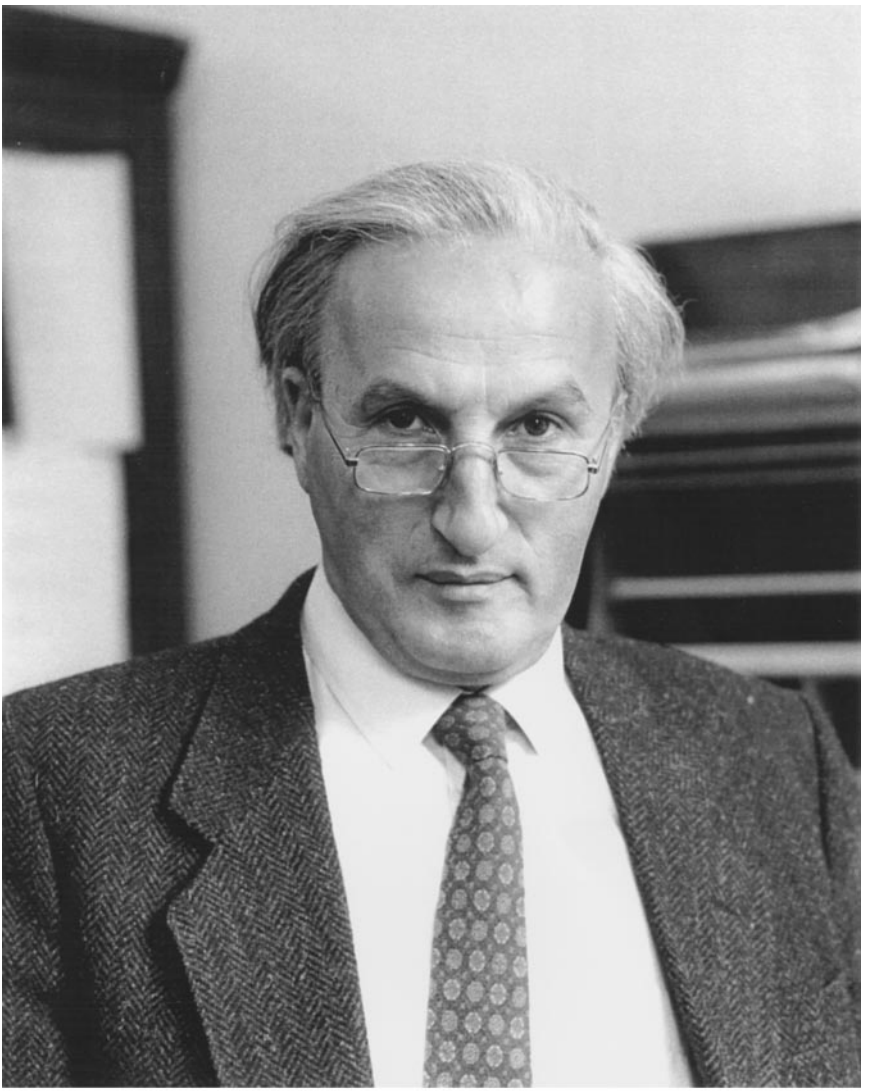

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A HUNDRED YEARS OF SODIUM PUMPING

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■ **Abstract** This article gives a history of the evidence (*a*) that animal cell membranes contain pumps that expel sodium ions in exchange for potassium ions; (*b*) that the pump derives energy from the hydrolysis of ATP; (*c*) that it is thermodynamically reversible—artificially steep transmembrane ion gradients make it run backward synthesizing ATP from ADP and orthophosphate; (*d*) that its mechanism is a ping-pong one, in which phosphorylation of the pump by ATP is associated with an efflux of three sodium ions, and hydrolysis of the phosphoenzyme is associated with an influx of two potassium ions; (*e*) that each half of the working cycle involves both the transfer of a phosphate group and a conformational change—the phosphate transfer being associated with the occlusion of ions bound at one surface and the conformational change releasing the occluded ions at the opposite surface.

INTRODUCTION

It has been the convention in recent years for the author of the first chapter in each *Annual Review of Physiology* to take a more historical and more personal view of the topic being discussed than would be appropriate in the rest of the volume. Since it is now seven years since I forsook the sodium pump for the less tractable problems of the origin and machinery of the mind, I am glad to be able to make a virtue of necessity and follow that convention.

