# Enzymatic Basis for Active Transport of $Na^+$ and $K^+$ Across Cell Membrane

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## INTRODUCTION

Experiments on nerve, muscle, frog skin, red blood cells, and cells from a number of other tissues have shown that $Na^+$ is transported from the cytoplasm to the interstitial fluid against an electrochemical gradient (for ref. see 112). By the definition given by Ussing (111), this is called active transport.

A transport against an electrochemical gradient requires energy, and it has been shown by experiments on nerve (14–18) and on red blood cell membranes (31, 54, 114) that the energy for active transport of $Na^+$ comes from adenosinetriphosphate (ATP), and that inosinetriphosphate (ITP), guanosinetriphosphate (GTP), or uridinetriphosphate (UTP) cannot replace ATP (54).

The active transport of $Na^+$ is dependent on the concentration of $K^+$ in the extracellular fluid, and there seems to be some kind of a coupling between the active outward transport of $Na^+$ and inward transport of $K^+$ (38, 47, 79, 104).

It is also characteristic of the active transport of $Na^+$ that it is inhibited by cardiac glycosides (39, 68, 85, 114).

The results of the above-mentioned experiments on the intact cell have made it possible to formulate a number of requirements of a $Na^+$ transport system and have thus given the basis for identification and isolation of the transport system (98–100).

The transport system must at least fulfill the following requirements. It should *1*) be located in the cell membrane, *2*) have an affinity for $Na^+$ that is higher than for K at a site located on the inside of the cell membrane, *3*) have an affinity for

$K^+$ that is higher than for $Na^+$ at a site located on the outside of the membrane, *4*) contain an enzyme system that can catalyze the hydrolysis of ATP and thus convert the energy from ATP into a movement of cations, *5*) be capable of hydrolyzing ATP at a rate dependent on concentration of $Na^+$ inside the cell and also on concentration of $K^+$ outside the cell, and *6*) be found in all cells in which an active, linked transport of $Na^+$ and $K^+$ occurs.

In the search for the transport system, a (Na + K)-activated, ATP-hydrolyzing enzyme system was found (93) that has been shown to fulfill requirements 1–6 for a transport system and, furthermore, meets two additional requirements: *7*) that there is a close correlation between the effect of cardiac glycosides on the cation transport in the intact cell and their effect on this system, and *8*) that this enzyme system has the same quantitative relation to $Na^+$ and $K^+$ as the transport system in the intact cell.

## AFFINITY FOR MONOVALENT CATIONS

The enzyme system is located in a microsomal particle that can be isolated from a cell homogenate by differential centrifugation. It hydrolyzes adenosinetriphosphate (ATP) to adenosinediphosphate (ADP) and orthophosphate (Pi), and it differs from other ATP-hydrolyzing enzymes in that in addition to $Mg^{++}$ it requires both $Na^+$ and $K^+$ in the medium for maximum activation (93). With $Mg^{++}$ alone, the enzyme system has a low activity. The addition of one of the cations $K^+$, $Rb^+$, $Cs^+$, or $NH_4^+$ besides $Mg^{++}$ leads to a very slight increase in activity or to no increase at all; the addition of $Na^+$ gives some increase, and the magnitude of this increase varies for different enzyme preparations (3, 80, 93, 94, 96). $Li^+$ has an effect that lies between that of $K^+$ and $Na^+$ (93).

If, however, the medium contains $Na^+$ as well as $Mg^{++}$, the addition of one of the cations $K^+$, $Rb^+$, $Cs^+$, $NH_4^+$, or $Li^+$ leads to a considerable increase in activity (93, 94). The highest activity is obtained with $NH_4^+$, followed by $K^+$, $Rb^+$, $Cs^+$, and lowest with $Li^+$. The enzyme system, however, has the highest affinity for $K^+$, followed by $Rb^+$, $NH_4^+$, $Cs^+$, and $Li^+$ in that order (94). In order to obtain this effect of the monovalent cations $Na^+$ must be in the medium; it cannot be replaced by any of the other cations. Maximum activity is obtained when the $Na^+$ concentration is 100 mM/liter or higher (3, 89, 93, 94).

With $Mg^{++}$ + $Na^+$ in the medium, increasing concentrations of $K^+$ increase the activity up to a maximum, and then the activity decreases down to the $Mg^{++}$ level. The activity at the maximum and the concentration of $K^+$ that gives the maximum increase with increasing concentrations of $Na^+$ (3, 6, 80, 93, 103).

A kinetic analysis of the effect of $Na^+$ and of $K^+$ on the enzyme activity suggests that the enzyme system has two sites with affinities for cations (94)—one site where the affinity for $Na^+$ is 6–8 times as high as the affinity for $K^+$ and where $K^+$ by competition can replace $Na^+$, and a second site with high affinity for $K^+$ and a very low affinity for $Na^+$. Maximum activity of the enzyme requires $Na^+$ at the first site and $K^+$ at the second. At concentrations of $K^+$ that are high compared

with the concentration of $Na^+$, $K^+$ will displace $Na^+$ from the first site by competition and thus decrease the activity.

It has been questioned (44) whether there are two sites or only one with affinities for $Na^+$ and $K^+$, but a more detailed kinetic analysis has not revealed evidence in favor of either of the two possibilities. On the other hand, experiments have shown that the ($Na^+ + K^+$)-activated enzyme system in the intact cell requires $K^+$ on the outside and $Na^+$ on the inside of the membrane for activity (7, 41, 115). This seems to lend support to the assumption that two sites exist and that the activation requires $Na^+$ at one and $K^+$ at the other. However, the possibility still exists that there is only one site, which shifts from the inside to the outside of the membrane and has different affinities for cations in the two different positions.

The competition of $K^+$ with $Na^+$ at the $Na^+$ site, which leads to a decrease in activity when the $K^+$ concentration becomes high compared with the $Na^+$ concentration, is correlated with the effect of $K^+$ on the active transport of $Na^+$ in the intact cell. In experiments on red blood cell membranes it was found not only that the active transport of $Na^+$ was dependent on the external $K^+$ concentration, but also that an increase in the concentration of $K^+$ in the internal solution at a constant concentration of $Na^+$ leads to a decrease in the $Na^+$ transport (56).

The activity of the ($Na^+ + K^+$)-activated enzyme system is strongly inhibited by $Ca^+$ in low concentrations (57, 93, 102), and so is the active transport of $Na^+$ in the red blood-cell membranes when $Ca^+$ is in the inside solution (57).

The inhibition of the enzyme system by $Ca^+$ can to some extent be reversed by an increase in the $Mg^{++}$ concentration (94) and also by an increase in the $Na^+$ concentration (76).

