Frontispiece: I. M. Glynn

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'ALL HANDS TO THE SODIUM PUMP'

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In 1941 we were at war with Germany, most of continental Europe was under Nazi occupation, there was still a threat of invasion, and shipping losses were frightening – at one point reaching half a million tons in three months. In this country no one was thinking about academic physiology. But until the Japanese attacked Pearl Harbor, which was in December of that year, life in the United States went on as normal. And in the University of Rochester in upstate New York, a young American, Robert Dean, published an article pointing out that, when muscles were recovering from a period of activity or a period of cold-storage, the loss of sodium and the gain of potassium that occurred were thermodynamically uphill movements (Dean, 1941). So, he argued, the muscle membrane must contain a pump that pumps sodium out or potassium in or both. But though Dean's article contains the first use of the phrase 'sodium pump', and he deserves full credit for seeing the significance of the ion movements in recovering muscles – something that his contemporaries failed to see – his argument had, in fact, been anticipated – anticipated and then forgotten.

At the beginning of this century we were on good terms with Germany, and in the university town of Würzburg an Englishman, Ernest Overton, was working in von Frey's laboratory. This was the von Frey who made sets of calibrated bristles to test touch, but Overton was not interested in touch receptors; he was interested in the permeability of animal and plant membranes. In 1902 he published an article in *Pflügers Archiv* (Overton, 1902) which contained the following paragraph (the translation is by Bernard Katz):

> 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.

Overton goes on to say that this is a serious difficulty but not more serious than trying to understand other examples of transport and secretion in the body. The difficulties arise, he pointed out, 'because we are as yet rather ignorant of the chemical make-up of protoplasm and because it is very hard to disentangle a chain of interwoven processes which take place in a system of minute structural dimensions'.

Overton was right, of course, and I shall want to spend a good part of this talk discussing the disentangling of the chain of interwoven processes involved in

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pumping sodium and potassium. But, because the appetite of most physiologists for details of the unravelling of enzyme mechanisms is understandably limited, I want to begin with a topic of more general interest. How did the sodium pump evolve in the first place?

Until quite recently, most of us believed a theory that was proposed in the 1950s by E. J. Conway, whom many here will remember as the Professor of Physiology at University College, Dublin. Conway (1957) pointed out that any cell which contains proteins and other large impermeant organic molecules, and which is bathed by a fluid lacking or poorer in such constituents, has an osmotic problem; and he suggested that early in evolution cells solved this problem by expelling sodium ions to maintain a concentration difference across the membrane which balanced the osmotic effect of the impermeant molecules. Because sodium ions are positively charged, their expulsion from the cell would create an electrical potential across the membrane – positive outside – and this potential would tend to drive both sodium and potassium ions into the cell. Because hydrated potassium ions, being smaller than hydrated sodium ions, move faster in an electric field, and because the sodium ions are being expelled anyway, the cells would end up rich in potassium and poor in sodium.

This ingenious theory has had to be given up, because it now turns out that the sodium pump of animal cells is closely related, both in its structure and in the way it works, to a whole family of pumps, the so-called P-type ATPases. The family includes not only the sodium pump, the calcium pumps in sarcoplasmic reticulum and in the plasma membrane, and the hydrogen–potassium pump that is responsible for acid secretion in the stomach, but also pumps that expel hydrogen ions from fungal cells and from the cells of higher plants, and some bacterial pumps including one that pumps potassium ions into *E. coli*. They are called P-type ATPases because their mechanism involves the phosphorylation and dephosphorylation of the pump during each working cycle.

The resemblances between the sodium pump and the hydrogen and potassium ion pumps in bacterial, fungal and plant cells make Conway's theory implausible, and the best guess at a history of pumping would be something like this.

(1) Probably the first pump was an ATP-driven pump that expelled protons from unicellular anaerobic organisms, so preventing them from being killed by the acid products of their own fermentation. This primitive pump would have set up a pH gradient and an electrical gradient across the membrane, and that would have opened the way for the evolution of coupled transport systems that used the energy stored in these gradients to accumulate nutrients or expel wastes (see Fig. 1).

(2) Two other systems capable of expelling protons then seem to have evolved. In the first of these systems, the energy was supplied by light absorbed by a coloured membrane protein. Later, probably with the development of the cyanobacteria about three billion years ago, organisms evolved in which photosynthesis led to the production of oxygen from water, and the world became aerobic. That made possible the evolution of systems in which the energy for pumping protons came from respiration.

Cells that possessed either of these new systems did not need to use their primitive ATP-driven proton pumps to expel protons, and it is thought that, instead, they

allowed the pH and potential gradients, generated by their new pumps, to drive their old ATP-driven pumps backwards, synthesizing ATP (see Fig. 2). This is, of course, precisely what happens in chloroplasts and mitochondria today, and it is now generally believed that both of these organelles originated as independent organisms