THE FIRST FIFTY YEARS

In 1902, Ernest Overton (1) reported a discovery that, he tells us, left him so totally staggered (*vollständig verblüfft*) that he took several hours in the open air to think about it. Overton was an Englishman, and grandson of the man who as a boy had introduced Charles Darwin to beetles. Working in Würzburg, he had been studying the osmotic behavior of frog muscles, and what left him so *verblüfft* was the observation that muscles suspended for some time in an isotonic solution of sucrose were inexcitable. Adding sodium chloride to the solution restored excitability, and although the nature of the anion was not critical, only lithium could substitute for sodium. He concluded that sodium ions must have a specific function, and

he suggested that, during a very brief period following stimulation, the muscle membrane becomes permeable to both sodium and potassium ions, leading to an exchange of intracellular potassium for extracellular sodium. There was, he pointed out, a corollary to this hypothesis:

> Consider that, in the course of 70 years, heart muscle cells contract about 24×10^8 times and respiratory muscles about 6×10^8 times. If some sodium ions enter and some potassium ions leave during each contraction, then the differences between internal and external cation concentrations would gradually be levelled out unless there is some mechanism at work which opposes this equilibration. In actual fact, our muscles contain, so far as I am aware, just as much potassium and as little sodium in old age as they do in early youth. (Translation by Bernard Katz.)

What makes this argument for the existence of a sodium pump so remarkable is not the argument itself, which is straightforward, but the premises on which it is based. For Overton was writing before the publication of the famous paper by Bernstein (2), which suggested that the resting potential was roughly a potassium equilibrium potential and that the action potential was the result of a transient loss by the membrane of its selective permeability to potassium ions. How Overton arrived at his hypothesis he doesn't tell us, but I suspect that, like Bernstein's, it owed a great deal to work by Ostwald (3) on so-called precipitation membranes—artificial membranes formed by the precipitation of insoluble material at the interface of two solutions. Ostwald studied the permeability and electrical properties of membranes of copper ferrocyanide, which he formed by putting solutions of copper sulfate and potassium ferrocyanide on opposite sides of thin pieces of parchment. He found that such membranes were permeable to potassium and chloride ions but not to the larger barium or sulfate ions. What is more, when the fluids bathing the membrane contained only a single species of penetrating ion, present in different concentrations on the two sides, he detected transmembrane potentials whose magnitudes fitted the Nernst equation, published the previous year. And Ostwald predicted that analogous behavior in natural membranes would explain not only the electric currents in muscles and nerves but also the mysterious workings of the electric organs of electric fish.

Whether this notion of Overton's debt to Ostwald is right or not, Overton seems to have been equally prescient in his hypothesis and in its corollary. Prescience, though, is recognized only with hindsight, and for Overton's work that hindsight came in the mid-1940s, a decade after his death. Bernstein's paper was published just a month after Overton's and, of course, contains no reference to it; his 1912 book, *Elektrobiologie* (4), has only a brief account of Overton's experiments and no mention of their possible implications. Bayliss's influential *Principles of General Physiology* (5), published in 1915, is similarly inadequate, and Fenn's exhaustive (and exhausting) 1936 review on electrolytes in muscle (6) does not mention the relevant experiments. Andrew Huxley (7) has said that he and Alan Hodgkin were not aware of these experiments when they discovered the overshoot of the action

potential in 1939, and he believes that they would have thought of the correct explanation of the overshoot much sooner if they had been.

By 1940, the widely held notion that the high concentration of potassium and the low concentration of sodium within muscle fibers were maintained simply by the impermeability of the membrane to sodium ions was no longer tenable. In experiments on anesthetized rats, Fenn & Cobb (8) had shown that stimulation of the sciatic nerves for 30 min caused the gastrocnemius muscles to lose part of their potassium and take up a roughly equivalent amount of sodium; both changes were largely reversed during a few hours of rest. Using the then newly available radioactive ^{24}Na, Heppel (9) had shown that it took less than an hour for sodium in the muscles of potassium-deprived rats to equilibrate with sodium in the bathing solution. And Steinbach (10) had shown that frog muscles soaked in potassium-free Ringer's solution gradually lost potassium and gained sodium and that the exchange was reversible. Since, in the experiments of both Fenn & Cobb and of Steinbach, the movements of both potassium and sodium during the recovery phase were against the concentration gradients, and these movements were in opposite directions, it seemed to follow either that at least one species of ion can be pumped through the membrane or that much of the potassium inside the fibers is bound to molecules that are unable to penetrate the membrane and prefer potassium to sodium.

Curiously, none of the experimentalists who produced these striking results showed any enthusiasm for the notion of ion pumps, and their papers contain no mention of the relevant work of Overton. Fenn & Cobb suggested that, following excitation, perhaps "only the surface of the fiber breaks down and exchanges its potassium for sodium, the lower layers being still impermeable to sodium"—a suggestion later dismissed by Fenn under the umbrella of "some rather artificial hypotheses." Steinbach has only a brief discussion of the causes of ion movements during the recovery phase, and favors selective binding of potassium to indiffusible organic molecules within the cell. In a slightly later paper (11), he justifies this rejection of the notion of ions being pumped across the membrane, explaining that to postulate the existence of such a pump "removes much of the charm of the old selective permeability idea, since once an auxiliary mechanism must be assumed, the simplicity and clarity of the scheme is destroyed."

