters as the energy source [22], and that the substrate is ATP was suggested from experiments by Straub in 1953 [23] who showed that there was no active transport when formation of ATP is inhibited in red blood cells. Straub described a method for reversible opening of red blood cells, which made it possible to change their content [23]. The method was used by Gárdos in 1954 [24] to load red blood cells with ATP and to show that ATP supports the uptake of K^+. This was substantiated by Hoffman [25] who showed that there is a specific requirement for ATP for the active transport in red blood cells in the sense that ATP cannot be replaced by ITP, GTP or UTP. The effect of metabolic inhibitors on the active transport in giant axons suggested that also in nerves are energy rich phosphate esters the energy source [10], and microinjection of ATP into poisoned giant axons showed that ATP supports the active efflux of Na^+ [26]. Of the phosphate donors it is only ATP and to a lesser extent deoxy-ATP which has this effect [27].

4. ENERGY REQUIREMENT AND ELECTROGENIC EFFECT

In 1956 Zerahn [28] measured 18 molecules of sodium transported per molecule of oxygen consumed above the resting level in the short-circuited isolated frog skin. With a P/O ratio of 3 for oxidative phophorylation this means 3 Na^+ transported per ATP hydrolysed. In agreement with this Sen and Post in 1964 [29] in experiments with red blood cells found 1.16 ATP hydrolysed per 3 Na^+ transported out of and 2 K^+ transported into the cell. The 1:3:2 stoichiometry has since been found by others in experiments on red blood cells, and in the above mentioned experiments with the purified reconstituted enzyme. It means that the efficiency of the transport is high; whith a normal membrane potential and normal physiological Na^+ and K^+ concentrations it is 75–85%.

More sodium transported out of than potassium into the cell raises the question whether the transport is electroneutral or electrogenic. Is there a transport of another cation together with the 2 K^+, or an anion with the 3 Na^+? Or is there a net transfer of positive charge from inside to outside the cell in each transport cycle?

The first evidence that the Na,K-pump is electrogenic was given by Kernan in 1962 [30]. Frog sartorius muscles which were Na^+ loaded and K^+ depleted by soaking in cold K^+ free Ringer solution hyperpolarized beyond the K^+ equilibrium potential when transferred to a K^+ containing Ringer solution. From injection of Na^+ into the ganglion of *Helix aspera* with the membrane potential clamped, Thomas [16] showed that the current necessary to clamp potential is opposite to and equal to the current generated by the pump. It amounts to about 0.3 of the injected charge indicating that about ⅓ of the sodium ions are transported out electrogenically, while ⅔ are transported electrically neutral in agreement with a 3 Na^+ to 2 K^+ ratio. The electrogenic effect of the pumping has been confirmed from experiments on a number of different tissues [31], and from experiments with the purified reconstituted system [19]. Recent experiments suggest that it is the Na^+ translocation step which is electrogenic, carries one net positive charge, while the K^+ translocation step is electroneutral [32].

5. NATURE OF THE ACTIVE TRANSPORT SYSTEM

In 1895 Overton observed that the rate of penetration of organic substances into the cell protoplasm increased with their lipid solubility, suggesting that the protoplasm is surrounded by a barrier of lipids [33]. Thirty years later, in 1925, Gorter and Grendel extracted the lipids from red blood cells, spread the lipids as a monomolecular layer on a water surface, and measured the area at a surface pressure at which the monolayer started to exert a resistance to compression [34]. The area of the monolayer was close to twice the surface area of the red blood cells from which the lipids were extracted. The conclusion was that the lipids in the membrane are ordered in a 2 molecule thick structure with the head groups facing the waterphase on the two sides of the membrane and with the hydrocarbon chains in contact, a bimolecular layer. However, the surface tension of invertebrate eggs and other cells is 0.1–0.2 dyne/cm, which is 2 orders of magnitude lower than for a lipid-water interphase. A candidate for this effect was surface spread protein, and this lead Danielli and Dawson to suggest in 1935 that the cell membrane was a bilayer of oriented lipids with an adsorbed layer of extended protein on each side of the bilayer [35]. They also introduced a layer of non oriented lipids in between the bilayer.

A bilayer of lipids has a very low permeability to ions. A problem was therefore to explain the cation permeability, not to speak of active transport. In 1940 Lundegårdh [36] suggested that the ions which combined with the lipids in the monolayer are transferred across the membrane by turning of the lipid molecules. Lundegårdh had given good evidence that anions are transported actively into the protoplasm in plant roots, and suggested that the release of the anions to the root protoplasm from the lipids is dependent on expenditure of energy, while the release of the cations, which are not transported actively, is dependent on electrostatic forces [36].

The idea of a sodium pump in the membrane was introduced by Dean in 1941. Referring to experiments by Heppel (1938), by Heppel and Schmidt (1939) and by Steinbach (1940) (for reference see [37]) on the exchange of Na^+ and K^+ in muscle Dean, in a paper 'Theories of electrolyte equilibrium in muscle' concluded, 'that the muscle can actively move potassium and sodium against concentration gradients..., this requires work. Therefore there must be some sort of a pump, possibly located in the fiber membrane, which can pump out the sodium or, what is equivalent, pump in the potassium' [37].

A suggestion of the nature of a sodium pump came from experiments on crab nerve membranes in 1957 [38]. They were initiated by an observation by Libbet in 1948, that the sheath part of giant axons has a Mg^{2+} activated ATPase [39]. Also in the crab nerve membrane there is an ATPase which requires Mg^{2+} for activity. Characteristic for this enzyme is, however, that it is activated by a combined effect of Na^+ and K^+. From this and referring to the observations by Hodgkin and Keynes [10] that triphosphates are the substrate for the transport in giant axons it was suggested that the membrane bound ATPase is involved in the active transport of Na^+ and K^+ across the cell membrane [38]. Gárdos' observation that ATP supported the active uptake in red blood cells was unknown to the author.