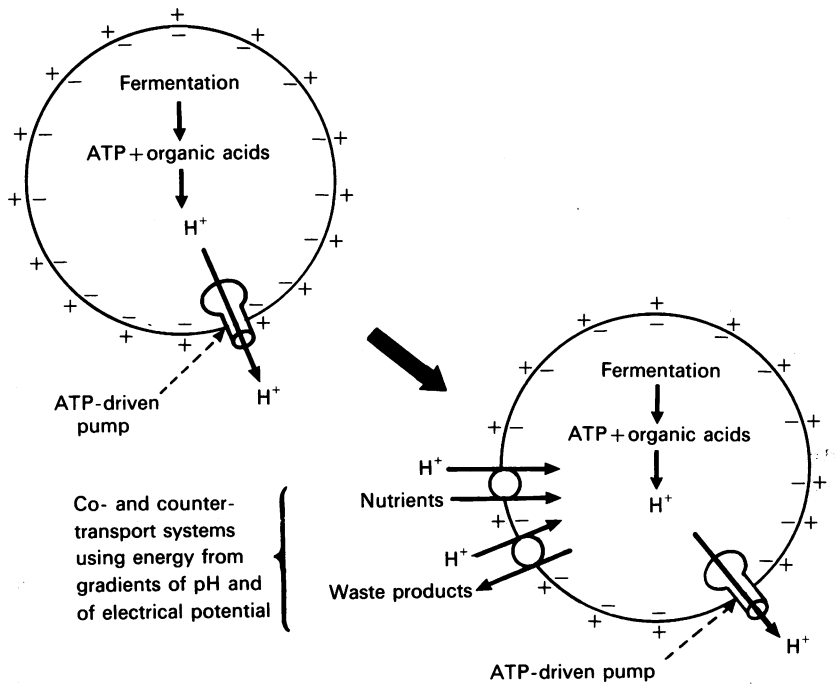

Fig. 1. Suggested evolution of the first pump and co- and counter-transport systems.

living symbiotically in larger cells, chloroplasts evolving from photosynthetic blue–green bacteria, and mitochondria from bacteria that obtained their energy from respiration. The existence in both mitochondria and chloroplasts of separate DNA and of protein-synthesizing machinery supports this notion (see Margulis, 1981).

(3) Now it is very well established that the working cycles of the ATP-driven proton pumps in mitochondria and chloroplasts – better called ATP synthases, since they normally run backwards – do not involve phosphorylation and dephosphorylation. It is therefore unlikely that the P-type ATPases have evolved from the primitive ATP-driven pumps. Nor is there any resemblance between the P-type ATPases and the mechanisms that couple light energy or energy from respiration directly to cation transport. So we have to assume that at an early stage in evolution, before the separation of bacteria, fungi, plants and animals, a second kind of ATP-driven pump evolved, which differed from the original ATP-driven proton pump in that its working cycle did include phosphorylation and dephosphorylation. We do not know whether this pump was originally evolved to pump protons or other cations; and we do not know what advantage it had over existing pumps. Until recently one might have suggested that the advantage was versatility, since it looked as though the mitochondrial/chloroplast type of ATPase could transport only

protons. Recently, though, it has been shown that the alkali-tolerant marine bacterium *Vibrio alginolyticus* possesses an ATP synthase which is structurally of the mitochondrial type but which can use as a source of energy either a pH gradient or a gradient of sodium ions (Dibrov, Lazarova, Skulachev & Verkhovskaya, 1986) (see

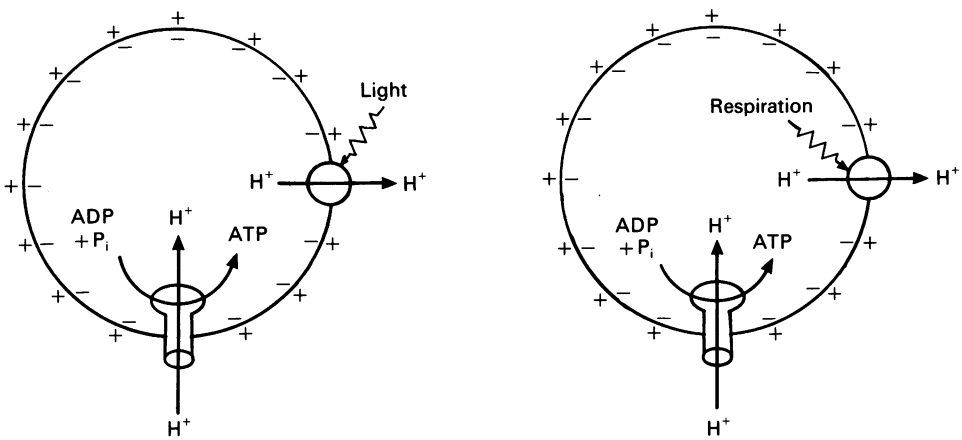

Fig. 2. The evolution of new proton pumping mechanisms using energy from light or respiration is thought to have made the primitive ATP-driven proton pump redundant as a pump. Driven backwards by the pH and electrical gradients created by either of the new mechanisms, however, it would have acted as an ATP synthase, and it is thought to have survived in this role in chloroplasts and mitochondria.

Fig. 3). This bacterium lives in algal mats whose pH varies with the amount of photosynthesis going on. When the surroundings are neutral, respiration is used to drive an outward movement of protons, and the resulting pH and electrical gradients are used by the ATP synthase. When the surroundings are alkaline, and it is impossible to build up a pH gradient acid on the outside, respiration is used to expel *sodium* ions, and the resulting *sodium* gradient and electrical gradient are used by the synthase. (For further discussion and references see Skulachev, 1989; Glynn & Karlish, 1990.)

Isoenzymes

Until the 1980s, it was customary to regard the sodium pump as a single enzyme, consisting of two catalytic α-subunits which carry all the ligand binding sites, and two β-subunits which are glycoproteins and are thought to facilitate the correct assembly and transport of the enzyme into the cell membrane. More recently it has been discovered that, like many other enzymes, the sodium pump consists of a family of isoenzymes (Sweadner, 1989, 1991). Three isoforms of the α-subunit and three of the β-subunit have now been identified, their distribution depending on the species, the tissue, the cell type, and the stage of development. Very surprisingly, one of the isoforms of the β-subunit turns out to be identical with an adhesion molecule found on neuroglial cells; and a monoclonal antibody to that adhesion molecule has been

shown to stimulate rubidium uptake by intact cultured astrocytes (Gloor, Antonicek, Sweadner, Pagliusi, Frank, Moos & Schachner, 1990). Intriguing though these results are, their significance is obscure; ATPase activity is *not* required for adhesion, and there is no obvious reason why the same molecule should be involved in the two roles.