Why selective binding is less destructive of the simplicity and clarity of the scheme than ion pumping is not clear, and in the following year, Dean (12), basing his conclusions on much the same experiments, argued strongly for the existence of an outwardly directed sodium pump.

Work on the storage of blood during the Second World War, by Maizels & Patterson in London, and by both Danowski and Harris in the United States, provided independent evidence for the pumping of both sodium and potassium (13–15). During cold-storage, red cells gradually lose potassium and gain sodium, but it was found that these changes could be reversed by replacing the cells in the circulation or by incubating them with glucose at 37°C. Reversal seemed to depend on energy from glycolysis because it was prevented by fluoride or iodoacetate, but not by cyanide or dinitrophenol.

What looked like a serious objection to the idea that the low sodium content of muscle was the result of an outward pumping of sodium was a calculation by Conway (16), in Dublin, suggesting that the energy required would greatly exceed that available. This objection lost its force when Ussing (17), in Copenhagen, suggested that a substantial part of the sodium efflux from muscle might represent a linked one-for-one exchange of intracellular and extracellular sodium ions, which need not require significant energy. Evidence for such an exchange was later found by Keynes & Swan (18) in experiments with frog muscle.

THE LAST FIFTY YEARS

I don't know when I first decided that I wanted to be a doctor. It may have been at the age of eight, when I walked into my grandmother's kitchen and saw one of my aunts sitting at the table dissecting a human brain. (The rules about disposal of body parts were more lax in those days.) She was the second member of the family to "do medicine," her younger brother having led the way, and there was a feeling in the family, as in many Jewish immigrant families, that medicine, both in its worthwhileness and its professional prospects, was the ideal career. We lived in Hackney, and I went to the local elementary school, which had a narrow curriculum but excellent teaching, and where large classes seemed to be compatible with good discipline. From there, through the generosity of the socialist but not too doctrinaire London County Council, I went to the City of London School—one of the few English so-called public (i.e., private) schools that was not a boarding school—where, looking back, I realize that the teaching was always good and that in mathematics, physics, and English it was superb.

When I came up to Cambridge as a medical student in 1946, I was a member of Trinity College. This was not the college my headmaster had suggested, but my mother's greengrocer had a son who was (and is) a distinguished mathematician, and his advice—routed to me through our respective mothers—was "Tell Ian to apply to Trinity because, if he wants to do research later, they have more research Fellowships." This advice was even better than he realized because, as well as research Fellowships, Trinity had Alan Hodgkin as a tutor in physiology. It was only in the previous year that he and Andrew Huxley had returned to Cambridge after their war service and had resumed their work on the nature of conduction in nerves, which they had had to break off at a very exciting stage when Hitler invaded Poland in 1939. By the time I had completed my medical course and returned to Cambridge as a graduate student in 1953, they had essentially solved the nerve problem. The Hodgkin-Huxley theory was as convincing as it was elegant, but it left unanswered an intriguing subsidiary problem. If the energy for each nerve impulse came from the downhill entry of sodium ions and loss of potassium ions, how were the gradients of these ions to be maintained? The question that Overton had raised half a century earlier, almost as an aside in discussing an unsupported and speculative hypothesis, suddenly became pressing. It was the question I decided to work on.

Defining the Problem

Before investigating the mechanism of a pump, it is as well to know just what it pumps and what its immediate source of energy is. Fifty years ago, it was not certain that the same mechanism was responsible for pumping sodium ions outward and potassium ions inward, or that the pumps in nerve and in muscle, which depend on respiration, were the same as the pump in mamalian red cells, which depends on glycolysis.

A possible link between the active movements of sodium and potassium had been suggested by the observation—in human red cells, frog muscle, and invertebrate nerve axons—that sodium efflux was reduced when the extracellular potassium concentration was lowered (19–21). Proof that the linkage not only existed but was tight came from experiments in which both glucose-dependent sodium efflux and glucose-dependent potassium influx were measured in batches of red cells incubated in media with potassium concentrations ranging from 0 to 5 mM (22). When there was no potassium outside the cells, there was no glucose-dependent sodium efflux; as the potassium concentration was increased, there were increases in the glucose-dependent fluxes of both sodium and potassium, and these increases seemed to fit Michaelis curves with similar K_m values. That the linked fluxes were not, as had been thought, $1Na^+:1K^+$ but $3Na^+:2K^+$ was shown by Post & Jolly (23), who monitored the net movements of sodium and potassium when cold-stored human red cells were incubated in media containing concentrations of sodium and potassium similar to those in the cells, so that net passive movements could be assumed to be small.

The identity, or near identity, of the pumps in different tissues became clearer following the discovery by Schatzmann (24) that the cardiac glycoside strophanthin inhibited the reuptake of potassium and expulsion of sodium by cold-stored red cells, without affecting either oxygen consumption or lactic acid production. This strongly suggested that the action was on the pumping mechanism rather than the energy supply; so when others found that active transport of sodium and potassium in other tissues was also sensitive to cardiac glycosides, it seemed likely that a similar mechanism was involved. The potency and specificity of the cardiac glycosides also made it possible to use them to estimate the number of sodium pumps on a cell membrane (25), and they have, of course, been a major tool for investigating the pump mechanism.