An important observation in this context was made by Schatzmann in 1953 who showed that cardiac glycosides inhibits the active transport in red blood cells. Since there is no known effect of these drugs on metabolism it was concluded that the inhibition of the transport is due to an inhibition of the sodium pump [40]. This has since been confirmed, and cardiac glycosides have been very important tools in all subsequent work on active transport of Na^+ and K^+.

Due to lack of knowledge of the effect of the cardiac glycosides on the active transport of Na^+ and K^+, the inhibitory effect was not tested in the first publication on the $Na^+ + K^+$ activated ATPase, but in a following paper in 1960 it was shown that *g*-strophantin (ouabain), which is the most water soluble of the cardiac glycosides, inhibits the activity [41]. It was furthermore shown that there is a specific requirement for Na^+ for activation while all the other monovalent cations have a K^+ effect but with lower affinity and with lower activating effect than K^+. The experiments also suggested that the activation by K^+ is at a site different from the site for Na^+, and that K^+ competes for the effect of Na^+ just as Na^+ competes for the effect of K^+. All these observations supported the view that the ATPase was involved in the active transport of Na^+ and K^+. Important further support was given from experiments on red blood cells by Post and coworkers [42], who showed that the Na^+ and K^+ effect on the fluxes of the cations correlated to their effect on the ATPase activity in broken membranes.

In the early 1960's so much evidence was collected from many laboratories that it was possible in a review paper in 1965 ([43] and references therein) to conclude that the Na,K-ATPase fulfills the following requirements to a system responsible for the active transport of Na^+ and K^+ across the cell membrane:

(1) it is located in the cell membrane,
(2) on cytoplasmic sites it has a higher affinity for Na^+ than for K^+,
(3) on extracellular sites the affinity for K^+ is higher than for Na^+,
(4) it has enzymatic activity and catalyzes the hydrolysis of ATP,
(5) the rate of hydrolysis depends on cytoplasmic Na^+ as well as extracellular K^+,

(6) it is found in all cells that have a coupled active transport of Na^+ and K^+,
(7) the effect of Na^+ and of K^+ on transport in intact cells and on the activity of the isolated enzyme correlates quantitatively, and
(8) the enzyme is inhibited by cardiac glycosides; the inhibitory effect on the active fluxes of the cations correlates with the inhibitory effect on the isolated enzyme system.

The enzyme system was named the (Na^+ + K^+) activated ATPase, or Na,K-ATPase.

An unsolved problem was whether the Na,K-ATPase is only the catalytic part of the transport system which converts the chemical energy to a movement of a carrier for Na^+ and K^+ in the membrane, the engine so to speak. Or is it the complete transport system, i.e. can the Na,K-ATPase activity measured in the test tube be taken as an indication of transport through the membrane fragments? The answer that it was the complete system was given when it became possible to reincorporate the purified Na,K-ATPase in the membrane of liposomes and measure transport as well as hydrolysis [17].

The identification of the pump as a membrane bound enzyme system brought active transport into the realms of biochemistry. But where and how to place a protein in the lipid bilayer of the membrane? In the unit membrane as it was formulated by Robertson in 1959 [44] the proteins are not in the bilayer, but arranged asymmetrically on the two sides of the membrane. The pump must, however, have access to both sides of the membrane. An answer to the problem was given by the fluid mosaic model of the membrane formulated by Singer and Nicolson in 1972 [45], in which globular proteins with their non-polar parts are embedded in the fluid bilayer with the polar part facing the interior as well as the exterior side of the membrane. The proteins form the pathways, channels, or carriers for the non lipid substances and ions for membrane passage. The proteins have lateral mobility, but do not flip-flop.

6. THE Na,K-ATPASE

The characteristic feature of the Na,K-ATPase is that in the precense of ATP and Mg^{2+} it is activated by a combined effect of Na^+ on cytoplasmic sites and of K^+ on extracellular sites, and that cytoplasmic K^+ inhibits by competing for binding of cytoplasmic Na^+, just as extracellular Na^+ inhibits by competing for binding of extracellular K^+. The apparent affinity for cytoplasmic Na^+ is with saturating ATP about 3 times higher than for cytoplasmic K^+, while the apparent affinity for extracellular K^+ is about 100 times higher than for extracellular Na^+. Due to this large difference in the Na^+–K^+ affinity ratios on the two sides of the membrane it is possible in the test tube with broken membrane pieces with the same concentrations of Na^+ and K^+ on the two sides of the membrane to observe the combined effect of the two cations (Fig. 1); and to test the extracellular K^+-Na^+ competition under conditions where the cytoplasmic sites are practically saturated with Na^+ and also to test the cytoplasmic Na^+-K^+ competition with the extracellular sites practically saturated with K^+.