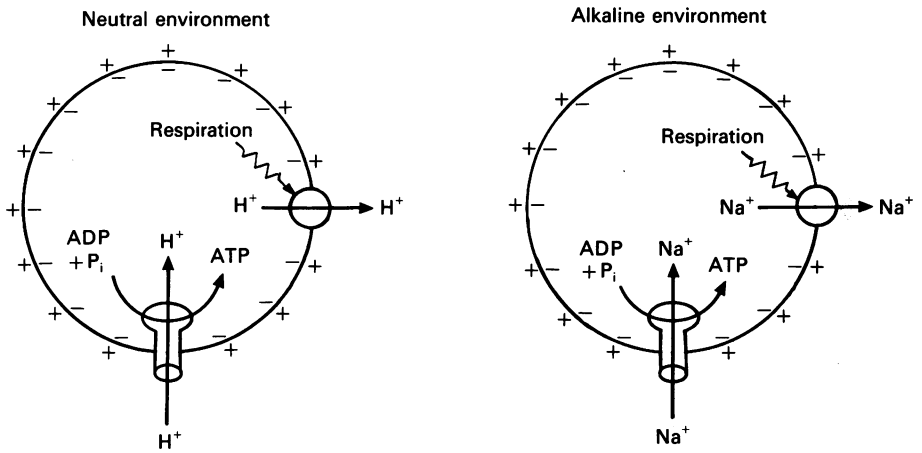

Fig. 3. ATP synthesis in the alkali-tolerant marine bacterium *Vibrio alginolyticus*. This bacterium has an ATP synthase which is of mitochondrial type except that it can use either a pH gradient or a sodium gradient as a source of energy. For further details see text.

But leaving aside that particular and peculiar problem, why should there be several variants of the sodium pump, differing slightly in their subunits? Isoforms can, of course, arise simply from gene duplication followed by genetic drift, but that is not an adequate explanation for the sodium pump isoforms. Fambrough and his colleagues have recently looked at the nucleotide sequences in the genes that encode three isoforms of the α-subunit that are found in birds, and have compared them with the sequences in the genes encoding the three isoforms of the α-subunit that are found in mammals (Fambrough, Wolitzky, Taormino, Tamkun, Takeyasu, Somerville, Renaud, Lemas, Lebovitz, Kone, Hamrick, Rome, Inman & Barnstein, 1991). It turns out that each of the avian isoforms has a direct homologue in mammals, and the sequence identity between the corresponding avian and mammalian isoforms – about 93–96% – is much greater than the sequence identity between the different mammalian or the different avian forms – 82–83%. The implication of these findings is that the three isoforms must have arisen not later than the date of the last common ancestor of birds and mammals – thought to be about 200 million years ago – and their primary structure must have been maintained fairly rigidly ever since. Such conservatism implies that each of the three forms has been preserved by natural selection. But why?

One obvious advantage of the existence of different isoenzymes encoded by different genes is that their synthesis and degradation can be regulated independently. In developing rat heart, for example, it has been shown that the ratio of the different isoenzymes changes with time, and the changes are greatly affected

by the levels of thyroid hormones and glucocorticoids (Lingrel, Orlowski, Price & Pathak, 1991). It also seems to be possible to target the different isoenzymes to different sites within a single cell; this has been seen, for example, in hippocampal pyramidal cells (McGrail, Phillips & Sweadner, 1991).

Another possible advantage in having different isoenzymes is that their activities can be separately controlled. A few years ago, Lytton, Lin & Guidotti (1985) reported that, in the fat cells of rats, insulin stimulates only one of the two Na^+,K^+-ATPase isoenzymes that are present. However, later work from the same laboratory has shown that the story is more complicated: it seems that both isoenzymes are stimulated though in different ways (McGill & Guidotti, 1991).

A third possible advantage in having several different isoenzymes is that their properties might be tailored for different jobs. For example, a cell such as a nerve cell, which needs to keep its sodium concentration low and within fairly narrow limits, but which from time to time has to cope with sudden large increases in sodium influx, will find it advantageous to have an additional high-capacity, low-affinity sodium pump that turns over slowly when the sodium concentration is near the desired level but fast when the sodium concentration rises greatly. It is therefore interesting that Jewell & Lingrel (1991) recently showed that Na^+,K^+-ATPase containing the $\alpha 3$-isoform (an isoform which occurs predominantly in nerve cells; see Sweadner, 1991) has an affinity for sodium ions only about a third as great as the affinity of Na^+,K^+-ATPase containing either $\alpha 1$ or $\alpha 2$.

Although this seems a very simple and pleasing result, it was not at all easy to get, and required an ingenious and elegant approach. Comparing the properties of the different isoenzymes is difficult, first because only one of the isoenzymes occurs unmixed with the others, and secondly because it is difficult to know whether differences between different tissues reflect differences in the isoenzymes or differences in their environment. Jewell & Lingrel got round these difficulties by (1) cloning the cDNAs that code for the three isoforms in the rat, (2) making appropriate point mutations to ensure that all three gene products would be ouabain resistant – ouabain resistance depends on having charged groups at just two points in the polypeptide chain, (3) expressing the isoforms in different batches of HeLa cells, and (4) looking at the properties of the expressed enzymes in the presence of a concentration of ouabain sufficient to knock out the HeLa cells' own pumps but insufficient to affect the inserted pumps.