The results of the above-mentioned experiment show that the isolated ($Na^+ + K^+$)-activated enzyme system, by the asymmetry in affinity for $Na^+$ and $K^+$, fulfills requirements 2 and 3 of a system involved in the active transport of $Na^+$ and $K^+$, and by the asymmetry in the activating effect of the cations it fulfills requirement 5 of an active transport system. Furthermore, experiments show that the enzyme system also fulfills the requirements of a system involved in the active transport of $Na^+$ and $K^+$ from the point of view of competition between $Na^+$ and $K^+$ for the $Na^+$ site and of the effect of $Ca^+$.

## RELATIONSHIP OF ENZYME SYSTEM TO ATP

The substrate for the ($Na^+ + K^+$)-activated enzyme system is ATP; with ITP as substrate (57, 80, 94) or with GTP or UTP as substrate (57), there is only a very slight or no activation by $Na^+ + K^+$. This is in agreement with findings that active transport in the intact cell requires ATP as the energy source and with ITP as substrate there is only a very slight transport of $Na^+$ and with GTP and UTP none at all (54).

The submicroscopic particle that contains the ($Na^+ + K^+$)-activated, ATP-hydrolyzing enzyme system also contains an enzyme that can catalyze an ATP-ADP but not an ATP-Pi exchange reaction (94). There is a certain similarity between the effect of cations and of g-strophanthin on the exchange reaction and

enzymatic hydrolysis of ATP. This indicates that the same enzyme is responsible for the two reactions. On the basis of these results it was suggested that an intermediary step in the hydrolysis of ATP is formation of a phosphorylated compound due to transfer of an energy-rich phosphate bond from ATP to the enzyme system.

This assumption seems to be supported by experiments showing that there is a labeling with $P^{32}$ of the microsomal particle in which the enzyme system is located when $ATP^{32}$ is used as substrate and that this labeling is dependent on the cations in the medium (1, 21, 22, 35, 48, 81, 83, 100). With $Mg^{++}$ the labeling is slow and increases to a certain level at which it remains after complete hydrolysis of ATP (Fig. 1). $Na^+$ increases the rate of labeling and, in contrast to the labeling with $Mg^{++}$ alone, it reaches a certain maximum after which it decreases to a low level when all ATP is hydrolyzed. The addition of $K^+$ besides $Mg^{++} + Na^+$ decreases the labeling to a very low level. To some extent, g-strophanthin reverses the effect of $K^+$, but it does not bring the labeling back to the level with $Mg^{++}$ alone nor to the level with $Mg^{++} + Na^+$ but somewhere in between.

The decrease in labeling when all the ATP is hydrolyzed in the presence of $Mg^{++} + Na^+$ shows that the phosphate transferred from ATP is again split off the enzyme system. The labeling in the presence of $Mg^{++}$ alone, which does not decrease when the hydrolysis of ATP is complete, may be due to an isotope exchange.

There is only a small amount of labeling of the lipids in the microsomal particles that contain the enzyme system; the most labeling is in the protein fraction (1, 21, 83, 100).

Fractionation of the proteins has shown that the highest specific activity is in the phosphoprotein phosphate (83, 100); these experiments have been done under conditions where it is reasonable to assume that the labeling found is activated by $Mg^{++}$ and not by $Mg^{++} + Na^+$.

In other experiments, the protein was digested with pepsin and the electrophoretic pattern showed three radioactive peaks when the enzyme system had been incubated with $Mg^{++}$ alone, whereas there was an additional fourth peak when incubated with $Mg^{++} + Na^+$ (1). None of these labeled compounds have been isolated.

The results of the exchange reaction and of the experiments with $ATP^{32}$ suggest that the breakdown of ATP is a two-step reaction in which the first step is a $(Mg^{++} + Na^+)$-requiring transfer of an energy-rich phosphate bond to a compound in the system, and the second step is a dephosphorylation due to the effect of $K^+$.

There are, however, some results that may suggest another explanation.

The first is that the initial rate of phosphorylation with $Mg^{++} + Na^+$ in the medium has not been found to be more than a small fraction of the initial rate of hydrolysis with $Mg^{++} + Na^+ + K^+$ in the medium (Skou, unpublished data).

The second is that the decrease in phosphorylation due to the effect of $K^+$ seems to be independent of the hydrolysis of ATP. At 37 C the addition of $K^+$ besides $Mg^{++} + Na^+$ increases the hydrolysis of ATP and decreases the labeling. At 0 C there is a $(Mg^{++} + Na^+)$-requiring labeling as at 37 C; at this temperature $K^+$ has no effect on the hydrolysis and still decreases the labeling. With $ITP^{32}$ as substrate at 37 C there is also a $(Mg^{++} + Na^+)$-requiring labeling as with $ATP^{32}$ as substrate; in these experiments $K^+$ also decreases the labeling due to $Mg^{++} + Na^+$

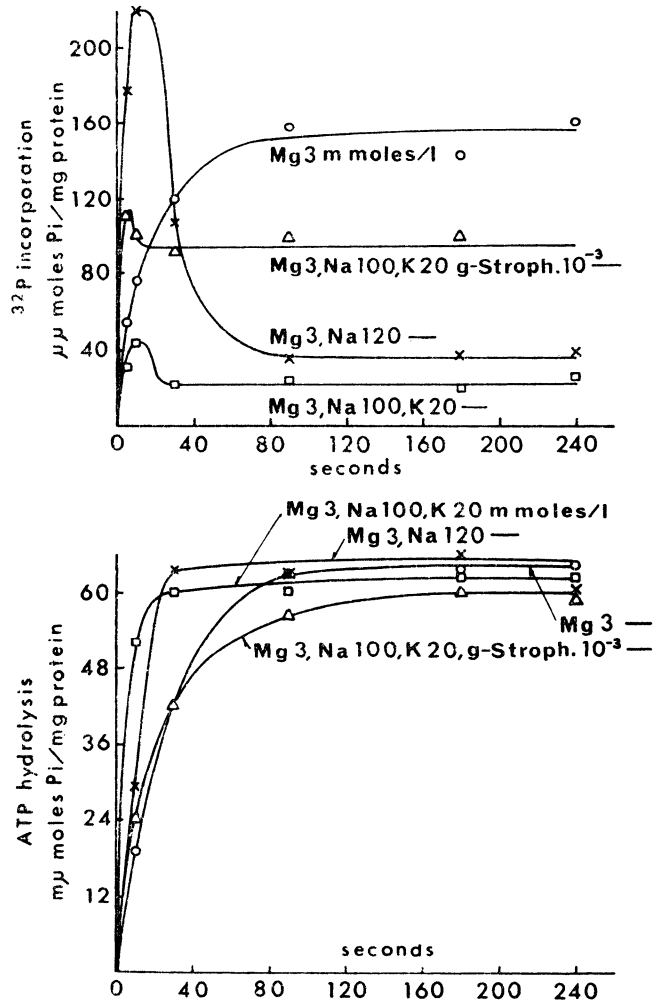
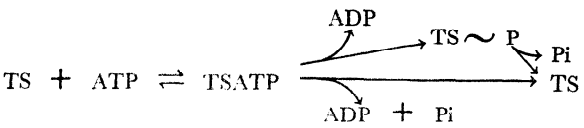