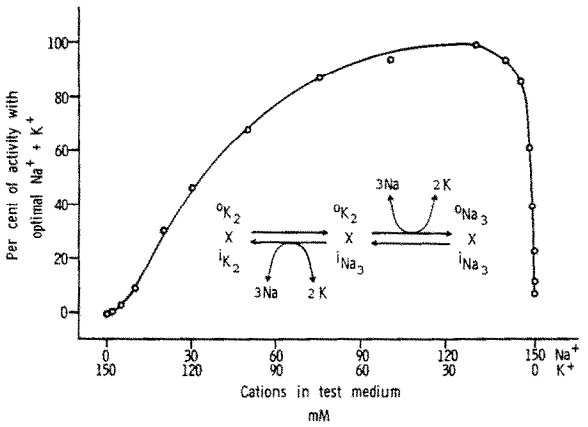

Fig. 1. ATP hydrolysis as a function of $Na^+ + K^+$ concentrations by Na^+,K^+-ATPase. The enzyme containing plasma membrane pieces are isolated from ox brain. Tested in 30 mM histidine HCl buffer, pH 7.4, 37°C with 3 mM ATP and 3 mM Mg^{2+}. The symbol i is for inside (intracellular), o for outside (extracellular).

Na^+ cannot be replaced by any other monovalent cation for activation on the cytoplasmic sites, whereas K^+ can be replaced by any of the other monovalent cations for activation on the extracellular sites including Na^+. The apparent affinity for extracellular activation is $K^+ > Rb^+ > NH_4^+ > Cs^+ > Li^+ > Na^+$ [41]. With Na^+ on the cytoplasmic as well as on the extracellular sites the activity is about 5% of the activity with optimal Na^+ plus K^+ (Fig. 1).

The cytoplasmic activation by Na^+ follows an s-shaped curve as does the activation by extracellular K^+, suggesting that more than one Na^+ and one K^+ is necessary.

Fig. 1. shows that with the normal intracellular Na^+ concentration, 10–20 mM, and about 120 mM K^+, the Na^+ activation is 15–20% of maximum, and with the normal extracellular about 4 mM K^+ and 140 mm Na^+, the K^+ activation on the extracellular sites is about 85% of maximum. This means that in the intact cell membrane the transport system operates with an activity which is a low fraction of maximum, it has a big reserve power. The turnover is with optimal ligand concentrations at 37°C about 10 000 per min.

The enzyme system is tightly bound to the membrane, and can be purified in the membrane by removal of other proteins from the membrane by treatment with ionic detergents like DOC and SDS under well controlled conditions (temperature, pH, time, detergent concentration) [46]. By choosing tissues with a high capaci-

ty for active transport like outer medulla of kidney, rectal glands from shark, electric eel tissue, or salt glands from marine birds, the enzyme can be purified to the extent that practically all the protein in the membrane is Na,K-ATPase. It consists of two polypeptides, α with 1016 amino acids and a molecular weight of 112 000 and β which is a glycoprotein with 302 amino acids and a protein molecular weight of 38 000. The ratio between the two on mole basis is 1:1. α is the catalytic unit, β seems to be necessary for insertion in the membrane. Attempt to separate α and β always leads to inactivation, but so far no function has been attributed to β in the transport reaction.

From a partly purified membrane preparation, the Na,K-ATPase is dissolved in an active form by non ionic detergents like lubrol or $C_{12}E_8$ [47]. The oligomeric structure of the Na,K-ATPase is under debate [48]. The preferred structure in the membrane seems to be an $(\alpha\beta)_2$ structure. However, the $C_{12}E_8$ dissolved enzyme which at the lower detergent concentration is $(\alpha\beta)_2$ is dissociated into $(\alpha\beta)$ by a higher concentration of detergent and is still catalytically active but very labile [49]. However, the negative cooperative interaction between binding of K^+ and ATP which is observed with the membrane bound enzyme and with the dissolved enzyme in the $(\alpha\beta)_2$ structure disappears by dissociation to $(\alpha\beta)$ [50]. It has so far not been possible to reconstitute the system in liposomes under conditions in which the system without doubt is in an $(\alpha\beta)$ form and measure if it has translocation properties. Recent radiation inactivation experiments on the membrane bound system suggest that the activity of $(\alpha\beta)$ requires close contact with another $(\alpha\beta)$, i.e. an $(\alpha\beta)_2$ structure, but $(\alpha\beta)$ has the normal Na,K-ATPase activity even if the other $(\alpha\beta)$ is partly inactivated [51]. However, the effect of partial inactivation of the one $(\alpha\beta)$ on transport by the other of an $(\alpha\beta)_2$ has not been measured. Judged from these experiments a structural contact between two $(\alpha\beta)$ seems to be necessary, but the above mentioned disappearance of cooperativity when the dissolved $(\alpha\beta)_2$ is dissociated into $(\alpha\beta)$ shows that the structural contact also has a functional implication.

Lipids are necessary for the activity [52]. The detergent-dissolved fully active enzyme has about 50 molecules of phospholipids and about 40 molecules of cholesterol per enzyme molecule [53]. This may represent a maximum number, but if the detergent used for solubilization can replace some of the necessary lipids it may represent less than the necessary amount. Fifty phospholipid molecules is about what is necessary for a bilayer annulus around the molecule [54]. There seems to be a requirement for acid phospholipids for activity [55] and in agreement with this the presence of the acid phospholipid, phosphatidylinositol, is necessary for full recovery of the activity after reconstitution into liposomes of $C_{12}E_8$ dissolved enzyme [56]. However, as only 1 of the 50 phospholipids in the active detergent dissolved $(\alpha\beta)_2$ preparation is acid, one per molecule seems to be enough [53].

The lipids anchor the protein in the membrane and are so to say the solvent for the protein; but beside this, what is their effect? Has this solvent a structural effect on the protein, and is it only in this solvent that the protein can undergo the conformational transitions which gives the translocation of the cations?

