

# THE IDENTIFICATION OF THE SODIUM-POTASSIUM PUMP

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by

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Looking for the answer.

You hunt it,  
you catch it,  
You fool yourself;  
the answer,  
is always,  
a step ahead.

J. C. S.

## INTRODUCTION

The cell membrane separates the cell from the surrounding medium. In 1925 Gorter and Grendel<sup>1</sup> extracted the lipids from red blood cells, spread them in a monomolecular layer on a water phase, and measured the compressed area. It was about the double of the surface area of the extracted red blood cells. They suggested that the cell membrane is a bilayer of lipids with the charged head groups of the phospholipids facing the water phase on the two sides of the membrane, and with the hydrocarbon chains meeting in the middle of the membrane. The thickness of the membrane is about 40 Å, much too small to be seen under a microscope.

The surface tension of invertebrate eggs and other cells is however, much lower than for a water-lipid interphase. To explain this, Danielli and Davson in 1935<sup>2</sup> suggested that there is an adsorbed layer of proteins on each side of the lipid bilayer. They also introduced a layer of non-oriented lipids in the middle of the bilayer.

In the cytoplasm there are proteins for which the cell membrane is impermeable. At the cell pH they carry negative charges, neutralized by potassium,  $K^+$ , which in the cell is at a concentration of about 150 meq/l, while outside the cell the  $K^+$  concentration is 4 meq/l. The difference in  $K^+$  concentration was explained as due to a Donnan effect of the proteins, and that the membrane is permeable to  $K^+$ . The Donnan effect gives an osmotic pressure, which is higher inside the cell than outside, and since the membrane is permeable to water, water will flow in, and as the lipid bilayer cannot resist a hydrostatic pressure the cell will swell and finally burst.

The higher osmotic pressure inside the cell is opposed by a high concentration of sodium,  $\text{Na}^+$ , outside, 140 meq/l, while the concentration inside is low, about 10–20 meq/l. A problem is to explain how this difference in the  $\text{Na}^+$  concentration on the two sides of the membrane can be maintained. There are two possible explanations:

One is that the membrane is impermeable to  $\text{Na}^+$ , that it is an equilibrium situation. This view was advocated by Conway, an Irish biochemist from Dublin. Boyle and Conway published in 1941 a paper in which they showed that for muscle fibers soaked in solutions with varying  $\text{K}^+$  concentrations, the calculated intracellular  $\text{K}^+$  concentrations, based on this assumption agreed with the measured<sup>3</sup>. There were, however, two problems. One was that this agreement did not hold at the normal physiological concentrations of  $\text{K}^+$ , only at higher concentrations. The other was the requirement of  $\text{Na}^+$  impermeability. But how then to explain that there is sodium in the cell, even if it is at a low concentration. Conway gave no answers to these problems.

The other possibility is that the membrane is permeable to  $\text{Na}^+$ , and that there are secretory, energy dependent processes in the cell, which compensate for the steady influx of  $\text{Na}^+$ , a steady state distribution. This view was advocated by R.B. Dean in a paper also published in 1941 entitled: "Theories of Electrolyte Equilibrium in Muscle"<sup>4</sup>. Referring to investigations by L.A. Heppel (1939,1940), by L.A. Heppel and C.L.A Schmidt (1938), and by H.B. Steinbach (1940) (see ref. 4) on muscle fibers, which had shown that the muscle membrane, contrary to the view held by Conway, is permeable to  $\text{Na}^+$ , Dean concluded: "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 sodium or, what is equivalent, pump in potassium."

In the following decade, helped by the introduction of radioactive isotopes of  $\text{Na}^+$ , and  $\text{K}^{45}$ , it was shown not only from experiments on muscle fibers, but on red blood cells, on nerves, and on frog skin that the membrane is permeable to  $\text{Na}^+$  as well as to  $\text{K}^+$ , (for references see an extensive review by Ussing<sup>6</sup>). An energy dependent efflux of  $\text{Na}^+$  is therefore necessary.

However, Conway strongly defended his view about the impermeability to sodium, and only reluctantly gave way. He admitted that there may be a certain permeability for  $\text{Na}^+$  and thereby a need for a pump, but that low permeability for  $\text{Na}^+$  is the main explanation of the concentration gradient. Conway's concern was, that it is a waste of energy to have a membrane permeable to  $\text{Na}^+$ , and then spend energy to pump  $\text{Na}^+$  out. Krogh<sup>7</sup> had 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" criticised Conway's view about impermeability to sodium. Krogh concluded: "The power of active transport of ions is of a common occurrence both in the vegetable and the animal kingdom and is possibly a general characteristic of the protoplasmic surface membrane". Conway replied the same year in a paper in *Nature*<sup>8</sup>: "Krogh ... considers the apparent impermeability to sodium as due to an active extrusion, sodium ions entering

the (muscle) fibers as fast, if indeed not faster, than potassium. 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 the energy requirement was about twice the resting metabolism of the muscle. Ussing<sup>9</sup> came to the same result, but he explained what the apparent problem was. Only a part of the measured  $\text{Na}^+$  flux is due to active transport of  $\text{Na}^+$ , the other part is due to an exchange across the membrane of  $\text{Na}^+$  from the one side for  $\text{Na}^+$  from the other side, a Na:Na exchange, which is energetically neutral, and which gives no net flux of  $\text{Na}^+$  across the membrane. Taking this into account, the energy available is more than sufficient. However, Conway's concern was relevant in the sense that with the knowledge at that time about membrane function, an active transport seemed energetically an expensive way to solve the osmotic problem of the cell.

In the 1940s and first half of the 1950s the concept of active transport developed<sup>6</sup>. It was defined as a transport against an electrochemical gradient<sup>10</sup>. It was shown that the active efflux of sodium was coupled to an influx of potassium, a pump, and that the substrate for the transport was energy rich phosphate esters (for references see<sup>11</sup>).

But what was the nature of the pump? With the information available in the beginning of the 1950s it was possible to foresee that the pump is a membrane bound protein with enzymatic activity, which has ATP as substrate and is activated by  $\text{Na}^+$  on the cytoplasmic side, and by  $\text{K}^+$  on the extracellular side. But nobody apparently thought that way. A reason may be that the membrane according to the model by Danielli and Davson is a bilayer of lipids with no room for proteins inside the bilayer spanning the bilayer. On the contrary it was assumed that protein in the bilayer would destabilize the cell membrane. It was assumed that the protein was in the interphase between the lipids and the water on the two sides of the membrane.

#### THE WAY TO THE SODIUM PUMP<sup>12-13</sup>.

My scientific interest was the mechanism of action of local anaesthetics. I held a position at the Institute of Physiology at the University of Århus, and was using this problem as subject for a thesis. In 1953, I had finished a series of experiments on the problem, which was published in 1954 in book form in Danish, and accepted by the faculty to be defended for the medical doctor's degree<sup>14</sup>. It was also published as 6 papers in English<sup>15</sup>.