FIG. 1. *Above:* effect of $Mg^{++}$ 3; $Mg^{++}$ 3; $Na^{+}$ 120; $Mg^{++}$ 3, $Na^{+}$ 100, $K^{+}$ 20; and $Mg^{++}$ 3. $Na^{+}$ 100, $K^{+}$ 20 g-strophanthin 1 mM/liter; respectively, on incorporation of $P^{32}$ from ATP intc microsomal particle that contains ($Na^{+}$ + $K^{+}$)-activated enzyme system. Microsomal particle was isolated from ox brain (96). ATP concentration 25 μM/liter, ATP was labeled in γ position and specific activity was 40,000 counts/min/μM ATP; pH 7.6, 37 C. *Abscissa:* time in sec; *ordinate.* μμmole of $P^{32}$ incorporated/mg protein. *Below:* hydrolysis of ATP measured in same experiment as shown above. *Abscissa:* time in sec; *ordinate:* mμM $P^{32}$ hydrolyzed from ATP/mg protein.

to the same level as found with ATP as substrate, but at the same time $K^{+}$ decreases the hydrolysis of ITP (101). The addition of $K^{+}$ thus decreases the labeling whether $K^{+}$ increases, has no effect, or decreases the hydrolysis. This suggests that the decrease in labeling with $K^{+}$ is not due to an increased hydrolysis of the phosphorylated compound. It indicates that the decrease in labeling with $K^{+}$ is due tc a decreased transfer of phosphate from ATP to the enzyme system and that there is no phosphorylation with $Mg^{++}$ + $Na^{+}$ + $K^{+}$ in the medium.

It must therefore be considered whether there are two pathways for the

hydrolysis of ATP—one that goes via a phosphorylated intermediate and another that does not:




where TS is the enzyme system.

With $Mg^{++}$ + $Na^+$ in the medium the hydrolysis goes via TS ~ P; with $Mg^{++}$ + $Na^+$ + $K^+$ it follows the other pathway.

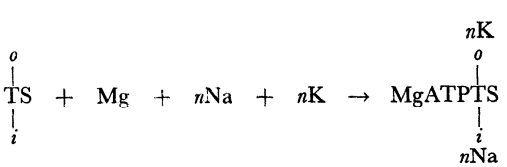
The enzyme system cannot only catalyze an ATP-ADP exchange reaction, but also an ITP-ADP exchange reaction, and the rate of this exchange is 75–80% of the rate of the ATP-ADP (94). This is supported by experiments with $ITP^{32}$ as substrate. As mentioned above, it was found that there is a transfer of $P^{32}$ from $ITP^{32}$ to the enzyme system, and this requires $Mg^{++}$ + $Na^+$ just as the $P^{32}$ transfer from ATP; $K^+$ decreases this phosphorylation (Skou, unpublished data).

There is a pronounced difference in the hydrolysis of ITP and of ATP by the enzyme system. With ITP as substrate the affinity for $Na^+$ is lower, and the activating effect of $K^+$ with $Mg^{++}$ + $Na^+$ in the medium is very much lower. The optimum $Mg^{++}$/ATP ratio was found to be 2/1 with $Mg^{++}$ + $Na^+$ + $K^+$ in the medium and 1/1 for $Mg^{++}$/ITP (94).

The observation that ITP can phosphorylate the enzyme system to nearly the same extent as ATP correlated with the finding that there is a pronounced difference in the affinity for $Na^+$ and in the effect of $Na^+$ + $K^+$ on the hydrolysis with ITP and ATP as substrate indicates that even if the phosphorylation might be a normal step in the breakdown of ATP, the effect of ATP cannot be only to transfer an energy-rich phosphate bond to the system. ATP as such must have an effect that seems to be to increase the affinity for $Na^+$ and to increase the activating effect of $K^+$ for the hydrolysis of ATP. This suggests that the binding of $Na^+$ and possibly also of $K^+$ takes place when ATP is bound to the enzyme system before ATP is hydrolyzed.

The increase in affinity of the enzyme system for $Na^+$ due to the effect of ATP and the concentration of $Na^+$ + $K^+$ required to activate the system seems to be independent of the ATP concentration (107; Skou, unpublished data). As pointed out by Tosteson (107), this indicates that $Na^+$ and $K^+$ are not bound to the enzyme system as part of an enzyme substrate complex but to sites removed from the catalytic center of the enzyme and that there is a mutual interaction between the effect of $Na^+$ and $K^+$ at these sites and the effect of ATP on the catalytic center.

The reaction sequence accordingly seems to be as follows [modified from Skou (94)]:



where $n$ is the number of cations bound to the system (see later), and where $i$ and $o$ are the sites with affinities for monovalent cations.

At the moment $Na^+$ is at site $i$ and $K^+$ at site $o$, ATP is hydrolyzed and the hydrolysis does not lead to a phosphorylation. With $Na^+$ but no $K^+$ in the medium there is $Na^+$ at both site $i$ and site $o$, and due to this there is an abnormal hydrolysis of ATP that leads to a phosphorylation.

The knowledge we have at present does not allow any final conclusions to be drawn about the intermediary steps in the breakdown of ATP by the enzyme system. The experiments reported have, however, shown that the enzyme system from the point of view of substrate specificity fulfills requirement 4 of an enzyme system involved in the active transport of $Na^+$ and $K^+$.

## ISOLATION OF ENZYME SYSTEM FROM OTHER CELLS WITH ACTIVE TRANSPORT OF $NA^+$ AND $K^+$

The main characteristics of the enzyme system are: that it is located in a submicroscopic particle; that it hydrolyzes ATP to ADP and Pi; that the activity is dependent on a combined effect of $Na^+ + K^+$; and (see below) that the activity due to $Na^+ + K^+$ is inhibited by g-strophanthin (93, 94).

Enzyme systems with these characteristics have been isolated from a large number of tissues—red blood cell membranes (32, 54, 78, 80, 106), brain (3, 30, 50–53, 60, 61, 89, 96, 103, 118), nerve (7, 9, 26, 93), kidney (65, 66, 69, 96, 116), muscle (5, 6, 10, 77, 87, 95, 96, 119), liver (34, 88), intestine (105), electric tissue (2, 10, 40), parotid gland (90), frog skin (10, 95), ciliary body (12), lens (12), retina (64), thyroid tissue (108, 109, 117), and toad bladder (10).

The most extensive studies on the occurrence of the $(Na^+ + K^+)$-activated enzyme system were made by Bonting et al. (13). They found the enzyme system in 29 of 36 tissues from the cat. It was undetectable only in tissues without cells or with a very low cell density; the highest activity was found in nervous tissue and in tissue concerned with secretory functions.