Since, in rat, pumps containing the $\alpha 1$-isoform are relatively insensitive to ouabain, the difference in the sodium affinities of pumps containing $\alpha 1$ and $\alpha 3$ can explain the observation of Inoue & Matsui (1991) that exposure of cultured rat brain neurones to glutamate, which increases sodium influx and therefore $[Na^+]_i$, greatly increases the sensitivity of potassium influx to ouabain.

How does the sodium pump pump?

We are, of course, still a long way from being able to give the kind of detailed account of the mechanism of the sodium pump that can be given, for example, of the mechanism that allows haemoglobin to combine reversibly with oxygen. This is partly because in the sodium pump we are dealing with a much more complicated process, but mainly because we lack the detailed knowledge of molecular structure. Nevertheless, there is now a widely accepted view of the way the pump works. I want

to describe that view and to talk about some of the varied experimental approaches that have led to it. Finally, I shall try to link the mechanism and the structure, to the rather limited extent that this can be done.

When it is working normally, the sodium pump pumps three sodium ions out of the cells and two potassium ions into it for each molecule of ATP hydrolysed. It is not

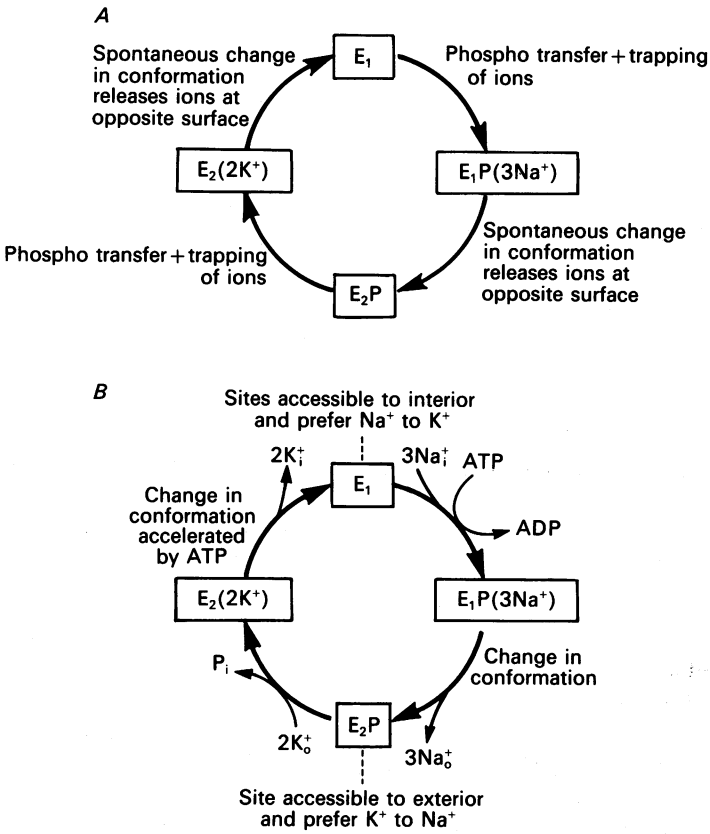

Fig. 4. The basic cycle of the sodium pump. *A*, a diagram showing the alternation of steps in which the transfer of a phospho group (to or from the pump) is associated with the trapping of ions, and steps in which spontaneous changes in conformation release the trapped ions at the face of the pump opposite to that from which they were trapped. Ions shown within brackets are trapped within the pump molecule. *B*, a more detailed diagram showing the binding and release of substrates and products.

likely that an enzyme which has four substrates (ATP, water, internal sodium and external potassium), and four products (ADP, inorganic phosphate, external sodium and internal potassium) has a single-step reaction mechanism; we therefore want to know: What are the individual steps in the overall reaction? Which of them are associated with the movements of sodium and potassium? And how are these movements brought about?

It is easier to follow an argument if one knows where it is leading; so let me start with a diagram (Fig. 4*A*) that gives an outline of the answers to those questions.

There are four basic steps. In two of the steps, the transfer of a phospho group to the pump, or from the pump, is accompanied (or is preceded) by a change in

conformation that traps within the pump cations that have bound at one of the faces of the pump. In the intermediate two steps, spontaneous changes in conformation lead to the release of the trapped ions at the opposite face of the pump.

Now, let me be more specific (Fig. 4*B*). In the first step, binding of ATP and of three sodium ions to the inner face of the pump is followed by the transfer of a

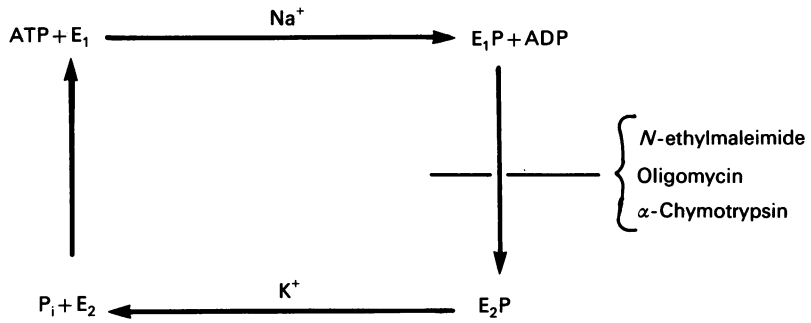

Fig. 5. The original Albers–Post scheme. For further details see text. The blocking action of α-chromotrypsin was discovered much later (Jørgensen *et al.* 1982).