7. CRYSTALLIZATION

With the enzyme purified in the membrane to the extent that practically all the protein in the membrane is Na,K-ATPase, the enzyme can be crystallized in the membrane in two dimensions. Dependent on the conditions for crystallization, the enzyme is crystallized in an $(\alpha\beta)$, or in an $(\alpha\beta)_2$ form [57]. It is not possible from the conditions needed to form the monomer or the dimer to find support for conclusions regarding the oligomerization of the noncrystallized system in the membrane.

A three-dimensional model of the Na,K-ATPase has been constructed from a Fourier analysis of tilted membrane preparations of negatively stained $(\alpha\beta)_2$ crystals [58,59]. The resolution is about 20 Å. The molecule consists of two symmetrical rodlike structures which each seem to consist of an $(\alpha\beta)$ protomer with a height of about 100 Å perpendicular to the membrane. The two protomers are separated by a 20 Å wide cleft, but on the cytoplasmic side connected by an about 20 Å high area, a part of which seems to be inside the lipid bilayer on the cytoplasmic side. The molecule protrudes about 40 Å on the cytoplasmic side of the lipid bilayer, and about 20 Å on the extracellular side. The intramembraneous part of each protomer is about 25% of the mass of the α as well as of the β subunit. Nearly all of the rest of the α unit is on the cytoplasmic side while that of the β unit is on the extracellular side of the lipid bilayer.

8. AMINO ACID SEQUENCE

The amino acid sequence of the α as well as of the β unit has been determined from cDNA [60]. The α chain consists of 1016 amino acids and has 8 hydrophobic regions. The N-terminal hydrophilic region which consists of 92 amino acids is on the cytoplasmic side. This is followed by 4 transmembrane segments with a cytoplasmic loop between segments 2 and 3 of about 145 amino acids. Between transmembrane segments 4 and 5 there is a bulky cytoplasmic part of about 439 amino acids which contains the ATP binding site and the phosphorylation site (Asp-369). The location of the C terminal is uncertain and so is the total number of transmembrane segments, 6, 7 or 8. The protein part of the β chain consists of 302 amino acids with one transmembrane segment located near the

cytoplasmic N-terminal and with the major, hydrophilic part of the molecule on the extracellular side.

9. ISOFORMS

In 1979, Sweadner showed that there are two isoforms of the α-chain, α and $\alpha(+)$ in brain tissue [61]. It was named $\alpha(+)$ because it migrated slower than α in SDS gel electrophoresis [61]. This is not due to a higher molecular weight as originally assumed. A third isoform, αIII, is also found in brain tissue and the notation used now is α_1, α_2 and α_3. The isoforms are found but in different proportions in many other tissues. They differ in their sensitivity towards cardiac glycosides; for a given tissue α_2 and α_3 being more sensitive than α_1. However the sensitivity towards cardiac glycosides for a given isoform differs for different species, and also for different tissues, which means that sensitivity towards cardiac glycosides for a given preparation cannot be used to identify the isoform. The M_w of the three isoforms is closely the same, but there are differences in the amino acid composition [61]. Substitution of the neutral glutamine and asparagine in the extracellular junction between the first and the second transmembrane segment with the charged amino acids arginine and aspartate decreases the sensitivity for the cardiac glycosides. There are quantitative differences in ligand affinities of the three isoforms. Apart from an increase in affinity for Na^+ by insulin of the α_2 isoform in muscle and in adipocytes no differences in function have been ascribed to the three isoforms [61].

10. REACTIONS WITH ATP AND CONFORMATIONAL TRANSITION

An intermediary step in the hydrolysis of ATP by the Na,K-ATPase is the formation of an acid stable phosphoenzyme with the phosphate bound to an aspartic acid (nr. 369 of the amino acids) in the α chain. The phosphorylation is suggested from ADP-ATP exchange experiments, but more direct evidence is given from experiments with [^{32}P]ATP, which shows that the phosphorylation is Na^+-dependent and the dephosphorylation is K^+-dependent [62,63]. In the presence of Na^+ the first step in the phosphorylation is formation of an ADP sensitive phosphoenzyme and this is converted to a K^+ sensitive phosphoenzyme. This suggests two different conformations, denoted E_1-P, which has a high energy phosphate bond and is ADP sensitive, and E_2-P, which has a low energy phosphate bond and is K^+ sensitive. With 150 mM Na^+ the major part of the phosphoenzyme is on the E_2-P form, but is shifted towards E_1-P by an increase in the Na^+ concentration. The rate of phosphorylation is high in the presence of Na^+, but the rate of dephosphorylation is low. Addition of K^+ increases the rate of dephosphorylation.

A more detailed investigation shows that the transition from the ADP sensitive to the K^+ sensitive phosphoenzyme involves at least one extra conformational step with formation of a phosphoenzyme which has a fast rate of transition to the ADP sensitive phosphoenzyme, i.e. both disappear fast when ADP is added [64]. In the following the three conformations of the phosphoenzyme are denoted E_1'-P, E_1''-P and E_2-P, respectively.

In 1972 Post et al. made the important observation that dephosphorylation by K^+ of E_2-P leads to a conformation of the enzyme from which K^+ has a low rate of release, but the rate of release is increased by ATP [65]. This led to the concept of occluded K^+. It was later shown by Glynn et al. that phosphorylation of the enzyme leads to occlusion of Na^+ [66].