Bonting et al. (11) studied the occurrence of the enzyme system in tissues in which an active $Na^+ + K^+$ transport sensitive to cardiac glycoside is known to occur. They investigated 21 tissues (nerve, muscle, secretory tissue, erythrocytes, leukocytes, and ascites cells) from 10 different species and found in all the tissues the $(Na^+ + K^+)$-activated enzyme system in significant quantities. The activity, measured in millimoles of ATP hydrolyzed per gram wet weight of tissue per hour at 37 C, ranged from 1.24 in human erythrocytes to 1825 in pig brain.

In all the tissues mentioned, the enzyme systems have all the main characteristics. There are, however, quantitative differences in the pH optimum, in the affinities for $Na^+$ and $K^+$, in the activating effect of $Na^+$ alone or $K^+$ alone, in the $(Mg^{++} + Na^+ + K^+)/Mg^{++}$ activity ratio, and in the concentrations of g-strophanthin that inhibit the activity due to $Na^+ + K^+$.

The pH optimum for the enzyme system from crab nerve is 7.2 (93), whereas it is 7.5–7.6 for the enzyme system isolated from mammalian tissue (13, 32, 60, 95).

The concentration for half maximum activity with $Na^+$ alone is 1.4 mM/liter

for the crab nerve enzyme (93), whereas it is 0.4 mM/liter for the enzyme system from mammalian brain and 0.6 for the enzyme system from mammalian kidney (96).

The concentration of $K^+$ for half maximum activity with $Mg^{++} + Na^+$ in the medium is 1.6 mM/liter for the crab nerve enzyme (93), whereas it is 1.0 mM/liter for rabbit brain enzyme, 0.5 for rabbit kidney enzyme (96), and 1.0 for rabbit heart muscle (6).

The differences in the activating effect of $Na^+$ alone with $Mg^{++}$ in the medium (mentioned on p. 597) may not only be due to differences in the enzyme systems from different tissues, but may, according to Aldridge (3), be explained by the presence of $Na^+$ and $K^+$ in the submicroscopic particle that contains the enzyme system. By passing the suspension of submicroscopic particles through a Dowex 50 column (tris-form), the stimulation obtained by $Na^+$ alone was lowered but not prevented, without affecting the degree of stimulation obtained on addition of $Na^+ + K^+$.

The activating effect of $Na^+$ alone seems, however, also to depend on the buffer system used. In a histidine buffer, the stimulation is higher than in a tris buffer at the same pH. This is not due to a depressing effect of the tris buffer, as the same activity as in the tris buffer is found in other buffers; it seems as if the histidine somehow increases the activating effect of $Na^+$ (100).

The concentrations of $K^+$ needed to change the activation by $K^+$ to an inhibition at a given concentration of $Na^+$ in the medium and the slope of the curve for the inhibition are different for enzyme systems isolated from crab nerve (93), red blood cell membranes (80), rabbit brain (96), rabbit kidney (96), and rabbit heart muscle (6). This seems to indicate that the $Na^+/K^+$ affinity ratio for the $Na^+$ site, which for the crab nerve enzyme was about 6–8 (95), is of a different size for the enzymes from the other tissues. These affinity ratios have not been calculated.

The main difficulty in isolation of the enzyme system is to get rid of a $Mg^{++}$-activated, ATP-hydrolyzing enzyme. In the tissues from the crab nerve this can be done by differential centrifugation of a homogenate; in this way it is possible to obtain a preparation in which the activity is low with $Mg^{++}$ alone and in which the addition of $Na^+ + K^+$ in optimum concentrations gives a 6–20-fold increase in activity (93). The same holds for enzyme prepared from electric tissue from *Electrophorus electricus* (2, 10, 40).

In all the other tissues mentioned, the procedure used for the preparation of the crab nerve enzyme gives a $(Mg^+ + Na^+ + K^+)/Mg^{++}$ activity ratio of only 1.5–3.0.

For enzyme from mammalian brain and kidney, the addition of deoxycholate (DOC) and ethylenediaminetetraacetate (EDTA) to the medium in which the tissue is homogenized increases the $(Mg^{++} + Na^+ + K^+)/Mg^{++}$ activity ratio to 6–8 and at the same time increases the specific activity of the isolated $(Na^+ + K^+)$-activated enzyme system (96).

In cardiac muscle, the DOC procedure gives preparations that only in a few cases have an activity ratio higher than 2.0 (6, 87, 96). Without the use of DOC, the preparation has a high activity with $Mg^{++}$ alone, and there is practically no

increase in the activity by the addition of $Na^+ + K^+$. The activity ratio of the DOC-prepared heart muscle enzyme system can be increased by storing the enzyme preparation at $-5$ C for a number of days (87); this leads to a decrease in the activity, but the activity with $Mg^{++}$ alone decreases faster than the activity with $Mg^{++} + Na^+ + K^+$, i.e. the $(Mg^{++} + Na^+ + K^+)/Mg^{++}$ activity ratio is increased.

The supernatant of the heart muscle homogenate, after centrifugation at 250,000 *g* for 60 min, contains a heat-stable factor. When this is added to the heart muscle enzyme in suitable concentrations, it decreases the activity of the enzyme with $Mg^{++}$ alone more than the activity with $Mg^{++} + Na^+ + K^+$ and thus, like storage of the enzyme, increases the activity ratio (100). The same heat-stable factor is found in the supernatant from the preparations of the enzyme system from parotid gland (90).

$Na^+$ iodide in a 2-M concentration seems to have an effect on the enzyme system prepared from erythrocyte membrane similar to that of the heated supernatant on the enzyme system prepared from muscle (74).

It has been shown that storage at 2 C for several days of the enzyme system from kidney prepared without DOC will increase the $Na^+ + K^+$ activation and will at the same time decrease the activation with $Mg^{++}$ alone. The effect can be accelerated by the addition of urea in a 1.3-M concentration (66).

The results of the experiments mentioned show that the $(Na^+ + K^+)$-activated enzyme system can be isolated from all tissues in which an active transport of $Na^+ + K^+$ is known to occur, and even if there are quantitative differences, the enzyme systems from different tissues all basically have the same characteristics. The enzyme system thus fulfills requirement 6 of a system involved in the active transport of $Na^+$ and $K^+$.

## LOCATION OF ENZYME SYSTEM IN THE CELL

The enzyme system is located in a subcellular fragment found in the sediment of differential centrifugation that, according to Hanzon and Toschi (46), contains the broken cell membranes. The assumption of the location of the enzyme in the cell membrane is supported by experiments on red blood cells in which it has been shown that the $(Na^+ + K^+)$-activated, g-strophanthin-sensitive enzyme system is in the membrane (41, 54, 55, 115), and that in the broken red cells the enzyme is located in the membrane fragments (41, 54, 55, 80, 107, 115). The assumption of this location is also supported by experiments on squid axons performed by Bonting and Caravaggio (9), who found that the enzyme system is located in the sheath part of the squid axons, and by the observations of Cummins and Hyden (26), who showed that in the neuron the enzyme system is located in the membrane part.