The work of Kalckar, Lipmann, and others in the 1930s and 1940s had made it clear that energy from both glycolysis and respiration was made available as the energy-rich phosphate bonds of ATP. The most straightforward interpretation of the dependence of sodium pumping in some tissues on glycolysis and in other tissues on respiration was, therefore, that pumps in both kinds of tissue used ATP as a fuel. Support for this view came from experiments showing that dinitrophenol, which uncouples respiration from ATP synthesis, inhibited pumping in tissues that rely on respiration, whereas arsenate, which uncouples glycolysis from ATP synthesis, inhibited pumping in tissues that rely on glycolysis (21, 26, 27). Direct evidence that ATP fueled the pump came first from experiments by Gárdos (28),

working in Budapest, who showed that resealed red cell ghosts containing trapped ATP could accumulate potassium actively in the absence of any other substrate. Although Gárdos's resealed ghosts were heavily contaminated with intact cells, these were unlikely to have been responsible for most of the accumulation since it continued even in the presence of arsenate. Later, Dunham (29) showed that removal of ATP from iodoacetate-poisoned red cells by the addition of glucose prevented cation transport, and Caldwell & Keynes (30) showed that injecting ATP or arginine phosphate into cyanide-poisoned squid axons restored cation transport.

The Identity of the Sodium Pump and the $(Na^+ + K^+)$-Activated ATPase—"All Roads Lead to Rome"

In 1956, like others working on the sodium pump, I was thinking mainly in terms of circulating, lipid-soluble, cation-selective carriers. But because the sodium pump seemed so much better at discriminating between sodium and potassium ions than any known cation-binding agent, I wondered whether this discrimination might depend not (or not only) on differences of affinity, but on differences of the reactivity of an enzyme depending on whether sodium or potassium ions were bound to it. (A somewhat analogous hypothesis would be that most of us are dark-haired because, as Anita Loos tells us, "Gentlemen Prefer Blondes," "But-Gentlemen Marry Brunettes.") In my first full paper (22), I therefore listed eight enzymes that showed striking discrimination between sodium and potassium ions, and I noted that several of them catalyzed reactions that involved the transfer of large amounts of energy. The following year, in a long review on red cells written while I was doing my national service as an under-employed doctor in the Royal Air Force, I made the same point, and finished the article by suggesting that investigating the role of the ATPase in the red cell membrane might be fruitful (31).

What I did not know, when I wrote that, was that Jens Christian Skou, in Aarhus, had already done, and was about to publish, experiments showing that fragments of crab nerve membrane possessed Mg-ATPase activity that was stimulated greatly by the joint presence of sodium and potassium ions (32). Skou pointed out in the last paragraph of his paper, "the crab-nerve ATPase . . . seems to fulfil a number of conditions that must be imposed on an enzyme which is thought to be involved in the active extrusion of sodium ions from the nerve fibre." The case for a connection between this ATPase and the sodium pump was strengthened when, at the suggestion of Post, Skou tested the effect of ouabain on the ATPase (33, 34). Subsequently, work by Post and his colleagues at Vanderbilt, and by Ned Dunham and me in Cambridge, showed that similar Na,K-ATPase activity was present in the human red cell membrane and that the properties of the ATPase closely paralleled the properties of the pump (35, 36). In particular, experiments on resealed red cell ghosts showed that it was intracellular sodium ions and extracellular potassium ions that activated the ATPase (37). Skou himself found that Na,K-ATPases similar to that in crab nerve could be found in the mammalian brain and kidney (38), and Bonting & Caravaggio, at Nijmegen, found a striking correlation between the

fluxes of sodium and potassium and the activity of the membrane Na,K-ATPase in six different tissues of the cat (39). In the late 1960s and early 1970s, even more striking evidence for the identity came from experiments showing that the pump could be driven backward to synthesize ATP (40, 41); that an antibody to partially purified Na,K-ATPase inhibited the pump (42); and that, when ATP was added to a suspension of artificial lipid vesicles with partially purified Na,K-ATPase incorporated into their membranes, movements of sodium and potassium ions (with appropriate stoichiometry) could be detected (43, 44). By then, though, the identity of the pump and the ATPase was hardly in question.

An intriguing feature of Skou's important discovery is that, as he himself makes clear in a commentary written 32 years later (33), he had not been primarily concerned with the sodium pump. He had been investigating the mechanism of local anesthetics and had wanted a lipoprotein enzyme that would sit in a lipid monolayer and whose activity he could follow when the surface pressure in the monolayer was increased by the insertion of anesthetic molecules. Having discovered the properties of the enzyme, he realized that what he was looking at was probably the sodium pump, but he points out that the statement in the introduction to the 1957 paper, "A further study on the ATPase in nerves and its possible role in the active outward transport of sodium ions seems warranted," was "a subsequent rationalization." And in deciding the title of that paper, he rejected the phrase "sodium pump" as "too provocative."