The reactions with ATP and the cations Na^+ and K^+ can be described by the following scheme in which the parentheses show occlusion. Mg^{2+} which is necessary for the reaction is omitted.

$$E_1ATPNa_3 \rightleftharpoons E_1\text{-}PADPNa_3 \rightleftharpoons E_1'\text{-}P(Na_3) \rightleftharpoons E_1''\text{-}P(Na_3) \rightleftharpoons E_1''\text{-}P$$
$$E_1''\text{-}P \rightleftharpoons E_2\text{-}P \rightleftharpoons E_2\text{-}PK_2 \rightleftharpoons E_2'(K_2) \quad (1)$$

Na^+ on the cytoplasmic sites, which activates the hydrolysis, becomes occluded by the phosphorylation. This is followed by a molecular rearrangement of the phosphoenzyme with a transition into a conformation from which Na^+ has a higher rate of exchange, a deocclusion, and a transition to the K^+ sensitive E_2-P form. In the scheme it is assumed that it is the release of Na^+ which allows the conformational transition to the E_2-P form, which has two binding sites for K^+. Another possibility is that release of one of the three Na^+ is enough to allow the transition, and the transition decreases the affinity for the two remaining Na^+ and increases the affinity for K^+. The affinity of E_2-P for K^+ is high and comparable to the affinity for extracellular K^+ for hydrolysis, suggesting that the binding sites on E_2-P are facing the extracellular solution and that it is K^+ from the extracellular side which becomes occluded when E_2-PK_2 dephosphorylates. The rate of dephosphorylation of E_2-PK_2 is high.

In the nonphosphorylated form the enzyme binds ATP with high affinity in the presence of Na^+, K_D 0.1–0.2 μM, while in the presence of K^+ the affinity is lower, indicating two different conformations determined by the cation bound to the system, a Na^+ conformation, E_1, and a K^+ conformation, E_2 [67,68]. The two conformations can also be distinguished from their reactivity towards tryptic digestion [69], their fluorescence signal using intrinsic tryptophan fluorescence [70], or extrinsic probes like formycin triphosphate, which is an ATP analog [71], fluorescein

isothiocyanate which binds covalently to a lysine group near the ATP binding site [72], or eosin which binds non-covalently to the ATP site [73].

With no cations in the medium, and under conditions where the Na^+ effect of buffer cations on the conformation can be minimized, the enzyme is in the E_2 conformation [74]. Addition of Na^+ leads to a transition to E_1Na_3, with a $K_{0.5}$ for Na^+ of a few mM. Addition of K^+ to E_2 leads to a conformation from which K^+ has a very low rate of release, an occluded form, $E_2'(K_2)$ [75]. The affinity of E_2 for K^+ is low, but as the equilibrium between E_2K_2 and $E_2'(K_2)$ is poised towards $E_2'(K_2)$, the apparent affinity of E_2 for K^+ is high with a $K_{0.5}$ in the μM range.

$$E_2'(K_2) \rightleftharpoons E_2K_2 \rightleftharpoons E_2 \rightleftharpoons E_1Na_3 \quad (2)$$

In the nonphosphorylated form, it is K^+ bound to the cytoplasmic sites which becomes occluded [76]. The rate of transition from E_1Na_3 to $E'_2(K_2)$ is high, $t_{1/2}$ is a few ms at 22°C, while the rate of the reverse reaction is low with a $t_{1/2}$ of a fraction of a second. It is the rate of the deocclusion of K^+ from the K^+ occluded $E'_2(K_2)$ which is low. This rate is increased by ATP with a low affinity (K_D 0.45 mM) [70]; the rate of transition from $E'_2(K_2)$ to E_1Na_3 is also increased by an increase in pH [77], and by an increase in the concentration of K^+ as well as of Na^+ each of which binds to $E_2'(K_2)$ with a low affinity [78].

With Na^+ but no K^+ the K_M for ATP is 0.1–0.2 μM, and a Lineweaver-Burk plot is linear. With $Na^+ + K^+$, K_M increases to 0.1–0.2 mM, and the Lineweaver-Burk plot becomes curved and can be resolved in two affinities for ATP, one high as seen with Na^+ alone, and one low [79]. As mentioned above, ATP by a low affinity effect increases the rate of deocclusion of K^+ from $E_2'(K_2)$ [70]. The higher K_M for ATP in the presence of $Na^+ + K^+$ than in the presence of Na^+ is explained from the requirement for ATP to speed up the rate of transition from $E'_2(K_2)$ to E_1Na_3 in the hydrolysis reaction.

$$E_2'(K_2) \rightleftharpoons E_2'(K_2)ATP \rightleftharpoons E_2K_2ATP \rightleftharpoons E_2ATP \rightleftharpoons E_1Na_3ATP \quad (3)$$

It is unsolved whether the high and low affinity effect of ATP seen in the presence of $Na^+ + K^+$ is due to a consecutive effect of two ATP molecules, is due to two ATP binding sites, or to a shift in a rate limiting step.

A combination of (1) and (3) gives a scheme for the $Na^+ + K^+$ dependent hydrolysis of ATP by the enzyme system:

$$E_1ATPNa_3 \rightleftharpoons E_1\text{-}PADPNa_3 \rightleftharpoons E_1'\text{-}PNa_3 \rightleftharpoons E_1''\text{-}P(Na_3) \rightleftharpoons E_1''\text{-}P$$

$$E_1''\text{-}P \rightleftharpoons E_2\text{-}P \rightleftharpoons E_2\text{-}PK_2 \rightleftharpoons E_2'(K_2)$$

$$E_2'(K_2) \rightleftharpoons E_2'(K_2)ATP \rightleftharpoons E_2K_2ATP \rightleftharpoons E_2ATP \rightleftharpoons E_1Na_3ATP \quad (4)$$

11. COUPLING BETWEEN CHEMICAL REACTION AND TRANSPORT

It seems reasonable to assume that occlusion of the cations means transfer from the water phase to the membrane phase. In the occluded conformation the exposure of the cation sites is neither to the cytoplasmic nor to the extracellular side of the system, but the cations are inside 'gates' closed towards the media on both sides of the membrane.