These experiments indicate that the $(Na^+ + K^+)$-activated enzyme system is in the cell membrane (requirement 1), but it does not exclude the possibility that it may also be located in other parts of the cell.

EFFECT OF CARDIAC GLYCOSIDES

Schatzmann (85) has shown that cardiac glycosides in low concentrations are specific inhibitors of cation transport. This has been confirmed by a number of other investigators (39, 68, 114). This effect has been used to distinguish between active and passive transport of cations across the cell membrane.

Cardiac glycosides inhibit the activity of the enzyme system; they inhibit only the activity due to $Na^+ + K^+$ and not that due to $Mg^{++}$. This has been found for enzyme system isolated from all the tissues mentioned previously.

The sensitivity to the cardiac glycosides differs for enzyme systems from different tissues of the same animals and from the same tissue of different animals. This is not surprising since it is known that the sensitivity of different animals to cardiac glycosides varies within wide limits (for ref., see 100).

The concentration of g-strophanthin that gives half maximum inhibition of the active transport in the intact human red blood cells is $3-7 \times 10^{-8}$ M (37, 63, 101). This is not far from the concentration that gives half maximum inhibition of the enzyme system from red blood cells—$10^{-7}$ M (80), $1.3 \times 10^{-7}$ M (32).

It has also been shown in experiments on red blood cells that there is a close correlation between the inhibitory effect of different types of glycosides on the cation transport and on the activity of the enzyme system (32). Glycosides that are powerful inhibitors of the active cation transport are also powerful inhibitors of the enzyme activity, and glycosides that are less effective inhibitors of the cation transport are also less effective inhibitors of the enzyme activity. The experiments indicate that the same molecular configuration of the glycosides is important for the inhibition of both the active cation transport and of the $(Na^+ + K^+)$-dependent activity of the enzyme system.

The inhibition of the enzyme activity by very low concentrations of g-strophanthin can be prevented by an increase in the concentration of $K^+$ (32, 76), just as the inhibition of the cation transport by very low concentrations of cardiac glycosides in the intact blood cells can be prevented by an increase in the external concentration of $K^+$ (39, 55). Cardiac glycosides have no effect on the active transport of cations when they are added to the inside of the membrane; this has been shown in injection experiments on squid axons (19).

The effect of $K^+$ on the inhibition of the enzyme activity by g-strophanthin suggests that cardiac glycosides inhibit the enzyme activity by a displacement of $K^+$ from the $K^+$ site; however, the effect on the $K^+$ activation cannot be explained on the basis of simple competitive kinetics, and a displacement of $K^+$ must therefore be due to an indirect effect on the $K^+$ site. Another possibility is that the cardiac glycosides interfere with the coupling between $Na^+$ and $K^+$ (4).

The effect of cardiac glycosides on the activity of the enzyme system due to $Na^+ + K^+$ thus shows a close correlation with the effect on the active transport of cations in respect to *a*) the concentrations that inhibit the transport, *b*) the effect of different types of cardiac glycosides, and *c*) the site of action on the two systems. The enzyme system thus fulfills requirement 7 of a system involved in the active

transport of cations, and the experiments on the effect of cardiac glycosides lend strong support to the assumption that the enzyme system is involved in the active transport of $Na^+$ and $K^+$.

## QUANTITATIVE RELATION BETWEEN EFFECT OF $NA^+ + K^+$ ON ENZYME SYSTEM AND ACTIVE TRANSPORT IN INTACT CELL

The microsomal particle that contains the enzyme system seems to be disintegrated cell membranes. It has two sites with affinities for monovalent cations: one site with an affinity for $Na^+$ higher than that for $K^+$, and a second with an affinity for $K^+$ higher than that for $Na^+$. The experiments by Whittam (115), Glynn (41), and Baker (7) indicate that in the intact cell the $Na^+$ site of the enzyme system is located on the inside and the $K^+$ site on the outside of the cell membrane.

In experiments on the enzyme system isolated from crab nerve it was shown that the enzyme system has such affinities for $Na^+$ and $K^+$ at these two sites as would be appropriate if the function of the enzyme system was to control intracellular concentration of $Na^+$. With the $Na^+$ site in contact with concentrations of $Na^+$ and $K^+$ identical to the intracellular concentrations in the crab, and the $K^+$ site in contact with extracellular concentrations, the activity of the enzyme system is about 40% of maximum. A variation in the "intracellular" $Na^+$ concentration from 20 to 60 mM/liter, i.e. from below to above the normal intracellular concentration of 40 mM/liter, gives the highest change in activity of the enzyme system (93, 95, 98).

In experiments on red blood cell membranes it was found that the concentrations of $Na^+$ and $K^+$ that give half maximum activation of the active transport of the cations in red blood cells correspond to the concentrations of $Na^+$ and $K^+$ that give half maximum activation of the $(Na^+ + K^+)$-activated enzyme system from red blood cell membranes (80).

It has also been shown that there is a correlation between the activity of the enzyme system and the intracellular concentrations of $Na^+$ and $K^+$. The red blood cells of one type of sheep have a low $Na^+$ and a high $K^+$ concentration; the cells of another type have a high $Na^+$ and a low $K^+$ concentration. The activity of the $(Na^+ + K^+)$-activated enzyme system isolated from the red blood cells with the low $Na^+$ concentration is about 4 times as high as that of the enzyme system isolated from the red blood cells with the high $Na^+$ concentrations (106, 107). Human red blood cells contain 20 mM/liter of $Na^+$ and 100 mM/liter of $K^+$, whereas cat red blood cells contain 104 mM/liter of $Na^+$ and 6 mM/liter of $K^+$. The activity of the enzyme system isolated from human red blood cells is about 8 times as high as that of the enzyme system isolated from cat red blood cells (13).

In 8 of 12 tissues in which it was possible on the basis of data from the literature to calculate the cardiac glycoside-sensitive cation exchange, a correlation was found between the cation transport and the activity of the enzyme system (11). And in another series of experiments on 6 different tissues a significant correlation was revealed between the activity of the enzyme system and active cation transport over a 25,000-fold range (10).

The stoichiometry between the active transport of $Na^+$ and the hydrolysis of ATP due to the ($Na^+ + K^+$)-activated enzyme system has been found to be close to 3 $Na^+$ transported per energy-rich phosphate bond split (10, 41, 91, 92). This agrees with the finding in frog skin that the oxygen consumption is 1 equivalent for every 4–5 equivalents of $Na^+$ transported (71, 120). For a P/O ratio of 3, this means 2–3 $Na^+$ transported per energy-rich phosphate bond. The number of $K^+$ transported per energy-rich phosphate bond hydrolyzed seems to be lower than 3 (41, 79, 91).