phospho group from ATP to the pump and the trapping of the three sodium ions within the pump molecule. In the second step, which is a spontaneous step, these ions are released to the exterior. In the third step the binding of two potassium ions at the exterior face leads to transfer of the phospho group from the pump to water (to form inorganic phosphate), and this is accompanied by the trapping of the two potassium ions. In the fourth step – again a spontaneous step – these potassium ions are released to the cell interior. The conformational changes not only alter the chemical reactivity of the pump molecule and the accessibility of the binding sites to the media bathing the two faces of the pump, but also change the selectivity of those sites; so that each cation is picked up at a high affinity site on one face and discharged from a low affinity site on the opposite face. At this point I want to add only one other fact. The fourth step, the conformational change that releases the trapped potassium ions to the interior, is very slow unless ATP (or a non-phosphorylating analogue of ATP) is bound at a low-affinity site. So ATP has two separate roles: acting with a high affinity it phosphorylates the pump in the first step; acting with a low affinity, and without phosphorylating, it accelerates the change in conformation that releases potassium ions in the last step.

Let us look at some of the evidence for this complex story.

Phosphorylation studies

Once the identity of the sodium pump and a membrane ATPase had been established (Skou, 1957; Post, Merrit, Kinsolving & Albright, 1960; Dunham & Glynn, 1961), the most straightforward approach to investigating the pump mechanism was to expose the pump to [γ^{32}P]ATP, and to follow the phosphorylation and dephosphorylation under different conditions. Using this approach, and working with fragmented membranes from guinea-pig kidney or electric eel electric organ, i.e. membranes very rich in sodium pumps, Robert Post and his colleagues at Vanderbilt,

and Wayne Albers and his colleagues at NIH, found that sodium ions catalysed phosphorylation of the pump, and potassium ions dephosphorylation (Charnock & Post, 1963; Albers, Fahn & Koval, 1963; Post, Sen & Rosenthal, 1965) (see Fig. 5). The group that becomes phosphorylated is a β-aspartyl carboxyl group (Post & Kume, 1973), which is situated in a loop of the α-chain that projects into the cytoplasm.

To explain the properties of the phosphoenzyme under different conditions it is necessary to suppose that it can exist in two conformations: E_1P, which can hand its phospho group back to ADP to make ATP, but which is unaffected by potassium ions; and E_2P, which does not react with ADP, but which is hydrolysed in the presence of potassium ions (Fahn, Hurley, Koval & Albers, 1966*a*; Fahn, Koval & Albers, 1966*b*; Siegel & Albers, 1967; Post, Kume, Tobin, Orcutt & Sen, 1969). In the normal cycle, E_1P changes spontaneously to E_2P, but the conversion can be blocked by a number of inhibitors including oligomycin and *N*-ethyl maleimide (Fahn *et al.* 1966*a*, *b*; Siegel & Albers, 1967; Fahn, Koval & Albers, 1968). It can also be blocked by breaking a particular peptide bond in the α-chain using α-chymotrypsin (Jørgensen, Skriver, Hebert & Maunsbach, 1982).

The notion that the normal cycle also involves two different forms of the unphosphorylated enzyme was originally suggested by Siegel & Albers (1967), who thought it likely that if the enzyme changed conformation in going from E_1P to E_2P, then, after E_2P had been hydrolysed, it would need to change conformation again to get back to the original state. Evidence that the unphosphorylated enzyme can indeed exist in more than one form came from observations on ligand binding in different conditions. In particular, in sodium media the enzyme was found to have a high affinity for ATP, whereas in potassium media the affinity was low (Hegyvary & Post, 1971; Nørby & Jensen, 1971); and the difference was clearly associated with a change in conformation because the pattern of tryptic digestion in the two media was found to be different (Jørgensen, 1975, 1977).

But the first convincing evidence that a second form of unphosphorylated enzyme was actually involved in the pump cycle came from some very ingenious experiments by Post and his colleagues at Vanderbilt (Post, Hegyvary & Kume, 1972) (see Fig. 6). 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 ions. Post and his colleagues found that the rate at which it was possible to rephosphorylate enzyme that had just been dephosphorylated depended on whether rubidium or lithium had been used to catalyse the dephosphorylation. And 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 catalysed the dephosphorylation. To explain this memory, they suggested that the catalysing ions became occluded within the enzyme at the moment of hydrolysis and were released only later after a slow conformational change. And since they found that the enzyme was available for rephosphorylation sooner if higher concentrations of ATP had been used, they suggested that the binding of ATP at a low affinity site accelerates the conformational change that releases the occluded ions. We shall see later that this explanation is correct.

Flux studies

A limitation of the approach I have just been describing is that, because fragmented membranes are used, the experimenter can never be sure at which surface of the pump ions are acting; nor can the experimenter know whether the

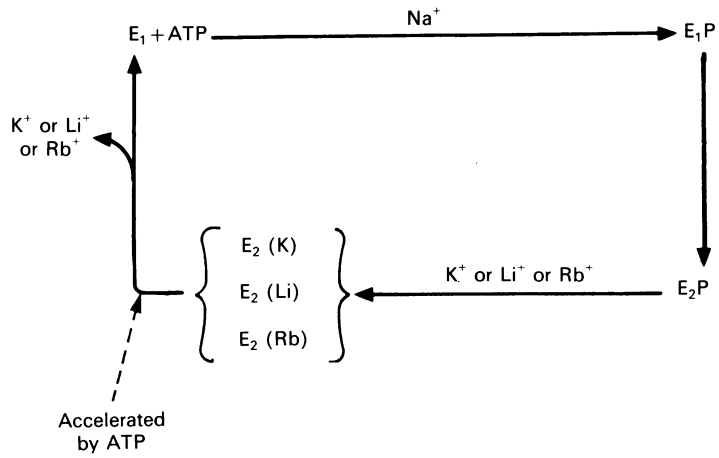

Fig. 6. Diagram to illustrate the hypothesis of Post *et al.* (1972) that cations catalysing the dephosphorylation of the pump become occluded within the pump molecule and are released only later after a slow conformational change that can be accelerated by ATP.