I had received my medical degree in the summer 1944, and started my internship at a hospital in Hjørring in the northern part of the country, six months in the medical ward followed by six months in the surgical ward. I became interested in surgery, and after my internship I continued for another year in the surgical ward. We had no anaesthetists, and to avoid the unpleasant ether narcosis, we used whenever possible spinal and local anaesthesia. From the teaching of pharmacology I knew the Meyer-Overton theory<sup>16-17</sup>, that there is a correlation between solubility of general anaesthetics in lipids,

and the anaesthetic potency. General anaesthetics are non-polar substances, while local anaesthetics are weak bases, which at the physiological pH exist as a mixture of charged and uncharged molecules. I wondered which of the two components is the anaesthetic component, and whether a correlation, similar to that for the general anaesthetics, existed for the local anaesthetics. I decided to use this problem as a subject for a thesis. After the two years at the hospital in Hjørring, I took up a position for a year at the Orthopaedic Hospital in Aarhus, and after this, in 1947 I applied for and received a position at the Institute of Medical Physiology at Aarhus University.

Aarhus University was young, founded 19 years earlier. There were Institutes for Anatomy, Biochemistry and Physiology as a beginning of a Medical Faculty, which was not completed until 1957. These were the only biological Institutes at the Campus, and the scientific biology milieu was poor, with little or no contact with the outside scientific world. We were three young doctors besides the Professor, Søren L. Ørskov, in the department each working on our thesis. None of us had any scientific background, but Ørskov was very helpful, patient and let us take the necessary time. We had an intake of 140 medical students a year so the teaching load was heavy. After having passed physiology the students had to continue at the medical faculty in Copenhagen for their medical degree.

I used the intact sciatic nerve of frog legs as a test object for measuring the blocking potency of five different local anaesthetics, which are weak bases, and of butanol as a representative of a nonpolar blocking agent. After removing the sheath around the nerve to get easier access to the single nerve fibers, I measured the blocking concentration as a function of time, and from this the minimum blocking concentration at infinite time of exposure could be determined. The concentrations necessary varied from the weakest to the strongest of the local anaesthetics by a factor of 1:920 (with butanol included, 1:13.500).

The order of anaesthetic potency and solubility in lipids were the same, but the quantitative correlation was poor, i.e. local anaesthetics did not follow the Meyer-Overton rule for general anaesthetics.

I was looking for another test object. As the cell membrane is a bilayer of lipids, I decided to use a monomolecular layer of lipids on a Krebs-Ringer water phase as a model for a water cell membrane interphase. The inspiration came from reading about Langmuir's work on monomolecular layers of lipids on a water phase in "The Physics and Chemistry of Surfaces" by N.K. Adam, and that Schulmann had applied capillary active drugs in the waterphase beneath the monolayer, and observed that they penetrated up into the monolayer<sup>18</sup>.

In a Langmuir trough the area of the monolayer can be measured as a function of the pressure that the monolayer exerts on a floating barrier, which separates the monolayer from the pure water phase without the monolayer. My first experiments were with a monolayer of stearic acid. At a given area per molecule, which also means at a given surface pressure, application of the local anaesthetics to the water phase gave an increase in pressure, in-

dicating that the local anaesthetics penetrated up into the monolayer, and the pressure increased with the concentration. There was a certain correlation between anaesthetic potency and pressure increase, but quantitatively not clear enough. However, with a monolayer of lipids extracted from the sciatic frog nerves there was a reasonably good correlation. The order of the concentrations necessary to increase the pressure followed the order of anaesthetic potency. And the minimum blocking concentration of the five local anaesthetics, which as mentioned varied by a factor of 1:920, gave a pressure increase in the monolayer at a certain area which was of the same order, they varied by a factor of 1:3.2. Also the effect of a change in pH on the local anaesthetic potency correlated reasonable well to the effect of pH on the pressure increase at a given area.

The rising phase of the nerve impulse, the depolarization is due to a transient increase in permeability of the membrane for  $\text{Na}^{+19}$ . The molecular basis for this was unknown, but it seemed unlikely that it was connected to the lipids. I assumed that the permeability increase was on proteins in the membrane. The monolayer results suggested to me, that the effect of the penetration of local anaesthetics into the lipid part of the nerve membrane was a blocking of the conformational change in proteins in the membrane, which gave the increase in permeability to  $\text{Na}^{+}$ .

To test this I wanted to see if pressure in a monolayer could influence the enzymatic activity of a protein in the monolayer, and take this as indication of an effect of pressure on conformation. And if there was an effect, then form a monolayer of a mixture of lipids and the enzyme, and test if penetration of local anaesthetics into the monolayer had an effect on the enzymatic activity. For this I needed an enzyme with high activity, which was related to membrane function. A candidate was acetylcholinesterase, which was then being prepared from electric eel by Professor Nachmansohn at Columbia University in New York. It had the further advantage that it involved a visit to New York.

The Professor of Physiology in Copenhagen Einar Lundsgaard was a close friend of David Nachmansohn and introduced me. I had planned to spend August in New York, take a break at the end of August and beginning of September to attend the 19th International Congress of Physiology in Montreal, and then return to New York in September\*. This would fit with my teaching schedule. Nachmansohn would not be in New York until September, as he spent the summer at the Marine Biological Station in Woods Hole, he therefore suggested I should join him there in August. In September he would return to New York. I agreed, although I did not know what to do in Woods Hole; there was no access to electric eel.

Scientists interested in the function of the nervous system came from all over the world to Woods Hole during the summer, to use the giant axons from squids as test objects. Coming from a young University with a poor scientific milieu, this was like coming to another planet. The place was bubbling with scientific activity. I realized that science is a serious affair and not just a

\* Not July and August as mentioned in references 12 and 13.

temporary hobby for young doctors writing a thesis in order to qualify for a clinical career. And also that it is competitive. I listened to lectures, met people whose names I knew from the textbooks, and from the literature, spent time in the laboratories looking on, and learning from the experiments.

In between I did some reading, and in a paper written by Nachmansohn, it was mentioned that B. Libet<sup>20</sup> in 1948 had shown that there is an ATP hydrolysing enzyme in the sheath part of the giant axon from squid: an ATPase. As ATP is the energy source in cells I wondered what the function could be of an ATPase in the membrane of a nerve. Situated in the membrane I assumed that it was a lipoprotein, and this was what I needed for the monolayer experiments. I decided to look for the enzyme when I came home.

I prepared acetylcholinesterase at Columbia University in September from the electric eel. Back in Aarhus I continued the monolayer experiments.