As mentioned previously, the enzyme systems prepared from mammalian brain and kidney have affinities for $Na^+$ and $K^+$ that are higher than the affinities of the crab nerve enzyme system for these cations. Considering that the concentrations of $Na^+$ and $K^+$ in the intra- and extracellular phases of mammals are lower than the concentrations of the same cations in the crab, the higher affinities were to be expected if the enzyme system was involved in the active, linked transport of $Na^+$ and $K^+$.

Accordingly, good quantitative agreement seems to exist between the effect of $Na^+$ and $K^+$ on the enzyme system and on the active transport system in the intact cell (requirement 8).

## NATURE OF ENZYME SYSTEM

The enzyme system is located in a microsomal particle that contains lipids in addition to proteins. Incubation of the particle with phospholipase A (77, 95) or phospholipase C (86) leads to a decrease of the activating effect of $Na^+ + K^+$, while the activity with $Mg^{++}$ is unchanged or decreased to a lesser degree. The same is found on incubation of the microsomal particle with lysolecithin (77, 100), unsaturated fatty acids like oleic acid (95), detergents like DOC (96), and lauryl sulfate (40, 42). Incubation of the enzyme system with oleic acid in a concentration of 0.5 mM/liter completely inhibits the $Na^+ + K^+$ activation, while stearic acid has no effect. Lauryl sulfate gave complete inhibition of the $Na^+ + K^+$ activity in a concentration of 0.5%. In low concentrations, DOC inhibits the activity with $Mg^{++}$ alone down to a certain level with no or only a slight effect on the $Na^+ + K^+$ activation. In higher concentrations, it also inhibits the $Na^+ + K^+$ activation. The concentration that gives inhibition differs for enzyme systems from different tissues (96).

The observation that phospholipase A as well as phospholipase C decreases the $Na^+ + K^+$ activation suggests that the effect is due to hydrolysis of lecithin more than to an effect of the products of the hydrolysis; but as lysolecithin and unsaturated fatty acids decrease the $Na^+ + K^+$ activation the products of the hydrolysis of lecithin may add to the effect of phospholipase A.

The result of these experiments may indicate that lipids are essential either for the affinity for $Na^+ + K^+$ or for the activating effect of $Na^+ + K^+$ and that the specificity for the cations requires an organized lipoprotein structure.

At present the number of enzymes involved in the breakdown of ATP by the enzyme system is unknown, but it is reasonable to assume that there is more than

one. In the first publication on this enzyme system it was referred to as an adenosinetriphosphatase (ATPase) (93). The involvement of an organized lipoprotein structure and perhaps more than one enzyme makes it more reasonable to call it an enzyme system instead of an ATPase and, in order to characterize it, to use the term ($Na^+ + K^+$)-activated enzyme system.

There is a source of error that has to be taken into consideration, especially in the experiments on the phosphate transfer, viz., that all the enzyme preparations have an activity with $Mg^{++}$ alone. It is important to know whether this activity is due to another enzyme that has nothing to do with the ($Na^+ + K^+$)-activated enzyme system, or whether it is the same enzyme system that either goes wrong when there is no $Na^+$ and $K^+$ in the medium or has been partially changed during the preparative procedure and has thus lost its specific requirements for $Na^+$ and $K^+$.

In experiments on red blood cell membranes it was found that the substrate site for the $Mg^{++}$-activated, ATP-hydrolyzing enzyme was on the outside of the membrane, and it was on the inside for the ($Na^+ + K^+$)-activated enzyme (57). This may indicate that there are two separate enzymes.

In a number of enzyme preparations from muscle there is no activation by $Na^+ + K^+$, but only by $Mg^{++}$. In these preparations, the addition of heated supernatant (see above) decreases the activity with $Mg^{++}$ alone, and the enzyme system is then activated by $Na^+ + K^+$, but always to a degree identical with, or lower than, the activity found with $Mg^{++}$ alone before the addition of the heated supernatant.

If there are two different enzymes, these results indicate that the $Mg^{++}$-activated enzyme inhibits the ($Na^+ + K^+$)-activated enzyme. Another explanation is that the $Mg^{++}$-activated enzyme is a partially changed ($Na^+ + K^+$)-activated enzyme, and that the addition of the heated supernatant gives back to the enzyme system its specific requirements for $Na^+ + K^+$. If this explanation is correct, one might expect that the treatment of the ($Na^+ + K^+$)-activated enzyme system with oleic acid or phospholipase A or C, which leads to a disappearance of the $Na^+ + K^+$ activation, should lead to an increase in $Mg^{++}$ activation; this has not been found.

On the other hand, the experiments with $ATP^{32}$ (Fig. 1) showed that the labeling found with $Mg^{++}$ alone in the medium disappears when $Mg^{++} + Na^+ + K^+$ are present in the medium. This seems to indicate that it is the same enzyme system that functions in one way when $Na^+ + K^+$ are present in the medium and in another when there is only $Mg^{++}$ in the medium.

At the present time it is not possible to determine the relationship between the two activities, but for the elucidation of the function of the ($Na^+ + K^+$)-activated enzyme system it is important to get more information.

The ($Na^+ + K^+$)-activated enzyme system is inhibited by a number of SH inhibitors (6, 42, 69, 82, 97), including *p*-chloromercuribenzoate (PCMB), N-ethylmaleimide (NEM), and 2,4-dinitrofluorobenzene (DNFB) (97).

The inhibition by PCMB is reversed by cysteine; NEM and DNFB decrease the activity due to $Mg^{++}$ much faster than the activity due to $Mg^{++} + Na^+ + K^+$. ATP protects at least to some extent against the effect on the $Mg^{++} + Na^+ + K^+$

activity, although it does nto protect against the effect of NEM and DNFB on the $Mg^{++}$ activity. The $(Mg^{++} + Na^+ + K^+)/Mg^{++}$ activity ratio with NEM + ATP or DNFB + ATP in the incubation medium is therefore increased. ADP, but not ITP, has the same effect as ATP (97).

Urea has the same effect as NEM and DNFB on the $Mg^{++}$ and on the $Mg^{++} + Na^+ + K^+$ activity, and ATP can also protect against the effect of urea on the activity with $Mg^{++} + Na^+ + K^+$, but not against the effect on the activity with $Mg^{++}$ alone (Skou, unpublished data). This suggests that the effect of NEM and DNFB is not due to their effect on the SH groups as such, but to a change in the steric configuration, which is secondary to the effect on the SH groups. ATP seems to be able to prevent the change in steric configuration that leads to the decrease in the activity with $Mg^{++} + Na^+ + K^+$ in the medium, but not that leading to a decrease in the activity with $Mg^{++}$ alone.