chemical changes observed are accompanied by the movement of ions across the membrane. Complementary information has come from experiments which looked at the fluxes catalysed by the pump in a wide variety of conditions. For experiments of this kind, what was important was not the density of pumps but the integrity of the cell membrane, and the tissues that proved most useful were therefore not kidney and electric organ but nerve, muscle, and, above all, red cells and the resealed ghosts made from them. More recently, it has become possible to use artificial lipid vesicles with purified Na^+,K^+-ATPase incorporated into their membranes.

For discussing these experiments it will be convenient to use a rather fuller version of the overall scheme (Fig. 7), which, as well as the four basic states, shows intermediate states with ions bound but not yet occluded or released. And although this is not, of course, how things happened historically, to make the story easier to follow I shall assume for the moment that the scheme is correct and show how the various flux modes fit into the picture.

Under physiological conditions, the cycle runs clockwise and energy from the hydrolysis of one molecule of ATP is used to drive three sodium ions outwards and two potassium ions inwards. If the concentration gradients are made so steep that the energy from ATP is insufficient, the whole system – unless it stops or changes stoichiometry – ought to run backwards, synthesizing ATP at the expense of downhill movements of the cations. In experiments on resealed red cell ghosts, we found that it did (Garrahan & Glynn, 1966, 1967*e*). Although predicted as a possibility, this was a startling result to get because one is not used to machines

driven by chemical fuels being reversible. It is as if one took a motor car with an empty petrol tank, let it run downhill, and found petrol spurting from the filler cap. But although this was a pleasing experiment to have done, if only for the embarrassment it caused to those who still did not believe in the sodium pump – this

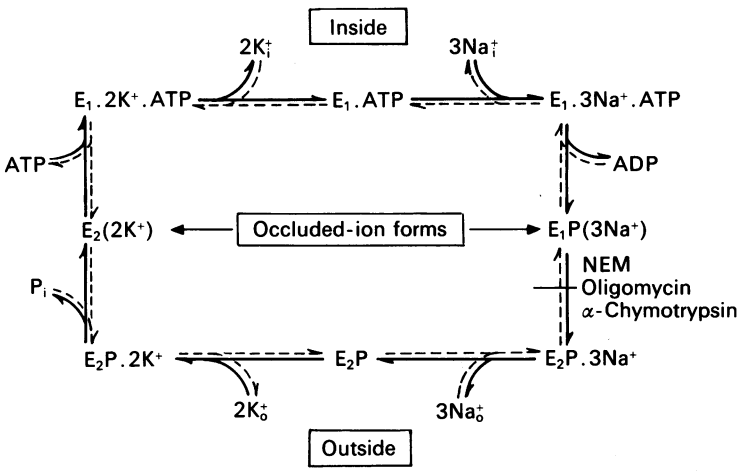

Fig. 7. The normal pump cycle. This is an expanded version of diagram *B* in Fig. 4, showing intermediate states with ions bound but not yet occluded or released. It is now believed that the release of sodium ions from $E_1P(3Na^+)$ to the exterior occurs in two stages, but for simplicity this has not been included in the diagram. Modified from Karlish *et al.* (1978).

was in 1966 – all it proved, so far as mechanism is concerned, is that all the steps in the pump cycle must be reversible, and in physiological conditions none can be too far from equilibrium. Fortunately, some of the other abnormal flux modes were more informative.

When red cells or resealed ghosts were incubated in media containing no potassium, it was possible to detect a one-for-one exchange of internal and external sodium ions, as if the right-hand part of the cycle were shuttling backwards and forwards. If that suggestion is correct, then, first, the exchange should require the presence of both ATP and ADP – we found that it did (Glynn & Hoffman, 1971; Cavieres & Glynn, 1979); secondly, the exchange should not be accompanied by the hydrolysis of ATP – it was not (Garrahan & Glynn, 1967*d*); thirdly, the exchange should have a high affinity for sodium inside and a low affinity outside – it had (Garrahan & Glynn, 1967*a*, *c*); and lastly, the exchange should be blocked by oligomycin – it was (Garrahan & Glynn, 1967*d*). The fact that oligomycin inhibited sodium–sodium exchange but did not inhibit (and in fact stimulated) [^{14}C]ADP–ATP exchange (Blostein, 1970) showed that both the phosphorylation step and the subsequent conformational change were necessary to get sodium ions across the membrane, and suggested to us that E_1P might contain occluded sodium ions (Glynn & Hoffman, 1971), a point that I will come back to later.