A Peculiar Difficulty

Why was Skou's 1957 paper so important? After all, a devil's advocate could argue that there was already both indirect and direct evidence that the linked Na^+/K^+ pump was fueled by ATP. If the energy in the terminal phosphoryl bond of ATP is to be made available for osmotic work, the end products are likely to be ADP and orthophosphate; so it should not have been surprising that the pumping machinery acted as an ATPase.

The answer is that Skou's work opened up a bundle of new approaches to elucidating the pump mechanism. Transport enzymes in general, and the sodium pump in particular, present a peculiar difficulty to the investigator. Most enzymes convert substrates into products that are chemically different from the substrates, and therefore easily distinguishable. In contrast, the sodium and potassium ions that are pumped across the membrane remain sodium and potassium ions, and the conversion of an intracellular ion into an extracellular ion, or vice versa, can be detected only by working with intact cells. As long as the only way of recognizing the presence of the pump was by observing the movements of the ions across the cell membrane, investigators were restricted to working with intact cells, which is, of course, why resealed red cell ghosts and squid giant axons, whose contents could be regulated to a considerable extent by the experimenter, were so important. But as soon as the pump was identified as an $(Na^+ + K^+)$-activated ATPase, with recognizably different substrates and products (and, as it later turned out,

with recognizably different intermediate forms), it could be studied in membrane fragments rich in pumps and, ultimately (45), in more-or-less purified and even solubilized pump preparations.

Working Out the Working Cycle

The reaction catalyzed by an enzyme that requires four substrates (Na^+_i, K^+_o, ATP and H_2O) and produces four products (Na^+_o, K^+_i, ADP and orthophosphate) is unlikely to occur in a single step. Understanding the mechanism of the pump, therefore, involves two stages: discovering the sequence of reaction steps that make up the working cycle and showing how the atomic structure of the pump makes these reactions possible. There is at present a great deal of sophisticated work going on concerned with the second stage, and accounts of this, together with accounts of recent work on the electrical properties of the pump, of the nature and functions of different isoforms of the Na,K-ATPase, and of the possible role of endogenous inhibitors of the pump, can be found in Taniguchi & Kaya's edited volume on Na/K-ATPase and related ATPases (46, see also 47; for recent work on the related calcium-ATPase, see 48). In the very limited space available here, only the history of the first stage is discussed. Even with this restriction, the relevant literature is extensive, and much important work will, inevitably, be omitted. For fuller reviews see 49–51.

By what sequence of steps is the hydrolysis of ATP coupled to the active efflux of three sodium ions and the active influx of two potassium ions? The answer to this question came largely from complementary studies of phosphate-group transfer in membrane fragments and of ion fluxes in intact cells.

The first suggestion that the enzyme becomes phosphorylated during the working cycle came from Skou, who found that membrane fragments from crab nerves catalyzed an ATP-ADP exchange (34). That exchange, however, appeared not to need sodium ions or to be inhibited by ouabain; its relation to the sodium pump was, therefore, uncertain. In the early 1960s, Wayne Albers and his colleagues at the National Institutes of Health, and Post and his colleagues at Vanderbilt, exposed membrane fragments from electric-eel electric organ or from guinea pig kidney to [γ^{32}P]ATP and followed the phosphorylation and dephosphorylation of the enzyme under different conditions (52–54). They found that sodium ions were necessary for (or greatly accelerated) phosphorylation, and potassium ions greatly accelerated the hydrolysis of the phosphoenzyme yielding orthophosphate. Later the phosphorylated group was shown to be a β-aspartyl carboxyl (55). Both groups pointed out that the dependence of phosphorylation on sodium ions and of dephosphorylation on potassium ions suggested that the outward movement of sodium involved a phosphorylation step and the inward movement of potassium involved hydrolysis of the phosphoenzyme. There were, though, interesting differences in their interpretations.

Albers and his colleagues thought that the enzyme functioned in turn as a Mg^{2+}-activated kinase, a Na^+-activated transferase, and a K^+-activated phosphatase.

Following phosphorylation of the enzyme, the negatively charged phosphate group was, they supposed, transferred to another site (or more probably a succession of sites) in a channel whose environment of fixed charges was such that only Na^+ ions could act as counter ions. At the outer end of the channel, the K^+-dependent phosphatase action of the enzyme released the phosphate group as orthophosphate, allowing the Na^+ ions to escape. They also suggested, more tentatively, that negatively charged sialic acids at the cell surface might prevent the orthophosphate from leaving and that its diffusion back to the cell interior along a K^+-selective channel could account for the coupled inward transport of potassium.