Assuming this and adding sidedness to the above described scheme for hydrolysis gives a scheme for the coupling between the ATP hydrolysis and the translocation (Fig. 2). ATP with a low affinity increases the rate of deocclusion of K^+ from $E_2'(K_2)$ to the cytoplasmic side, opens the 'gate' for K^+ for release from the membrane to the cytoplasmic side. The deoccluded E_2K_2ATP form has a low affinity for K^+ and with K^+ exchanged for cytoplasmic Na^+ the molecule is converted to the E_1 form which has three binding sites for Na^+, E_1ATPNa_3. The following phosphorylation leads to occlusion of Na^+, i.e., to transfer of Na^+ to the membrane phase and closing of the 'gate' to the cytoplasmic side. By a molecular rearrangement of the phosphoenzyme the 'gate' on the extracellular side is opened and the transition into the $E_2 \cdot P$ conformation shifts the cation affinity to a low Na^+ high K^+ affinity with a following exchange of Na^+ for extracellular K^+. Dephosphorylation of E_2-P by K^+ occludes K^+, transfers extracellular K^+ to the membrane phase and closes the 'gate' to the extracellular medium. The opening of the gate to the cytoplasmic side by ATP closes the cycle. The placement in the scheme in Fig. 2 of the enzyme intra- and extracellularly indicates the orientation of the cation binding sites, not a movement of the enzyme or of the binding sites for ATP and phosphate. The binding sites for ATP and phosphate are in all steps facing the intracellular solution. A major difference between the E_1, and the E_2 conformations, phosphorylated and nonphosphorylated, is that the E_1 conformations have three binding sites for the cations, while the E_2 conformations have two binding sites.

The main principles behind the coupling between the chemical reaction and the translocation as it is illustrated in the scheme in Fig. 2 is a shift in affinities for the cations and an occlusion – deocclusion of the cations governed by the stepwise degradation of ATP. The tight coupling between these reactions divides the translocation into steps in each of which a gradient is created along which the cations can flow. Thereby the cations are moved against the overall gradient, and the free energy from the hydrolysis of ATP is converted into another energy source, the gradient for the cations.

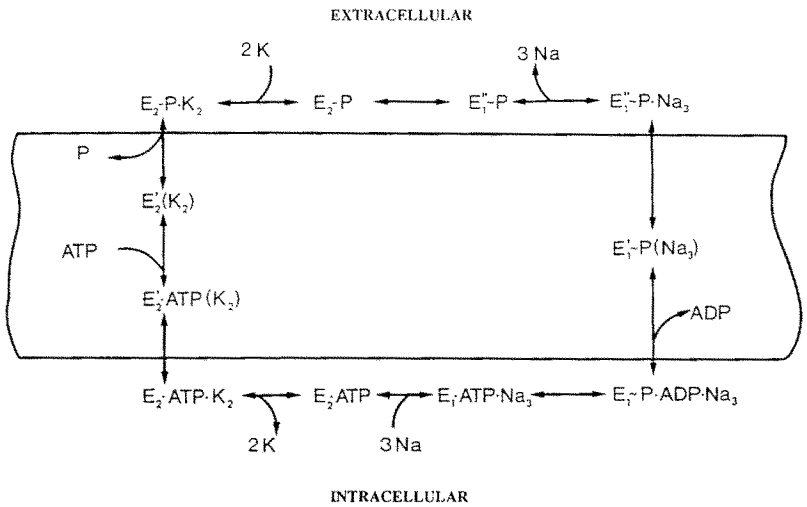

Fig. 2. A scheme for the Na^+-K^+ exchange across the cell membrane by Na^+,K^+-ATPase. The reaction with the cations is consecutive and the scheme is based on the Albers-Post scheme [80,81], modifications of this scheme by Karlish et al. [71], and on the scheme for formation of the phosphoenzymes by Nørby et al. [64]. The symbols E_1, E'_1, E''_1, E_2 and E'_2 refer to different conformations of the enzyme. Of the two major different conformations E_1 and E_2, E_1 has three monovalent cation binding sites while E_2 has two binding sites. E-P represents phosphoenzymes. Parentheses indicate that the cations are occluded, i.e. in the membrane phase inside 'gates' closed towards the intracellular as well as towards the extracellular medium. The placement in the scheme of the enzyme extra- and intracellular indicates the orientation of the cation binding sites, not a movement of the enzyme. The binding sites for ATP and phosphate are in all steps facing the intracellular solution. For further explanation see text.

Fig. 2 is based on the most widely used scheme to describe the transport reaction, the so called Albers-Post scheme [80,81], which is a modification of a scheme suggested by Shaw [13]. Included is the modification suggested by Karlish et al. [71], and the scheme for phosphorylation in the presence of Na^+ by Nørby et al. [64]. In the reactions described above (1–4) and in the scheme in Fig. 2 the phosphorylation is purely dependent on an effect of Na^+ and the dephosphorylation on an effect of K^+. The reaction with the cations is consecutive, Na^+ leaves before K^+ binds and vice versa. The experiments the observations are based upon have been performed by a consecutive addition of the cations, which must give as an answer a consecutive reaction. If, however, there is a simultaneous binding of Na^+ and K^+ at one or more steps in the reaction, this may influence the reaction with ATP.