I had no access to giant axons in Aarhus, but decided to look for the putative nerve membrane ATPase in crab nerves, because the crab nerve, like the giant axon, has no myelin sheath. In 1954 I arranged with a fisherman south of Aarhus to send me some crabs, and started to isolate the sciatic nerve from the legs. The nerves were homogenized and the membrane pieces isolated by a differential centrifugation.

The experiments showed that the membrane fractions had a low magnesium ( $Mg^{2+}$ ) activated ATP hydrolysing enzyme activity. Addition of  $Na^+$  besides  $Mg^{2+}$  gave a slight increase in activity.  $K^+$  had no effect in the presence of  $Mg^{2+}$ , Fig. 1. However the activity varied from experiment to experiment. Calcium ( $Ca^{2+}$ ) was excluded as the reason for the variations. After having spent November–December trying to find a solution I gave up and went on my Christmas holiday. I resumed the experiments in June the following year, but still without being able to get reproducible results, went on summer holidays. Returning in August, I made a  $Na^+$  salt of ATP and a  $K^+$  salt and found to my surprise that the activity with the  $K^+$  salt was higher than with the  $Na^+$  salt. This could not be due to a difference in ATP but to an effect of  $K^+$  which differed from that of  $Na^+$ . But why in this experiment and not in the previous experiments, where  $K^+$  had no effects in the presence of  $Mg^{2+}$ . The answer was that in the experiments with the  $K^+$  salt of ATP there was  $Na^+$  in the medium. In other words the enzyme needed a combined effect of  $Na^+$  and  $K^+$  for activation. I then started a systematic investigation of the combined effect of the two cations.

As seen from Fig. 1,  $K^+$  has two effects in the presence of  $Na^+$ . It activates, the higher the concentration is of  $Na^+$ . The  $K^+$  affinity for the activating effect is high. At higher concentrations the activating effect of  $K^+$  decreases, and the apparent affinity for  $K^+$  for this effect decreases with an increase in the  $Na^+$  concentration. With 3 mM  $Na^+$  it is seen that  $K^+$  not only inhibits its own activation, but also the small activation due to  $Na^+$ . The results suggest that there are two sites on the enzyme, one where  $Na^+$  is necessary for activation, and another where  $K^+$  activates when  $Na^+$  is bound to the former.  $K^+$  in higher concentrations competes for  $Na^+$  at the  $Na^+$  site, and by displacing  $Na^+$  from the site decreases the activity.

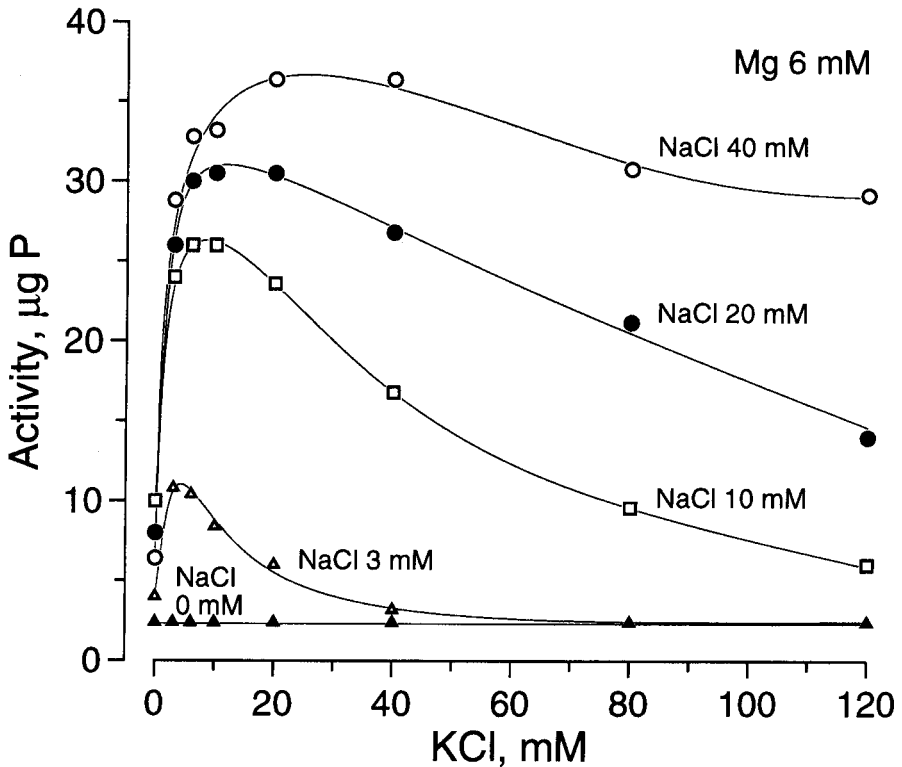


Figure 1. Effect of  $\text{Na}^+$ , and of  $\text{K}^+$  in the presence of  $\text{Mg}^{2+}$  on the hydrolysis of ATP by membrane fractions isolated from nerves of the shore crab *Carcinus maenas*. Test solution contained 30 mM histidine HCL as buffer, pH 7.2, 3 mM ATP, 6 mM  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$  in concentrations shown on the Figure. Activity is given as  $\mu\text{g P}$  (inorganic phosphate) hydrolysed from ATP in 30 min. at 36 °C (Reprinted by permission from.<sup>27</sup>)

I now understood the reason for the varying results. With little effect of  $\text{Na}^+$  on the activity, and of no effect of  $\text{K}^+$ , I had not bothered whether or not there was  $\text{Na}^+$  or  $\text{K}^+$  in the medium. I got ATP as an insoluble barium salt, which was converted to a soluble  $\text{Na}^+$  or  $\text{K}^+$  salt, sometimes the one sometimes the other, and in between instead of homogenising the nerves in glucose I used a 0.58 M KCl solution. It never occurred to me that there could be a combined effect of  $\text{Na}^+$  and of  $\text{K}^+$ .