Post and his colleagues combined the notion of a sodium-phosphoenzyme complex with Trevor Shaw's cyclical carrier model (see 31) to produce a scheme in which both the phosphorylated and unphosphorylated forms of the enzyme could exist in alternative configurations, one with the cation-binding sites facing inward, the other with these sites facing outward. Sodium ions at the inner face of the membrane were supposed to catalyze the formation of the phosphenzyme and be carried outward bound to it. Potassium ions at the outer face of the membrane were supposed to catalyze the hydrolysis of the phosphoenzyme and be carrried inward bound to the dephosphoenzyme.

Despite the differences between the two interpretations, the basic notion that the working cycle of the pump consisted of a phosphorylation step associated with sodium efflux and a hydrolysis step associated with potassium influx became known as the Albers-Post scheme and provided a fertile base for further elucidation of the working cycle.

The reversibility of the pump, discovered in 1966 (40, 41), implied that no individual step in the cycle could be too far from equilibrium to be reversed by appropriate changes in ligand concentrations. It followed that, if the Albers-Post scheme were correct, it should be possible to find conditions in which an inward movement of sodium ions was associated with the transfer of a phosphate group from the phosphoenzyme to ADP and other conditions in which an outward movement of potassium ions was associated with phosphorylation of the enzyme by orthophosphate.

It was already known, from the work of Albers and his colleagues at NIH (52), that when preparations of Na,K-ATPase from the *Electrophorus* electric organ are incubated in high-sodium, potassium-free media containing both ATP and ^{14}C-labeled ADP, they catalyze an ATP-ADP exchange, suggesting the shuttling of a phosphate group between nucleotide and enzyme. Within a few years, experiments on red cells, frog muscle, and squid axons showed that when these cells were incubated in high-sodium potassium-free media there was an ouabain-sensitive exchange of internal and external sodium ions (56–59). In red cells, the exchange was close to one-for-one and showed a marked asymmetry, with a high affinity for sodium at the inner face of the membrane and a low affinity at the outer face. Significantly, it did not occur unless the cells contained both ATP and ADP; and although the ATP was not consumed, replacement of ATP by its non-phosphorylating β,γ-imido analog was ineffective (60, 61). The implication was

that the sodium-sodium exchange was accompanied by an ATP-ADP exchange, and this was confirmed later (62, 63). The obvious interpretation was that the outward movement of sodium involved the transfer of a phosphate group from ATP to the enzyme, and the inward movement involved its transfer back to ADP to form ATP.

There was, though, an interesting and important twist to the story. In their 1966 experiments on ATP-ADP exchange by electric organ Na,K-ATPase, Fahn and his colleagues (64) noticed that the exchange was unaffected or slightly faster if the enzyme was pretreated with oligomycin, despite the fact that oligomycin inhibits electric organ Na,K-ATPase. They explained this by supposing (*a*) that the phosphoenzyme existed in two interconvertible forms (in a later nomenclature, E_1P and E_2P); (*b*) that only the first of these to be formed (E_1P) could react with ADP, and only the second (E_2P) was hydrolyzed in the presence of potassium; and (*c*) that oligomycin acted by preventing the conversion of E_1P to E_2P. So when Patricio Garrahan and I (65) found that oligomycin inhibited the ouabain-sensitive sodium:sodium exchange that occurs when red cells are incubated in high-sodium, potassium-free media, the implication was that the outward movement of sodium ions required not just phosphorylation but the conversion of E_1P to E_2P. That made sense if the conversion involved a conformational change in the phosphoenzyme, because such a change seemed to be just what was needed to release the sodium ions to the exterior.

What about the predicted association between an outward movement of potassium ions through the pump and phosphorylation of the pump by orthophosphate? Early experiments on the effects of cardiac glycosides on red cells showed that, under fairly physiological conditions, more than a fifth of the potassium efflux was inhibited by digoxin (25); but at that time there was no way of knowing whether this part of the potassium efflux was the result of a partial failure of discrimination between sodium and potassium by the forward-running pump or of occasional reversal of the part of the cycle concerned with potassium entry. Later experiments, both on intact red cells and on resealed red cell ghosts, showed that the glycoside-sensitive potassium efflux was part of an exchange of internal and external potassium ions (66–69). This exchange had interesting features: It was roughly one-for-one; it had strikingly asymmetric affinities for potassium (high outside, low inside); and it occurred only if the cells contained magnesium ions, orthophosphate, and a high concentration of ATP (or ADP, deoxy-ATP, CTP, or a non-phosphorylating analog of ATP). The need for orthophosphate strongly suggested that the outward movement of potassium involved a reversal of the dephosphorylation step, and studies on Na,K-ATPase preparations from pig kidney and from *Electrophorus* electric organ demonstrated the exchange of ^{18}O between orthophosphate and water that would be expected to result from the alternate formation of phosphoenzyme (from orthophosphate) and its hydrolysis (70). The ability of non-phosphorylating analogs of ATP to support potassium-potassium exchange pointed to a role for ATP in the second half of the pump cycle different from its phosphorylating role in the first half.