With Na^+ but no K^+ the rate of phosphorylation is high while the rate of dephosphorylation is low, which means that practically all the enzyme in steady state is in the phosphoform (E_2-P). According to the scheme in Fig. 2, addition of K^+ to the phosphoenzyme formed in the presence of Na^+ should give a rate constant for the dephosphorylation high enough to account for the rate of overall hydrolysis in the presence of $Na^+ + K^+$. It does not; there is a fraction of the enzyme which dephosphorylates with a much too low rate constant (see [64]). It suggests that K^+ present together with Na^+ has an effect on the phosphoenzymes formed. This is supported by kinetic experiments on the ATPase activity [82]. And also from the observation that K^+ in low, extracellular concentrations, in the presence of a high concentration of Na^+ inhibits the transition from the ADP-sensitive phosphoenzyme E'_1-P(Na_3) to E'_1'-PNa_3 (see Fig. 2) [64]. This must mean that K^+ is bound together with Na^+ to the enzyme, and that K^+ not only has an effect on the dephosphorylation but also on the formation of the phosphoenzyme. From kinetic experiments [83] and from the effect of Li^+ on the phosphatase activity [84] it has been suggested that there are simultaneously existing extracellular and cytoplasmic sites, and that the reaction which leads to the Na^+-K^+ exchange, occurs with simultaneous binding of K^+ to extracellular sites and of Na^+ to cytoplasmic sites. Simultaneously existing extracellular and cytoplasmic sites are supported by the observation with reconstituted enzyme that there is a transmembrane effect of extracellular not transported Na^+ on the affinity for cytoplasmic Na^+ in the Na^+-K^+ exchange [85], and of extracellular Na^+ on the affinity for cytoplasmic Na^+ in the ATP-dependent Na^+-Na^+ exchange [86], an allosteric effect of extracellular Na^+. Kinetic studies of the Na^+-K^+ exchange in red blood cells by Hoffman and Tosteson [87] and by Sachs [88] suggest simultaneous transport. However in the experiments by Sachs, when correction is made for an uncoupled Na^+ efflux the kinetics suggest a consecutive transport. A problem is however whether the uncoupled Na^+ efflux is also there when the Na^+-K^+ exchange is measured. In a reconstituted system in which the ATP hydrolysis-dependent Na^+-Na^+ exchange follows the same route as the Na^+-K^+ exchange, the kinetics suggest a simultaneous transport. In these experiments the uncoupled Na^+ efflux is practically nil [86].

None of the experiments cited above are conclusive,

but they show that the view which is often met in the literature, that the pump reaction is consecutive, may not be valid, or at least needs a more thorough investigation. It also means that the scheme in Fig. 2 must be taken as a working hypothesis. It is often used to explain results, while it should be the other way around, that the results should be used to correct the model.

But whatever the scheme is for the coupling between the ATP hydrolysis and the translocation of the cations it seems likely that the ATP-dependent shift in affinity for the cations and the occlusion – deocclusion is an important part of the coupling reaction. However, these terms give a description of the process. But what is the content on the molecular level? How does the system discriminate between Na^+ and K^+? The transition from the K^+ form $E_2'(K_2)$ to the Na^+ form E_1Na_3 involves a change in pK of amino acids in the system (lysine, α-amino acid and carboxyl groups) with a release of H^+ [89]. Modification of these groups decreases the affinity for K^+, with little or no effect on the affinity for Na^+. The experiments suggest that salt bridge formation as in haemoglobin is involved, a change in the quaternary and/or tertiary structure of the system, but it says nothing of the nature of the binding sites. Information on this may come from gene technology. Neither is it known what occlusion – deocclusion means on the molecular level. What kind of molecular rearrangement is it that excludes the cations bound to the sites from the water phase? What makes the cations move in the translocation step? It is obvious that they must move along a gradient created by the gradient for hydrolysis of ATP, but how. Is it by the gating combined with the affinity shift which follows from the reaction with ATP as described in Fig. 2? These and many other questions must be answered before the coupling between the ATP hydrolysis and the translocation is understood.

12. INHIBITORS

12.1. *Cardiac glycosides*

Cardiac glycosides are specific inhibitors of the Na,K-ATPase, and no other effect is known. They inhibit from the extracellular side of the Na,K-ATPase. Mg^{2+} is necessary, but binding and inhibition in the presence of Mg^{2+} is very slow. The rate is much increased by phosphorylation of the enzyme with formation of E_2-P. When the phosphoenzyme is formed with Mg^{2+}, Na^+ and ATP, addition of K^+ protects against the reaction with low concentrations of cardiac glycosides, probably due to the dephosphorylating effect of K^+. Mg^{2+} and Pi also promote the reaction with cardiac glycosides with formation of a phosphoenzyme, and Na^+, as well as K^+ protects against the reaction [90].

Cardiac glycosides differ in their affinity. With a given cardiac glycoside and a given combination of ligands, the inhibitory effect varies for different species and for a given species for different tissues. And as discussed above, for a given tissue there may be isoforms of the enzyme with different affinities. The most widely used cardiac glycoside, ouabain (g-strophanthin), which is the most water soluble, has a $K_{0.5}$ of the order of 10^{-5} to 10^{-7} M for enzyme from most tissues.

From the correlation between the inotropic effect of cardiac glycosides and their inhibition of the Na,K-ATPase, Repke in 1961 suggested that the Na,K-ATPase is the receptor for their inotropic effect on the heart [91]. This has since been substantiated. An explanation of the link between an inhibition of the sodium pump and an inotropic effect was given by Baker et al. in 1969 [92]. Increased intracellular Na^+ which follows from partial inhibition of the pump, leads to an enhanced Ca^{2+} influx and/or decreased Ca^{2+} efflux through the Na^+-Ca^{2+} exchange carrier in the cardiac muscle, and the increased intracellular Ca^{2+} concentration leads to increased contractility by the heart muscle.