The submicroscopic particle that contains the enzyme system also contains a system that can transfer electrons from DPNH to cytochrome *c* and to dyes. This has been found in submicroscopic particles isolated from brain, kidney, red blood cell membrane, and cardiac muscle. Both the DPNH-cytochrome *c* reductase and the diaphorase activity are inhibited by NEM. DPNH protects against this effect of NEM (97).

ATP affords protection, but to a lesser degree than DPNH, against the effect of NEM on the DPNH-cytochrome *c* reductase activity; DPNH seems to decrease the rate at which NEM inhibits the enzyme activity with $Mg^{++} + Na^+ + K^+$, but DPNH has no influence on the effect of NEM on the activity, with $Mg^{++}$ in the medium. At present, these are the only indications that the two systems may have any kind of relationship (97).

The absorption spectrum of the microsomal fraction of electric tissue that contains the $(Na^+ + K^+)$-activated enzyme system showed no trace of cytochrome, flavoproteins, ubiquinol, or pyridine nucleotide (42).

The enzyme system is inhibited by atebrin and by chlorpromazine (61, 98), and it seems as if the activity with $Mg^{++}$ alone is more sensitive to these drugs than the activity with $Na^+ + K^+$ (98). These drugs are known to be inhibitors of flavoenzymes (for ref., see 73), but so far there is no evidence that their inhibitory effect on the enzyme system is due to an effect on flavoenzymes.

Oligomycin, which in the mitochondria inhibits the reaction associated with the transfer of high-energy bonds in oxidative phosphorylation (59, 70), also inhibits the $Na^+ + K^+$ activation of the enzyme system (45, 62). The inhibition is higher at 25 C than at 37 C. The concentrations required are, however, much higher than those needed in the experiments with mitochondria. This effect of oligomycin might indicate that a high-energy intermediate is involved in the hydrolysis of ATP by the enzyme system.

2,4-Dinitrophenol in concentrations of 1 mM/liter has no effect on the $Na^+ + K^+$ activity (42, 98).

Reducing agents like dithionite, borohydride, ascorbic acid + *p*-phenylenediamine give partial inhibition; the same is found with oxidizing agents like hydrogen peroxide. This effect may be due to an effect on sulfhydryl groups (42).

Aldosterone and hydrocortisone, which increase renal excretion of $K^+$ and

reabsorption of $Na^+$, have no effect on the enzyme activity. Insulin, which affects uptake of $K^+$ by the cells, has no effect (13`

## RELATION OF ($NA^+$ + $K^+$)-ACTIVATED ENZYME SYSTEM TO ACTIVE TRANSPORT OF CATIONS

The experiments reported have shown that the ($Na^+$ + $K^+$)-activated enzyme system fulfills requirements 1–6 of a system involved in the active, linked transport of $Na^+$ and $K^+$ across the cell membrane, and that it behaves like the transport system in the intact cell, both in its relation to cardiac glycosides (requirement 7) and in its quantitative relation to $Na^+$ + $K^+$ (requirement 8). It is therefore reasonable to assume that this enzyme system is, in some way, involved in the active transport of $Na^+$ and $K^+$ across the cell membrane. But to understand how it functions in cation transport it is necessary to know the answer to a number of questions, including the following.

Is the catalytic activity measured in the test tube identical to the catalytic activity of the system when it is located in the intact cell membrane?

The treatment with DOC or the aging that seems to be necessary to obtain preparations with high $Na^+$ + $K^+$ activation suggests that it is a partially changed system. On the other hand, it is possible to obtain preparations with high $Na^+$ + $K^+$ activation without these procedures, viz. from crab nerve (93) and from electric tissue from the electric eel (1, 40). Furthermore, experiments on the red blood cell membrane seem to indicate that the enzyme system in the intact membrane behaves as it does in the test tube (41, 115).

Is ATP the only substrate that can activate the enzyme system? Or is it, as has been proposed for the transport of divalent cations in the mitochondria (8), possible to activate the system via an electron transport:

$$\xrightarrow{\text{electron transport}} TS\infty \leftarrow ATP + TS$$
$$\Big\downarrow \text{ion transport}$$
$$TS$$

It is suggestive that preparations from different tissues of the microsomal particles in which the enzyme system is located all show a DPNH-cytochrome *c* reductase activity. However, so far no relation has been found between the two systems, but we need more experiments before any conclusion can be drawn. First of all, we must know whether this electron-transferring system behaves in the intact cell as it does in the test tube, or whether it has been more or less changed during the preparative procedure, as seems to be the case with other DPNH-cytochrome *c* reductase activities (24, 113).

Is the ($Na^+$ + $K^+$)-activated enzyme system only part of the transport system for $Na^+$ and $K^+$, so to speak the "engine" that can convert the energy from ATP to a movement of a carrier for $Na^+$ and $K^+$, and if it is, does the microsomal particle in which the enzyme system is located contain the carrier, and of what nature is it? Is it the phosphatidic acid cycle as proposed by Hokin and Hokin (58)?

The observation that the microsomal particle contains sites with specific af-

finities for $Na^+$ and $K^+$ and that $Na^+$ and $K^+$ are bound to the sites and not as part of the enzyme substrate complex suggests that the particle contains the transport sites; they may either be part of the enzyme system or on a carrier intimately connected with the enzyme system.

The low amount of labeling of lipids and the high labeling of proteins in the microsomal particle with $P^{32}$ from ATP seems to exclude phosphatidic acid as a carrier, but this negative result is not conclusive.

What is the relation to transport of other substances that seem to require $Na^+$ in the medium for their transport, such as glucose (28), amino acids (23, 27, 36, 49), and iodide (for ref. see 117)? Is the enzyme system at the same time the "engine" and the transport system for $Na^+$ and $K^+$, and can carriers for these other substances be coupled to this "engine" whose activity is determined by the cation concentrations?

These and other questions of importance in the understanding of the function can only be answered by a more detailed knowledge of the components of the system, of the intermediary steps in the hydrolysis of ATP, of what it is that determines the affinities for cations, etc., than we have at the present time.

It is therefore not possible to answer the question how this enzyme system can participate in the transport of cations and other substances.

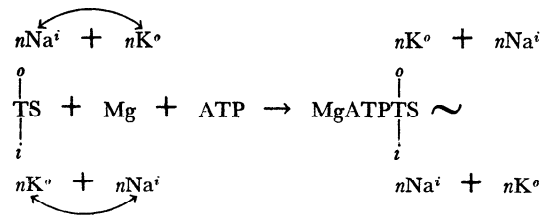
However, an attempt has been made to find out if our present knowledge can give any ideas of where to find the answer (98–100). In these papers it is claimed that whether the transport of cations is via a transport of sites with affinities for cations from the inside to the outside of the membrane (29, 43, 78, 84, 117), or via a transport of affinities for $Na^+$ and $K^+$ through fixed sites in the membrane, the transport requires sites that can shift their affinity for cations. This means that to understand the transport we must know what determines the affinity of a site for a monovalent cation and how this affinity can be changed.