In other conditions, it was possible to demonstrate a one-for-one exchange of internal and external potassium ions, as if the left-hand part of the scheme were

shuttling backwards and forwards. If the scheme is correct, we should expect the shuttling to require both inorganic phosphate and ATP – we found that it did (Glynn, Lew & Lüthi, 1970; Glynn, Hoffman & Lew, 1971); the shuttling should not be associated with the hydrolysis of ATP – it was not (Simons, 1974); and the

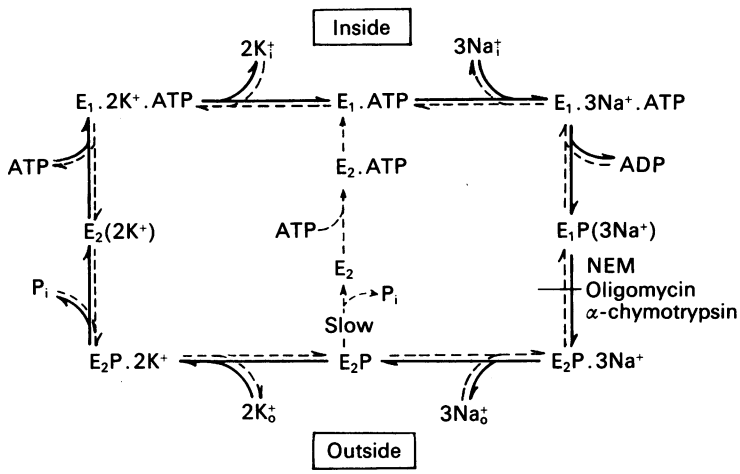

Fig. 8. An extra central pathway has been added to the scheme shown in Fig. 7, to explain the uncoupled efflux of sodium seen in media lacking both sodium and potassium.

exchange should have a high affinity for potassium ions outside and a low affinity inside – it had (Simons, 1974; Sachs, 1981). Lastly, since, according to the scheme, ATP acts merely by binding at a low affinity site, we should expect non-phosphorylating analogues of ATP to be capable of substituting for ATP – and they were (Simons, 1975).

Now we come to a deficiency of the original scheme. If red cells containing sodium are incubated in choline media lacking both sodium and potassium, the cycle should stop at the E_2P stage and there should be neither fluxes nor ATP hydrolysis. In fact what happens is that there is a slow 'uncoupled' efflux of sodium ions (Garrahan & Glynn, 1967*b*; Beaugé & Ortiz, 1973; Lew, Hardy & Ellory, 1973), which is accompanied by a slow hydrolysis of ATP, the stoichiometry being two to three sodium ions per ATP (Karlish & Glynn, 1974; Glynn & Karlish, 1976). To accommodate these findings we had to suppose that E_2P could break down slowly without leading to any inward movement of ions, as in the central dotted pathway in Fig. 8. But if such a pathway exists, just as it is possible for sodium ions to go out by the right-hand pathway and for the cycle to be completed by the central pathway, so it ought to be possible for potassium ions to go out by the left-hand pathway, and for the cycle to be completed by the central pathway. Arguing in this way, John Sachs predicted that in appropriate conditions an uncoupled efflux of potassium should occur, and he was able to demonstrate that it did (Sachs, 1986).

So the flux studies nicely complement the phosphorylation studies, and the scheme can account for all the flux modes that I have described. With a slight relaxation of assumptions about ion selectivity, and of other constraints, the scheme can also

account for five other flux modes that have been observed under different conditions: an exchange of internal hydrogen ions for external potassium ions (Hara & Nakao, 1986); an exchange of internal sodium ions for external hydrogen ions (Polvani & Blostein, 1988); a second kind of sodium–sodium exchange that occurs in the absence of ADP and is accompanied by the hydrolysis of ATP (Lee & Blostein, 1980); a second kind of uncoupled sodium efflux in which only one sodium ion is expelled for each molecule of ATP that is hydrolysed (Yoda & Yoda, 1987*b*); and a very slow rubidium–rubidium exchange that occurs in the absence of both nucleotides and phosphate (Karlish & Stein, 1982; Kenney & Kaplan, 1985). But for the story to be really convincing we want direct evidence for the existence of the hypothetical forms of the enzyme that are supposed to contain occluded ions; for it is these occluded-ion forms that are, literally as well as metaphorically, central to the pumping mechanism.

Direct evidence for ion occlusion

I have already mentioned the remarkable observations of Post *et al.* (1972) which suggested that, following the hydrolysis of the phosphoenzyme, the catalysing ions remained transiently occluded within the pump. At that time there was no reason to believe that the occluded-potassium form, if it existed at all, existed more than transiently. Some years after these experiments, however, Steven Karlish, David Yates and I were using stopped-flow fluorimetry, with a fluorescent analogue of ATP, to follow the change in conformation of the unphosphorylated kidney Na^+,K^+-ATPase when it was changed from a potassium medium to a sodium medium. We found, to our astonishment, that the change in conformation was extremely slow – the time constant was between 5 and 10 s at room temperature – and that it could be accelerated by increasing the concentration of the nucleotide (Karlish, Yates & Glynn, 1978; Karlish & Yates, 1978). But these were just the features of Post's hypothetical occluded-potassium form. So was the stable form of the pump in potassium media a form that contained occluded potassium ions?

To answer this question, Luis Beaugé and I did a very simple experiment (Beaugé & Glynn, 1979) (see Fig. 9*A*). We suspended a preparation of kidney Na^+,K^+-ATPase in a sodium-free solution containing a small amount of radioactive rubidium – rubidium rather than potassium because you can get it with a much higher specific activity – and we forced the suspension though a small column of cation exchange resin at a rate such that it was in contact with the resin for a little under 1 s, i.e. much less than the time constant of the conformational change seen in the fluorescence experiments. After correcting for the amount of rubidium carried through the column in control experiments, we calculated that the enzyme had carried approximately two rubidium ions through the resin per enzyme phosphorylation site. Later Donald Richards, Yukichi Hara, Marcia Steinberg and I varied the flow rate and so were able to estimate the rate of release of the occluded rubidium. By combining experiments of this kind with simultaneous measurements using fluorescent probes, we showed that there was a good correlation between the rate of release of occluded rubidium and the rate of the conformational change. And both were accelerated by ATP, which acted with a low affinity and without phosphorylating (Glynn & Richards, 1982; Glynn, Hara, Richards & Steinberg, 1987).