12.2. *Vanadate*

Vanadate was found as a contaminant in certain commercial preparations of ATP. In the presence of Mg^{2+} it inhibits the Na,K-ATPase in mM concentrations by binding to what seems to be the phosphorylation site which is on the cytoplasmic side of the system [93]. K^+ on the cytoplasmic sites of the system promotes vanadate binding while extracellular K^+ has no effect. Na^+ on the extracellular sites protects against vanadate binding, and the effect can be overcome by displacement of Na^+ from the sites by K^+ [94]. This means that with the unsided preparation in the test tube, K^+ in the presence of Mg^{2+} promotes binding, while Na^+ prevents binding, but the effect of Na^+ can be overcome by K^+.

Vanadate is not specific for the Na,K-ATPase like the cardiac glycosides, since vanadate also inhibits other ATPases.

12.3. *Oligomycin*

Like the cardiac glycosides, oligomycin inhibits the enzyme from the extracellular side, but in contrast to cardiac glycosides and vanadate, oligomycin does not give complete inhibition. It decreases the rate of hydrolysis and of transport [94]. Oligomycin decreases the rate of transition from E_1Na_3 to $E_2(K_2)$ with no effect on the reverse reaction [77]. This is due to an occlusion of Na^+ [95], which means that it is due to a transition of E_1Na_3 to $E_1(Na_3)$ with a low rate of release of Na^+. This suggests that oligomycin decreases the rate of hydrolysis by decreasing the rate of deocclusion of Na^+ from E_1'-$P(Na_3)$ to E_1''-PNa_3 and thereby

decreases the rate of formation of E_2-P, the phosphoenzyme which becomes dephosphorylated by K^+. This explains that the fractional inhibition of the hydrolysis by oligomycin increases when the rate of hydrolysis is increased by an increase in the ATP concentration. With a low ATP concentration it is the deocclusion of K^+ from the dephosphoenzyme which is rate limiting, and the overall rate of hydrolysis is little effected by a decrease in rate of formation of E_2 - P, while at a high ATP concentration with a high rate of deocclusion of K^+ the overall rate of hydrolysis becomes much more sensitive to a decrease in the rate of formation of E_2 - P. With optimum ATP the activity is inhibited down to about 20% of maximum.

13. REGULATION OF ACTIVITY

The Na,K-ATPase, the Na,K-pump is an energy transducer which converts the energy from the hydrolysis of ATP into a gradient for Na^+ and for K^+ across the cell membrane. As mentioned above the gradient is used as a free energy source for many different processes, and the transport system thereby has a key role in impulse transmission, in cell homeostasis and in function of organs like kidney, intestine and secretory glands.

The activation of the pump by a combined effect of cytoplasmic Na^+ and extracellular K^+ and the cytoplasmic as well as the extracellular competition between Na^+ and K^+ couples a change in passive fluxes of Na^+ and K^+ across the cell membrane directly to an opposite change in active transport. The steady state gradients for Na^+ and K^+ across the cell membrane are a complex function of the number of pump molecules in the membrane, of the membrane permeability not only for Na^+ and K^+, and of the membrane potential which again is a function of membrane permeability. The number of pump molecules varies considerably for different cell types [96], from about 200 in a red blood cell to several million pumps per cell in the thick ascending limb of Henles loop. With the normal cytoplasmic and extracellular Na^+ and K^+ concentrations the Na^+,K^+-pump operates as mentioned above with an activity which is a low fraction of maximum, 5–20%. The large reserve power is important, for example during exercise where the muscle gains Na^+ and looses K^+. During maximal exercise the net loss of K^+ to the extracellular space may amount to 40 mmol/min or more [97]. This will rapidly give a deleterious concentration of K^+ in plasma unless it is counteracted by an increase in the active uptake of K^+. An increase in the pump activity follows as discussed above directly from a change in the passive fluxes. But besides there is a stimulating effect of insulin, epinephrine and norepinephrine on pump activity, which may also play a role during exercise. These hormones have no direct effect on the isolated Na,K-ATPase, and mediation of their effect in the intact membrane from the hormone receptor seems to involve cyclic AMP. These hormones have an immediate effect on the pump activity. The maximal capacity for K^+ reabsorption in the total muscle pool is about 125 mmol/min [97].

Thyroid hormones and corticosteroids also increase the activity of the pump but the effect takes hours to develop and seems to be due to a de novo synthesis of enzyme molecules [97].

The Na,K-ATPase is the receptor for the therapeutic as well as the toxic effect of cardiac glycoside on the heart, and there is no other known effect of cardiac glycosides than their inhibitory effect of the transport system. This has raised the question [98] 'Is there an endogenous cardiac glycoside like factor in plasma of importance for regulation of pump activity?' As cardiac glycosides give vasoconstriction, the question has been extended, is a circulating cardiac glycoside like factor responsible for development of hypertension? Plasma inhibits the activity of isolated Na,K-ATPase. It has been known for long that unsaturated fatty acids are inhibitors of the enzyme system and this seems to explain the inhibition by plasma. The vasoconstrictor effect may at least partly be due to pump inhibition in nerve terminals with an increased transmitter release and/or decreased reuptake. So far no conclusive evidence has been given of a circulating cardiac like factor, neither of a connection between pump inhibition and hypertension, but the problem is still under debate [99].

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