The problem then was, what was the physiological function of the enzyme? I was interested in the effect of local anaesthetics on nerve conduction, and my first reaction was that this was the  $\text{Na}^+$  channel, which opens for the influx of  $\text{Na}^+$  leading to the nerve impulse. I soon rejected the idea because the opening of the channel is voltage dependent, and not dependent on ATP. The other possibility was that it was part of or the sodium pump. I had little knowledge about the active transport of  $\text{Na}^+$  and of  $\text{K}^+$ , I therefore started to look into the literature to see what the substrate was for the active transport in nerves. I had limited access to the literature, so there were few papers I read about active transport. The closest I could come was that A.L. Hodgkin and

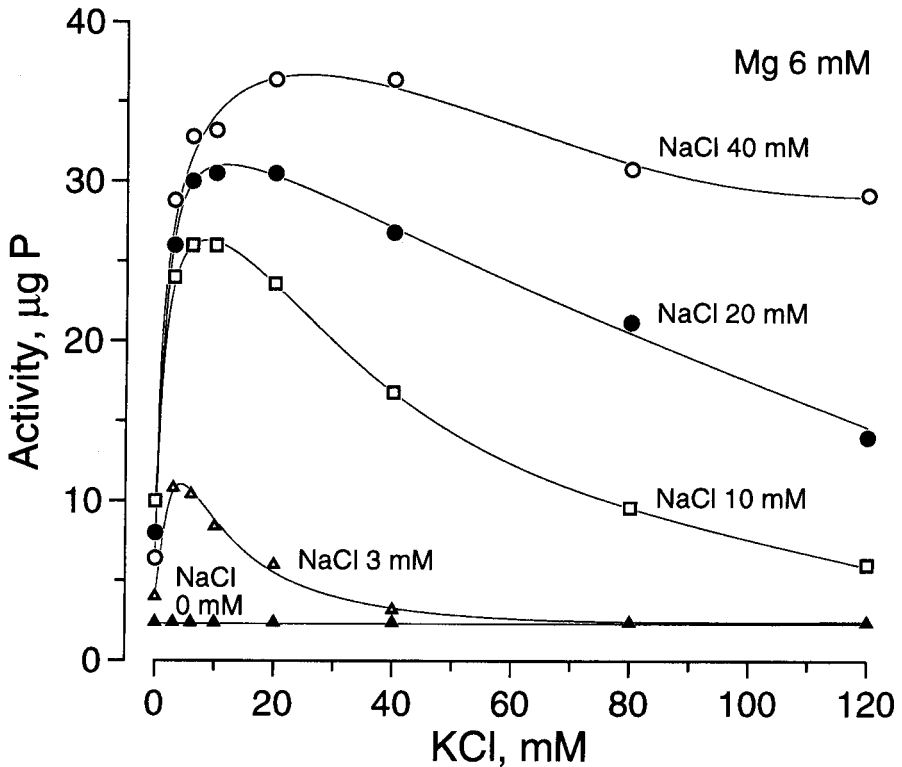


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R.D. Keynes<sup>21</sup> had shown that poisoning giant axons with dinitrophenol, cyanide or azide, decreased the active transport of sodium, suggesting that high energy phosphate esters are the substrate. And as ATP is a high energy phosphate ester, I thought it likely that it could be the substrate.

There were two papers I did not read, one was by G. Gardos<sup>22</sup> from Budapest published in 1954 in a Hungarian journal, which I did not have access to, and which was not cited in the paper by Hodgkin and Keynes. In this Gardos showed that ATP supported the active uptake of  $K^+$  in red blood cells. The other was a paper published in 1956 by Hodgkin and Keynes<sup>23</sup>, in which they reported that injecting ATP in a cyanide poisoned giant axon gave no dramatic recovery of the active extrusion of sodium. Fortunately, I did not see this last paper until I had sent my paper on the crab nerve enzyme for publication. In 1957, the experiment was repeated by Caldwell and Keynes<sup>24</sup>, with the result that ATP was the substrate for the active transport.

In a discussion after a paper by R.D. Keynes on "Electrolytes and Nerve Activity"<sup>25</sup> at an international symposium on Neurochemistry in Aarhus in 1956, I showed the results with the crab nerve ATPase<sup>26</sup>. The same year I wrote the paper, and suggested from the characteristics of the effect of the cations, and that fact that ATP was a substrate, that the enzyme was involved in the active transport of  $Na^+$  across the cell membrane. I considered putting the word pump in the title, but found it too provocative, so it became "The Influence of Some Cations on an Adenosine Triphosphatase from Peripheral Nerves". No wonder that few people noticed that this enzyme had to do with active transport of  $Na^+$ . It was published in 1957<sup>27</sup>.

With my little knowledge about active transport, I was unaware of the importance of the observation on the crab nerves. Parallel with the crab nerve experiments I continued the experiments on the monolayer with the acetylcholinesterase. In 1958, I presented a paper at the 4th International Congress of Biochemistry in Vienna on "The Influence of the Degree of Unfolding and the Orientation of the Side Chains on the Activity of a Surface Spread Enzyme".

There was one important experiment I had not done. I realized this when I met Robert Post at the conference. I knew Robert from Woods Hole. We had spent time in the same laboratory, and I had driven with him and his wife Elisabeth in their car from Woods Hole to the International Congress for Physiology in Montreal. He told me that he had since worked with active transport of  $Na^+$  and  $K^+$  in red blood cells, and had shown that the stoichiometry between the  $Na^+$  transport out of, and of  $K^+$  into, the cell was 3 to 2<sup>28</sup>.

I told him about the  $Na^+ + K^+$  activated crab nerve enzyme, and that it seemed to be part of or the sodium pump. His reaction suggested to me that this was more important than surface spread enzymes. "Is it inhibited by ouabain"? he asked. "What is ouabain" was my reply. He then told me that Schatzmann in Switzerland in 1954 had shown that cardiac glycosides, of which ouabain is the most water soluble, specifically inhibits the active transport in red blood cells<sup>29</sup>. When Robert Post came to Aarhus after the conference I had the answer. The enzyme was inhibited by Ouabain, even if the sensitivity of the crab nerve enzyme is much lower than the sensitivity of the

transport in red blood cells. It convinced Robert that the enzyme had to do with active transport. I had learned that red blood cells were a classical test object for experiments on active transport, and had started to look for the enzyme in these cells. Robert asked if he could go on with these experiments when he returned to U.S.. I had no experience with this test object, and as he had the experience I agreed, and continued the experiments with the crab nerve enzyme<sup>30</sup>, and looked for the enzyme in other tissues<sup>31</sup>.

In 1959, after I had given my first paper on the crab nerve enzyme at the 21th International Congress for Physiology in Buenos Aires, Professor Hodgkin, who was the great name in neurophysiology and came from the famous Cambridge University, invited me for lunch to hear more about the enzyme. His interest suggested to me that the observation was of a certain importance.

Looking back, it was a very simple experiment to identify the pump. Just break the membrane and by this gain access to the Na<sup>+</sup> site on the inside and the K<sup>+</sup> site on the outside, add some ATP and test for the combined effect of Na<sup>+</sup> and K<sup>+</sup>. It ought to have been done by someone who worked in the transport field and knew about active transport. I felt like an intruder in a field that was not mine.

There was however, much luck involved. First, that Nachmansohn had invited me to Woods Hole, where I learned about the giant axons and read about the observation by Libet. Next, that from the monolayer experiments I became interested in membrane proteins, especially lipoproteins, and therefore took notice of Libet's observation. Finally, that I chose crab nerves as a test object. I learned later, that after homogenization of most other tissues in order to break the membrane, the membrane pieces form vesicles, which must be opened by treatment with detergent, in order to get access to both sides of the membrane, and thereby to see the combined effect of Na<sup>+</sup> and K<sup>+</sup>. This is not the case with the crab nerve membranes.