Two Conformations of the Dephosphoenzyme

Work in the early 1970s (71, 72) had shown that the affinity of Na,K-ATPase preparations for ATP was high in potassium-free media and dropped dramatically when potassium ions were added. It seemed likely that this drop in affinity reflected a change in conformation, and proof came in 1975, when Peter Jørgensen, at Aarhus, showed that tryptic digestion of kidney Na,K-ATPase yielded different products depending on whether the enzyme was in a sodium medium (E_1 form) or a potassium medium (E_2 form) (73). Later, Jørgensen & Petersen (74) showed that trypsin attacks phosphoenzyme in the E_2P form in the same way as it attacks the E_2 form, implying that, so far as the accessibility of peptide bonds to trypsin is concerned, the conformations must be similar. Comparisons of intrinsic tryptophan fluorescence supported this view and also suggested a similarity between the conformations of E_1 and E_1P (74, 75).

Occlusion of Ions During Their Transport Across the Membrane

It has always seemed possible that, at some stage during their passage across the membrane, transported ions are occluded within the pump molecule, unable to escape to either surface without either a conformational or a chemical change in that molecule. (For a general review of occlusion in enzyme pumps, see 76). The first direct, or almost direct, evidence for occlusion in the sodium pump came from ingenious experiments by Post and his colleagues, published in 1972 (77). Although the sodium pump is rather specific for sodium, it is much less specific for potassium, so various congeners of potassium, including rubidium and lithium, can substitute for potassium. Post and his colleagues found that the rate at which it was possible to rephosphorylate kidney Na,K-ATPase that had just been dephosphorylated differed depending on whether lithium or rubidium had been used to catalyze the hydrolysis. This was true even if the experiment was done in such a way that the conditions during rephosphorylation were identical. In other words, the enzyme appeared to remember which ion had catalyzed the hydrolysis. To explain this memory, they suggested that the catalyzing ions became occluded within the enzyme at the moment of hydrolysis and were released only later after a slow conformational change. Because they found that the enzyme was available for rephosphorylation sooner if higher concentrations of ATP were used, they suggested that the binding of ATP at a low-affinity site accelerated the conformational change that released the occluded ions. Here, then, was the likely role of ATP (or its non-phosphorylating analogs) in supporting potassium-potassium exchange.

At that time there was no reason to suppose that the form of the dephosphoenzyme containing occluded potassium ions could exist more than transiently. In the late 1970s, Steve Karlish, David Yates, and I were measuring the rates of interconversion between the E_1 and E_2 forms of unphosphorylated pig kidney Na,K-ATPase when the sodium or potassium concentrations in the medium were rapidly

changed. Because the two forms have such different affinities for ATP, we could monitor the changes in conformation using stopped-flow fluorimetry and formycin triphosphate (FTP), a fluorescent analog of ATP that the enzyme treats much like ATP but which has the convenient property of fluorescing more strongly when it is bound. What we found was that the direction and magnitude of the changes in fluorescence that followed changes in the composition of the suspending medium were much as we expected, but the rates were not (78). In particular, when excess sodium was added to enzyme suspended in a low-potassium, sodium-free, magnesium-free medium containing a very low concentration of FTP, the rise in fluorescence was astonishingly slow, with a rate-constant, at room temperature, of about 0.25 s^{-1}. The rate increased, in a roughly linear fashion, as the concentration of FTP was increased up to 24 μM, a concentration beyond which measurements of binding became inaccurate because the fraction bound was so small.

The combination of a slow conformational change and its acceleration by a nucleotide acting with a low affinity and presumably without phosphorylating (since the medium lacked magnesium ions), was so reminiscent of Post's hypothetical occluded-potassium form that we wondered whether that was what we were looking at. In other words, did dephosphoenzyme in a low-potassium, sodium-free medium contain occluded potassium ions? To answer that question Luis Beaugé, and I suspended pig kidney Na,K-ATPase in a sodium-free solution containing radioactive rubidium and forced it through a small column of cation exchange resin at a rate that was slow enough for the resin to remove nearly all of the free rubidium ions yet fast enough for the enzyme to emerge in less than 2 s, i.e., within a period much smaller than the time constant for the conformational change in the fluorescence experiments (79). The enzyme did indeed carry rubidium ions through the column, about two rubidium ions per phosphorylation site, and a high concentration of ATP or of sodium ions prevented this effect. Later experiments (80), varying the rates of flow through the column and comparing the rates of release of different potassium congeners with the rates of conformational change determined by stopped-flow fluorimetry using several different fluorescent probes, supported the hypothesis that the conformational change releases the occluded ions.