Experiments on the enzyme system have shown that ATP increases the affinity for $Na^+$ and also the activating effect of $K^+$. This has led to the assumption that the shift in affinities is due to an effect of ATP on the enzyme system. To transport ions the transport system must be activated and this must either be due to an effect of ATP as such or to a phosphorylation. With $Na^+ + K^+$ in the medium (i.e. as it is when the transport system is in the cell membrane) there seems to be no phosphorylation. It thus seems likely that the activation is due to an effect of ATP on the transport system and that the hydrolysis of ATP leads to a deactivation. A scheme for the enzyme system as a transport system would then be the following [modified from Skou (100)].

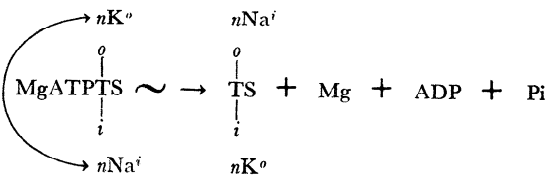
In the deactivated form of the enzyme system, site $o$, which is on the outside of the membrane, is occupied by $Na^i$, which has been transferred from site $i$ to site $o$ during the previous transport cycle; site $i$, which is on the inside of the membrane, is occupied by $K^o$, which has been transported from site $o$ to site $i$; $n$ is the number of ions transported per cycle, of the order of 2–3 [Post and Jolly (79), Sen and Post (91), Glynn (41)].

When bound to the enzyme system ATP activates the transport system, i.e. brings it into a form in which it can work—transport the cations. As a part of the

activation the affinities of site *o* change from a $Na^+$ to a $K^+$ affinity and $Na^i$ on site *o* will exchange with $K^o$ from the extracellular fluid; simultaneously ATP changes the affinity of site *i* from a $K^+$ to a $Na^+$ affinity and $K^o$ on site *i* exchanges with $Na^i$ from the cytoplasm.

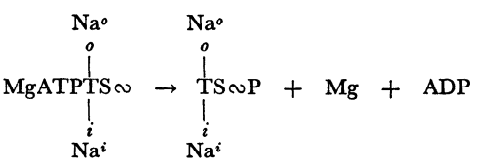



ATP is hydrolyzed when $K^+$ is at site *o* and $Na^+$ at site *i*, and this allows the transport system to deactivate and by that to work on the cations—transport them:

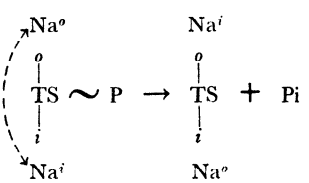



With a new ATP the cycle is repeated.

If $K^+$ is removed from the outside solution $Na^+$ at site *o* cannot exchange with K and there will be $Na^+$ at both site *i* and site *o*. Due to this there is an abnormal hydrolysis of ATP that leads to a phosphorylation.




The phosphorylation followed by the dephosphorylation gives an abnormal deactivation of the enzyme system and it will therefore not be able to work (transport the cations) in a normal way.




The scheme does not tell how ATP influences the affinities or how the cations are moved. It only says that it is the effect of ATP that is responsible for the change in affinities.

The above-mentioned results, that ATP seems to have an effect on the site with affinity for $K^+$, which must presumably be on the outside of the membrane

while ATP is on the inside, has led to the suggestion that the effect of ATP is due to an ATP-induced change in distribution of electrons inside the enzyme system. This assumption may find support in experiments on the electron transport in mitochondria in which it has been shown that ATP can induce a reverse transfer of electrons (20, 67).

But how can a change in distribution of electrons inside the enzyme system lead to a change in affinities for cations and bring the system into a state where it can do work?

The factor or factors determining the specificity of a system for monovalent cations are unknown. It may be the steric configuration of the anionic groups; or it may be the field strength of the same groups (33). According to the last hypothesis a low field strength of the anionic groups gives an affinity for $K^+$ higher than for $Na^+$, while a higher field strength gives an affinity for $Na^+$ higher than for $K^+$.

Both the steric configuration of the anionic group and the field strength of a negative charge are influenced by the density of electrons in the vicinity of the charge. A certain distribution of electrons on a macromolecule may therefore give one negative charge a higher affinity for $Na^+$ than for $K^+$ and at the same time another charge a higher affinity for $K^+$ than for $Na^+$, whether the selectivity is due to a certain steric configuration or to a certain field strength of the anionic groups. A change in the distribution of the electrons on the macromolecule may lead to a shift in the affinity of the first charge from a high $Na^+$ to a high $K^+$ affinity and simultaneously for the other charge from a high $K^+$ to a high $Na^+$ affinity. If the charges are close together, this may lead to an ion exchange where $Na^+$ is moved from the first charge to the second simultaneously with a movement of $K^+$ in the opposite direction. Based on this reasoning and on the assumption that the effect of ATP is due to an effect on the distribution of the electrons inside the enzyme system, a model has been proposed for the enzyme system as a transport system (for details see 98–100).

In this model, it is the distribution of the electrons on a macromolecule in a pore of a membrane that determines the affinity for cations of the negative charges on the molecule. One charge has a higher affinity for $Na^+$ than for $K^+$, while the others have a higher affinity for $K^+$. ATP induces a redistribution of the electrons on the macromolecule that leads to a stepwise change in affinity of the negative charges and thus to an ion exchange where $Na^+$ is moved outwards simultaneously with an inward movement of $K^+$. There must be at least three negative charges in the pore to give an unidirectional flow of the cations.

In contrast to the models proposed by Lundegårdh (72) and Conway (25) for an ion transport linked to an electron transport, there is no unidirectional flow of electrons through the membrane in this model. It is the electrons inside the macromolecule that are repeatedly redistributed due to the effect of ATP, and this gives an unidirectional flow of $Na^+$ in one direction and of $K^+$ in the opposite direction, and the number of $Na^+$ ions moved per ATP can be more than one.

At present we have no experimental evidence for the assumption that the distribution of electrons on a macromolecule can determine the affinity of negative charges for cations. This model is therefore mainly based on speculation. A more

detailed investigation of the enzyme system may, however, give information leading to a better understanding of the active transport of cations and perhaps also of other substances like glucose and amino acids.

## CONCLUSION

On the basis of our present knowledge of the active transport of $Na^+$ and $K^+$ in the intact cell it is possible to formulate a number of requirements of a transport system. The ($Na^+ + K^+$)-activated enzyme system that has been isolated from a large number of different tissues fulfill these requirements. This indicates that the enzyme system is involved in the active transport of $Na^+$ and $K^+$ across the cell membrane.

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