From then on the Na<sup>+</sup>,K<sup>+</sup>-ATPase took me away from the monolayer experiments. I never did the planned experiments on the effect of the local anaesthetics on a monolayer of a mixture of lipids and proteins; and I never used the Na<sup>+</sup>,K<sup>+</sup>-ATPase for monolayer experiments, first of all because it was not until 1980 that it became possible to extract the enzyme from a membrane in a pure stable water-soluble form, and secondly that was only possible with the use of detergent<sup>32</sup>. A problem would have been the detergent, but I had lost interest in monolayers.

In 1960, Robert Post published the paper on the red blood cell experiments, "Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocytes"<sup>33</sup>. In this he convincingly showed that in the red blood cells there is a Na<sup>+</sup>+K<sup>+</sup> activated ATPase also, and that the effect of the cations on the activity correlated with the effect on transport. Robert Post was known in the transport field, and his paper had a better title than mine, so it attracted more attention.

In the following few years many papers were published, which showed that the enzyme could be found in many different tissues, and evidence was given for its involvement in active transport (for references see<sup>34</sup>).

In 1965, so much evidence was at hand, that in a review paper I could conclude that the enzyme system fulfilled the following requirements for a system responsible for the active transport across the cell membrane: 1) it is located in the cell membrane; 2) on the cytoplasmic side, it has a higher affinity for  $\text{Na}^+$  than for  $\text{K}^+$ ; 3) it has an affinity for  $\text{K}^+$  on the extracellular side, which is higher than for  $\text{Na}^+$ ; 4) it has enzymatic activity and catalyzes ATP hydrolysis; 5) the rate of ATP hydrolysis depends on cytoplasmic  $\text{Na}^+$  as well as on extracellular  $\text{K}^+$ ; 6) it is found in all cells that have coupled active transport of  $\text{Na}^+$  and  $\text{K}^+$ ; 7) the effect of  $\text{Na}^+$  and of  $\text{K}^+$  on transport in intact cells, and on the activity of the isolated enzyme, correlates quantitatively; and 8) the enzyme is inhibited by cardiac glycosides, and the inhibitory effect on the active fluxes of the cations correlates with the inhibitory effect on the isolated enzyme system<sup>34</sup>.

The enzyme was named the  $\text{Na}^+$ - and  $\text{K}^+$ - activated ATPase, or  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase.

#### THE $\text{Na}^+$ , $\text{K}^+$ -ATPase AS AN ENERGY TRANSDUCER

Returning to Conway's concern about the waste of energy. Conway was right in the sense that even if the cell membrane is permeable to sodium, and therefore an energy requiring pump is needed, the ground permeability to sodium of the cell membrane is low, which is necessary in order that the maintenance of the gradient for sodium does not become energetically too costly for the cell. But what is the meaning of having a membrane permeable to sodium and then spend energy, 10–60% of the cell metabolism, to keep  $\text{Na}^+$  out of the cell and  $\text{K}^+$  within the cell?

1) As mentioned above it solves the osmotic problem due to the presence of impermeable protein anions in the cytoplasm.

But besides this, the gradients for  $\text{Na}^+$  into and  $\text{K}^+$  out of the cell sustained by the pump represent an energy source, which is used

2) for the creation of a membrane potential. The cell membrane is more permeable to  $\text{K}^+$  than to  $\text{Na}^+$ , which means that  $\text{K}^+$  flows out of the cell faster than  $\text{Na}^+$  into the cell. This leads to a diffusion potential across the membrane, negative on the inside, which slows the  $\text{K}^+$  outflux and increases the  $\text{Na}^+$  influx until the potential reaches a value of about  $-70$  mV, at which point the rates of the two fluxes are equal; a steady state situation. The membrane potential is the basis for the function of all excitable tissue. The nerve impulse is a depolarization of the membrane potential due to a transient increase in permeability to  $\text{Na}^+$ , with influx of  $\text{Na}^+$  followed by a repolarization due to an outflux of  $\text{K}^+$ <sup>35</sup>. This leads to an increase in intracellular  $\text{Na}^+$  and a decrease in  $\text{K}^+$ , which subsequently must be compensated for by the pump.

3) for transport of other substances in and out of the cell, see Fig. 2. In the cell membrane there are a number of protein molecules, which act as co- or countertransporters, or sym- and antiporters<sup>36</sup>. There are cotransporters which use the gradient for  $\text{Na}^+$  into the cell to transport glucose<sup>37</sup> or amino acids<sup>38</sup> into cells to a higher concentration inside than outside, a  $\text{Na}^+/\text{Cl}^-$ , a

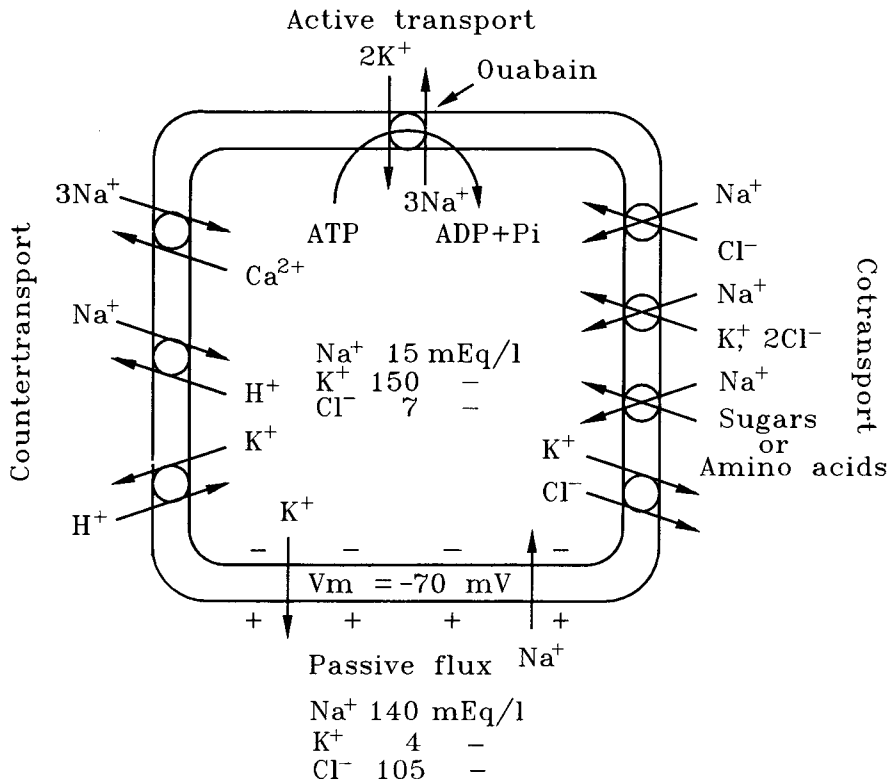


Figure 2. Transport across the cell membrane. For explanation see text. (Reproduced by permission from<sup>73</sup>).