In all these experiments, the occluded form was made directly from the unphosphorylated enzyme, but it was also possible to demonstrate its formation by the normal physiological route (81). Enzyme suspended in a Tris-medium containing magnesium, sodium, and a low concentration of radioactive rubidium was passed first through a thin layer of Sephadex containing 40 μM ATP (or ADP in the controls) and then through the cation exchange resin. With ATP, but not with ADP, roughly two rubidium ions per phosphorylation site were carried through the column. Clearly, there were two routes to the occluded-potassium form: the direct route by reversal of the final step of the normal cycle, picking up potassium ions on low-affinity sites at the inner surface of the membrane, and the physiological route, using the forward-running cycle and picking up potassium ions on high-affinity sites at the outer surface of the membrane (see 82).

The success of the rapid cation-exchange method for detecting occlusion of potassium congeners prompted us to use the same method to test the hypothesis that the E_1P form of the phosphoenzyme contains occluded sodium ions, a hypothesis that Joseph Hoffman and I had proposed 13 years earlier to explain the properties of the sodium-sodium exchange in red cells (60). The idea was to generate E_1P by phosphorylation of the enzyme in the presence of ^{22}Na-labeled sodium ions, to force the phosphorylated enzyme rapidly down a column of cation exchange resin, and to measure the radioactivity of the effluent. Because E_1P changes conformation to E_2P spontaneously, it was necessary to block that change, and this was done by pretreating the enzyme with either *N*-ethyl maleimide or α-chymotrypsin (83). Dephosphorylation of the E_1P by ADP was prevented by working close to 0°C and exposing the enzyme only very briefly to ATP at a low concentration in a thin layer of Sephadex above the resin. Evidence for the occlusion of sodium ions was found with both pretreatments, and the results with the α-chymotrypsin-treated enzyme were accurate enough to show that close to three sodium ions were occluded per phosphorylation site (84).

Later work has shown that the outward release of potassium ions from the occluded-potassium form of the enzyme, when orthophosphate is added, is an ordered release (85–87) and that the outward release of sodium ions from the occluded-sodium form of the enzyme occurs in two stages (88–90). Despite these complications, the working cycle remains an elaboration of the old Albers-Post ping-pong scheme. Another possible complication, the hypothesis that the pump is an $(\alpha\beta)_2$ diprotomer (or even a tetraprotomer) showing half-of-the-sites reactivity, has a long and interesting history, and there is evidence in its favor (for references see 91, 92 and papers in 46). But although it is clear that oligomer formation can occur, obligatory half-of-the-sites reactivity is difficult to reconcile with experiments showing (*a*) that the pump in the red cell membrane is a monomeric $\alpha\beta$ protomer (93); (*b*) that monomeric $\alpha\beta$ protomers and $(\alpha\beta)_2$ diprotomers, prepared from the same solubilized dog-kidney Na,K-ATPase, had the same specific activity (94); and (*c*) that, when a purified preparation of duck salt gland Na,K-ATPase with very high specific activity was exposed to $^{32}P_i$ in the presence of ouabain, close to one phosphate was incorporated per $\alpha\beta$ unit (95).

"Remembrance of Things Past"

Aaron Klug tells us that Rosalind Franklin once said to him, "What is the point of doing all this work if you don't get some fun out of it?" So, was the work I was engaged in fun? The answer is that it sometimes was, and the degree of enjoyment was not simply related to the significance of the results. The early experiments showing a tight link between active sodium efflux and active potassium influx, and the experiment to count the number of pumps in the red cell membrane, were particularly enjoyable because I was a research student and the whole business of discovery was new to me. On the other hand, proving that the pump could be driven backward, synthesizing its own fuel, was less exciting than it ought to have

been. This was partly because, initially, the effect was a small one; after a string of successful trials and no failures, Patricio Garrahan and I were convinced, but by then the novelty had worn off. A second reason was that Trevor Shaw had earlier attempted to prove the same point more elegantly by injecting an extract of firefly tails into a squid axon, arranging suitable gradients of sodium and potassium across the axonal membrane, and measuring the light emitted. Although our experiments were successful and his were not, we had the vague feeling that, where he had failed to produce a rabbit from a hat, we had succeeded only in producing a rabbit from a hutch. For me, the most enjoyable experiments were those using stopped-flow fluorimetry to follow the change in enzyme conformation that releases occluded potassium ions. For years I had worked surrounded by electrophysiologists whose exciting results flashed onto their oscilloscope screens during the course of their experiments. My exciting results came, if at all, during sessions with a slide-rule or calculator. So the pleasure of watching the spot on an oscilloscope screen languidly trace a perfect, if tremulous, exponential curve was a pleasure all the greater for being of a kind so long denied. And finally, of course, the occlusion experiments were a pleasure, both because such simple experiments provided such clear-cut results and because in examining the behavior of the occluded-ion forms we felt we were getting down to the nitty gritty of the pump. That is, though, probably the wrong metaphor to use in connection with a pump that is efficient enough to be thermodynamically reversible and that has now kept physiologists happily occupied for a century.

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