Na<sup>+</sup>/K<sup>+</sup> /2Cl<sup>-</sup> and a K<sup>+</sup>/Cl<sup>-</sup> cotransporter, which may be involved in volume regulation (for references see<sup>39</sup>). A 3Na<sup>+</sup>/Ca<sup>2+</sup> countertransporter of importance for regulation of the intracellular Ca<sup>2+</sup> concentration<sup>40</sup>, and a Na<sup>+</sup>/H<sup>+</sup> countertransport for regulation of internal pH. In addition, there are other examples.

4) for transepithelial transport in intestine, kidney and secretory glands.

The Na<sup>+</sup>, K<sup>+</sup>-pump thus acts as an energy transducer, that converts the chemical energy from the hydrolysis of ATP into another form of energy, a concentration gradient which is used for exchange of substances across the cell membrane. This is named a secondary active transport, while the transport, which is directly dependent on the energy from a chemical reaction, the active Na<sup>+</sup>, K<sup>+</sup> transport, is named a primary active transport. The Na<sup>+</sup>, K<sup>+</sup>-ATPase has thus a key function in the exchange of substances across the cell membrane.

A problem was where to place the Na,K-ATPase in the membrane. The transport system, which is a protein, must have access to both sides of the membrane. As mentioned previously there is no room for proteins in the bilayer of the Danielli-Davson membrane model. Neither is there in Robertson's unit membrane model from 1959<sup>41</sup>, which replaced it. The unit membrane was a bilayer of lipids with proteins arranged asymmetrically on the two

sides of the membrane, but still with no proteins spanning the membrane. It was not until 1972 that a suitable model was introduced by Singer and Nicolson<sup>42</sup>. In their fluid mosaic membrane model there are globular proteins embedded with their non-polar parts in the bilayer, and with the polar parts facing the two sides of the bilayer. They can move laterally in the membrane, but do not flip-flop. They form pathways for the transport of hydrophilic substances across the membrane.

Other mammalian ion transporting pumps were identified in the following years. A  $\text{Ca}^{2+}$ -ATPase in sarcoplasmic reticulum in muscle responsible for the transport of  $\text{Ca}^{2+}$  out of the muscle fiber<sup>43</sup>, a sarcolemma  $\text{Ca}^{2+}$ -ATPase isolated from red blood cells which transports  $\text{Ca}^{2+}$  out of the cell<sup>44</sup>, a  $\text{H}^+$ , $\text{K}^+$ -ATPase in the stomach, which transports  $\text{H}^+$  out of the cells in exchange for  $\text{K}^+$ , producing the stomach acid<sup>45</sup>. They all have in common with the  $\text{Na}^+$ , $\text{K}^+$ -ATPase that the reaction with ATP involves a phosphorylation, and they are therefore named P-type ATPases. A number of other P-type ATPases have been identified in bacteria and fungi (see<sup>46</sup>).

In the years that followed the identification of the sodium-potassium pump, many scientists from many countries took part in the elucidation of the structure of the system, and of the reaction steps in the transport process. This cannot be covered in the present lecture, but for those interested I shall refer to an extensive review by I. Glynn 1985<sup>11</sup>, to the Proceedings from the International Conferences on the  $\text{Na}^+$ , $\text{K}^+$ -ATPase held every third year, of which the latest is from 1996<sup>47</sup>, to recent reviews<sup>48-50</sup>, and to the recently published book by J. D. Robinson: "Moving Questions. A History of Membrane Transport and Bioenergetics"<sup>46</sup>.

There is, however, one question I would like shortly to touch, without going into details, namely this: what is our present view on the way the system transports the cations?

### A MODEL FOR THE TRANSPORT REACTION (see Figure 3).

The model in Fig. 3 is based on the so called Albers-Post scheme, a reaction scheme in which the system reacts consecutively with the cations, and in which the reaction with ATP in the presence of  $\text{Na}^+$  leads to a phosphorylation, and the following reaction with  $\text{K}^+$  to a dephosphorylation<sup>51-56</sup>.

The transport system consists of a carrier part located in the innermost part of the membrane and with the ATP binding part on the cytoplasmic side of the membrane (see Fig. 3). The carrier is in series with a narrow channel which spans half to two thirds of the membrane and opens to the extracellular side of the membrane<sup>57-58</sup> (in Fig. 3 the channel part is shown only as the opening to the outside). The carrier exists in two major different conformations<sup>51-56, 59-65</sup>,  $E_1$  which is the high affinity sodium form, and  $E_2$ , which is the high affinity potassium form. Each of the two conformations can exist in a phosphorylated<sup>51-56</sup>, and a non-phosphorylated form<sup>59-65</sup>, and each of the two major conformations has subconformations marked with primes in the

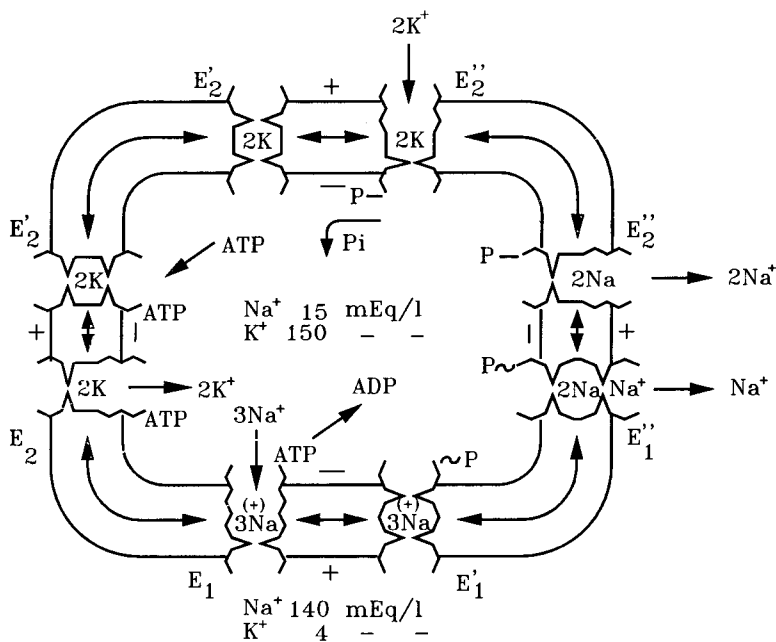


Figure 3. The active transport reaction. For explanation see text. (Reproduced by permission from<sup>73</sup>).

model. The carrier part has two gates, energy barriers, which can open and close access to the channel side and to the cytoplasmic side, respectively.

Referring to the model, with the system in the  $E_1$  conformation with the gate to the cytoplasmic side open but closed to the channel side,  $3 \text{ Na}^+$  are bound, two of them as a neutral complex and the third one as an ion. The system is catalytically active. The affinity for ATP is high (apparent  $K_D$  is about  $0.1 \mu\text{M}$ <sup>59-60</sup>), and with ATP bound the enzyme is phosphorylated<sup>51-56, 66-67</sup>, to an ADP sensitive form, which means that a high energy phosphate bond is formed,  $E_1' \sim P$ , which closes the gate to the cytoplasmic side. The  $3 \text{ Na}^+$  are occluded inside the carrier part<sup>68</sup>. The following two steps, involve conformational changes and rearrangements of the phosphate bond from a high energy to a low energy bond,  $E_1' \sim P$  to  $E_2'' \sim P$ <sup>51-56, 66-67</sup>, and with deocclusion and release of one  $\text{Na}^+$  from  $E_1' \sim P$ <sup>67</sup> to the extracellular side of the membrane. The transition to the  $E_2'' \sim P$  form deoccludes, opens the gate for the remaining  $2 \text{ Na}^+$ , and the affinity is shifted to a low  $\text{Na}^+$  high  $\text{K}^+$  affinity. By this, the  $2 \text{ Na}^+$  are exchanged for  $2 \text{ K}^+$  from the extracellular medium,  $E_2'' \sim P\text{Na}_2$  to  $E_2'' \sim P\text{K}_2$ . With  $2 \text{ K}^+$  bound the system is dephosphorylated, the extracellular gate closes and the  $2 \text{ K}^+$  are occluded,  $E_2'' \sim P(\text{K}_2)$ <sup>69-70</sup>. ATP bound to  $E_2''(\text{K}_2)$  on the cytoplasmic side, with an affinity which is low (apparent  $K_D$  is about  $0.1 \text{ mM}$ ), increases the rate by which the cytoplasmic gate opens and decreases the affinity for  $\text{K}^+$ ,  $E_2''(\text{K}_2)\text{ATP}$  to  $E_2\text{K}_2\text{ATP}$ <sup>69</sup>. The  $2 \text{ K}^+$  are exchanged for  $3 \text{ Na}^+$  from the cytoplasmic medium, and this closes the cycle.

The transport system is a very efficient pump. It pumps  $3 \text{ Na}^+$  out of and  $2$

$K^+$  into the cell for each ATP hydrolysed to ADP and Pi, and for this it uses 70%–85% of the free energy of the hydrolysis of ATP (see<sup>50</sup>). With the normal intra- and extracellular concentrations of  $Na^+$  and of  $K^+$ , the activity of the pump is 10–15% of maximum, i.e. the pump has a considerable reserve power. At 37°C, pH 7.4 the enzyme turns over at a rate of about 160 per sec.

In the transport reaction one net positive charge is carried to the outside. The transport is therefore electrogenic, but due to a high permeability of the cell membrane to Cl<sup>-</sup>, it only adds a few mV to the membrane potential. Each of the two conformations  $E_1$  and  $E_2$  seem to have 2 negative charged binding sites on which the positive charge of the cations are neutralized, in addition the  $E_1$  conformation mentioned above binds a third  $Na^+$  in ionic form<sup>71–72</sup>.

In order to obtain transport of the cations against an electrochemical gradient coupled to a chemical reaction, the hydrolysis of ATP, it is necessary that there is a tight coupling between each of the steps in the catalytic reaction, and each of the corresponding steps in the translocation reaction, as suggested in the model. The shift in affinities, and the opening and closing of the gates, are coupled to the stepwise degradation of ATP from phosphorylation with a high energy bond, to a low energy bond, and finally to dephosphorylation. This divides the translocation into steps in which local gradients are created along which the ions can flow, and thereby they are transported against their overall gradient.

The model is a working hypothesis, which explain a good deal of the experimental observations. There are however observations which do not fit with the model. And even though we have much information about the structure, there are many unanswered questions, especially at the molecular level. What is the nature of the gates, where are the binding sites for the cations, and of what nature, and how does the system discriminate between  $Na^+$  and  $K^+$ , etc.

## CONCLUSION

It may seem disappointing that 30 years of work, since the conclusion that the membrane bound  $Na^+,K^+$ -ATPase is identical with the  $Na^+,K^+$ -pump, has not given us an understanding of the basic molecular events behind the transport. However, considering that the problem is to reveal how 1320 amino acids inside a volume of  $60 \times 60 \times 100 \text{ \AA}^3$  can be assembled to a very efficient machine, which can convert the chemical energy from the hydrolysis of ATP into work, namely the transport of cations against their electrochemical gradient, and which can distinguish between so closely related cations as  $Na^+$  and  $K^+$ , it can be of no surprise that progress is slow.

Thirty years ago it seemed impossible to find a way to purify the enzyme not to speak of getting it into solution and reconstitute it into liposomes. Nobody dared to dream about knowing the sequence, and yet this and much much more have been accomplished.

## REFERENCES

1. Gorter, E., and F. Grendel, *J. Exp. Med.*, **41**, 439 (1925).
2. Danielli, J. F., and H. Davson, *J. Cell. Comp. Physiol.*, **5**, 495 (1935).
3. Boyle, P. J., and E. F. Conway, *J. Physiol.*, **100**, 1 (1941).
4. Dean, R. B., *Biological Symposia*, **3**, 331 (1941)
5. Hevesy, G., *Enzymologia*, **5**, 138 (1938)
6. Ussing, H. H., *Physiol. Rev.*, **29**, 129 (1949)
7. Krogh, A., *Proc. Roy. Soc. London B.*, **133**, 140 (1946).
8. Conway, E. J., *Nature*, **157**, 715 (1946).
9. Ussing, H. H., *Nature*, **160**, 262 (1947).
10. Rosenberg, T., *Acta Chem. Scand.*, **2**, 14 (1948).
11. Glynn, I. M., in: *The Enzymes of Biological Membranes*, edited by A. N. Martonosi, New York: Plenum, vol. **3**, 28 (1985).
12. Skou, J. C., *Biochim. Biophys. Acta*, **1000**, 435 (1989)
13. Skou, J. C., in: *Membrane Transport: People and Ideas* (ed. D. Tosteson), American Physiol. Soc. p. 155 (1989).
14. Skou, J. C., *Lokalanestetika*. Thesis, Universitetsforlaget Aarhus, (1954).
15. Skou, J. C., *J. Pharm. Pharmacol.*, **13**, 204 (1961).
16. Meyer, H. H., *Arch. Exp. Path. Pharmacol.*, **42**, 109 (1899).
17. Overton, E., *Studien über die Narkose zugleich ein Beitrag zur allgemeinen Pharmakologie*, Jena: Fisher (1901).
18. Adam, N. K., *The Physics and Chemistry of Surfaces*, London, Oxford Univ. Press (1941).
19. Hodgkin, A. L., and B. Katz, *J. Physiol.*, **108**, 37 (1949).
20. Libet, B., *Federation Proc.*, **7**, 72 (1948).
21. Hodgkin, A. L., and R. D. Keynes, *J. Physiol. Lond.*, **128**, 28 (1955).
22. Gardos, G., *Acta Physiol. Scient. Hung.*, **6**, 191 (1954).
23. Hodgkin, A. L., and R. D. Keynes, *J. Physiol. Lond.*, **131**, 592 (1956).
24. Caldwell, P. C. and R. D. Keynes, *J. Physiol. Lond.*, **137**, 12P (1957).
25. Keynes, R. D., in: *Metabolism of the Nervous System*, (ed. D. Richter). Pergamon, London, p. 159 (1957).
26. Skou, J. C., in: *Metabolism of the Nervous System*, (ed. D. Richter). Pergamon, London, p. 173 (1957).
27. Skou, J. C., *Biochim. Biophys. Acta*, **23**, 394 (1957).
28. Post, R. L., and P. Jolly, *Biochim. Biophys. Acta*, **25**, 118 (1957).
29. Schatzmann, H. J., *Helv. Physiol. Pharmacol. Acta*, **11**, 346 (1953).
30. Skou, J. C., *Biochim. Biophys. Acta*, **42**, 6 (1960).
31. Skou, J. C., *Biochim. Biophys. Acta*, **58**, 314 (1962).
32. Esmann, M., J. C. Skou, and C. Christiansen, *Biochim. Biophys. Acta*, 567, 410 (1979)
33. Post, R. L., C. R. Merritt, C. R. Kinsolving, and C. D. Albright, *J. Biol. Chem.*, **235**, 1796 (1960).
34. Skou, J. C., *Physiol. Rev.*, **45**, 596 (1965).
35. Hodgkin, A. L., and A. F. Huxley, *J. Physiol.*, **116**, 449 (1952)
36. Mitchell, P., *Biochem. Soc. Symp.*, **22**, 142 (1963).
37. Crane, R. K., *Comp. Biochem.*, **35**, 43 (1983).
38. Kromphardt, H., H. Grobecker, K. Ring, and E. Heinz, *Biochim. Biophys. Acta*, **74**, 549 (1963).
39. Hoffmann, E. K., and L. O. Simonsen, *Physiol. Rev.*, 69, 315 (1989)
40. Pitts, B. J. R., *J. Biol. Chem.*, **254**, 6232 (1979).
41. Robertson, J. D., *Biochem. Soc. Symp.*, **16**, 3 (1959).
42. Singer, S. J., and G. L. Nicolson, *Science*, **175**, 720 (1972).
43. Hasselbach, W., and M. Makinose, *Biochem. Z.*, **333**, 518 (1961).
44. Schatzmann, H. J., *Experientia*, **22**, 364 (1966).
45. Stewart, B., B. Wallmark, and G. Sachs, *J. Biol. Chem.*, **256**, 2682 (1981)



46. Robinson, J. D., *Moving Questions. A History of Membrane Transport and Bioenergetics*, American Physiological Soc., N.Y. (1997).
47. *Na/K-ATPase and Related Transport ATPases: Structure, Mechanism and Regulation* (eds. Beaugé, L. A., D. C. Gadsby, and P. J. Garrahan), *Annals New York Academy Sci.* vol. 834, New York (1997).
48. Jørgensen, P. L., in: *Molecular Aspects of Transport Proteins* (ed. De Pont J. J. H. M. M.), Elsevier Service Publisher B. V., p.1 (1992).
49. Glynn, I. M., *J. Physiol.*, **462**, 1 (1993).
50. Cornelius, F., *Biomembranes*, Vol. 5, 133 (1996).
51. Albers, R. W., *Annu. Rev. Biochem.*, **36**, 727 (1967).
52. Albers, R. W., S. Fahh, and G. J. Koval, *Proc. Natl. Acad. Sci. USA*, **50**, 474 (1963).
53. Fahh, S., G. J. Koval, and R. W. Albers, *J. Biol. Chem.*, **243**, 1993 (1968).
54. Post, R. L., and S. Kume, *J. Biol. Chem.*, **248**, 6993 (1973).
55. Post, R. L., S. Kume, T. Tobin, B. Orcutt, and A. K. Sen, *J. Gen. Physiol.*, **54**, 306S (1969).
56. Post, R. L., A. K. Sen, and A. S. Rosenthal, *J. Biol. Chem.*, **240**, 1437 (1965).
57. Läuger, P., *Biochim. Biophys. Acta*, **552**, 143 (1979).
58. Gadsby, D. C., R. F. Rakowsky, and P. De Weer, *Science*, **260**, 100 (1993).
59. Nørby, J. G., and J. Jensen, *Biochim. Biophys. Acta*, **233**, 104 (1971).
60. Hegyvary, C., and R. L. Post, *J. Biol. Chem.*, **246**, 5234 (1971).
61. Jørgensen, P. L., *Biochim. Biophys. Acta*, **401**, 399 (1975).
62. Karlsh, S. J. D., and D. W. Yates, *Biochim. Biophys. Acta*, **527**, 115 (1978).
63. Karlsh, S. J. D., D. W. Yates, and I. M. Glynn, *Biochim. Biophys. Acta*, **525**, 252 (1978).
64. Skou, J. C., and M. Esmann, *Biochim. Biophys. Acta*, **647**, 232 (1981).
65. Karlsh, S. J. D., *J. Bioenerg. Biomembr.*, **12**, 111 (1980).
66. Nørby, J. G., I. Klodos, and N. O. Christiansen, *J. Gen. Physiol.*, **82**, 725 (1983).
67. Yoda, S., and A. Yoda, *J. Biol. Chem.*, **26**, 1147 (1986).
68. Glynn, I. M., Y. Hara, and D. E. Richards, *J. Physiol.*, **351**, 531 (1984).
69. Post, R. L., C. Hergvary, and S. Kume, *J. Biol. Chem.*, **247**, 6530 (1972).
70. Beaugé, L. A., and I. M. Glynn, *Nature*, **280**, 510 (1979).
71. Nakao, M., and D. C. Gadsby, *Nature*, **323**, 628 (1986).
72. Goldschleger, R., S. J. D. Karlsh, A. Raphaeli, and W. D. Stein, *J. Physiol.* **387**, 331 (1987).
73. Skou, J. C., *News in Physiological Sciences*, **7**, 95 (1992).

## Erratum

Page 182, line 14 should read as:

little or no contact with the outside scientific world. We were three young medical