Clearly we were dealing with Post's occluded rubidium form; equally clearly we had not made it by the route used by Post and his colleagues, i.e. by the rubidium-catalysed hydrolysis of phosphoenzyme. Could we make it by this route, which I am afraid we took to calling the 'Postal route', though 'physiological route' would have been more appropriate?

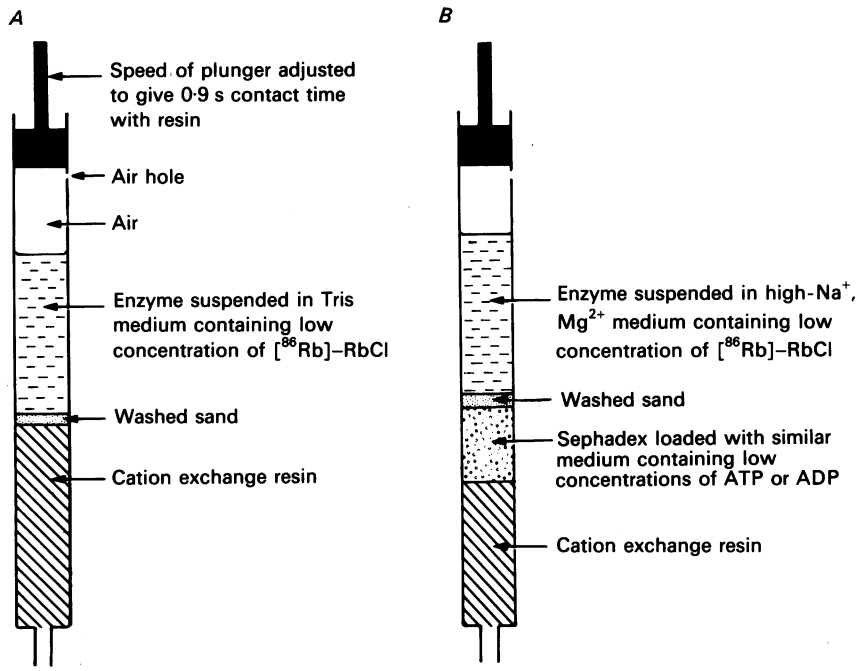

Fig. 9. Detection of occluded rubidium ions. *A*, method for detecting occlusion by the direct route, i.e. the binding of rubidium ions to the *unphosphorylated* pump followed by a change in conformation. *B*, method for detecting occlusion by the physiological route, i.e. the Rb^+-catalysed hydrolysis of the *phosphorylated* pump. For further details see text.

To answer this question we suspended a preparation of kidney Na^+,K^+-ATPase in a sodium medium containing a little radioactive rubidium, and passed it rapidly first through a layer of Sephadex containing a low concentration of ATP and then through a cation exchange column (see Fig. 9*B*). The idea was that, as it passed through the Sephadex, the enzyme would pick up sufficient ATP to be phosphorylated but insufficient to accelerate the conformational change leading to the release of the occluded rubidium. By comparing the amount of rubidium carried through the column with the amount carried through in control experiments (in which ATP was replaced by ADP or a non-phosphorylation analogue) we estimated that roughly two rubidium ions became occluded by the physiological route per phosphorylation site (Glynn & Richards, 1982).

It was clear that there were two routes to the occluded potassium form: the *direct* route, in which the binding of potassium ions to the unphosphorylated enzyme is followed by a spontaneous conformational change that occludes the ions; and the

physiological route, in which occlusion accompanies the potassium-catalysed hydrolysis of the phosphorylated enzyme (Fig. 10). There is, though, an important difference between the two routes. To enter by the physiological route, potassium ions must combine at high-affinity extracellular sites, since Blostein & Chu (1977)

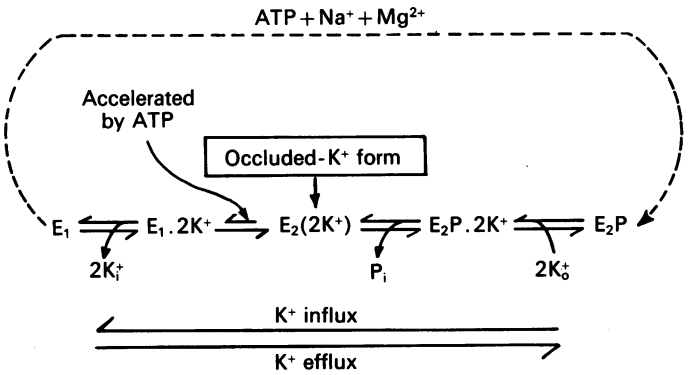

Fig. 10. The two routes to the occluded-K^+ form coupled back-to-back to form a pathway through the membrane. Modified from Glynn, Richards & Hara, 1985*b*.

showed that the potassium ions that hydrolyse E_2P act with a high affinity and at the external face of the pump. In contrast, when potassium ions combine with E_1 to form $E_2(2K^+)$, they act at low-affinity sites (Karlish, Yates & Glynn, 1978; Beaugé & Glynn, 1980), and these sites must be at the inner face of the pump since only potassium ions with access to the inner face can put the pump in the conformation that gives the trypsin-digestion pattern characteristic of Na^+,K^+-ATPase suspended in potassium-containing, sodium-free media (Karlish & Pick, 1981). The two routes coupled back to back, as shown in Fig. 10, therefore provide a pathway through the membrane with just the right qualities to transport potassium into or (in the unphysiological direction) out of the cell.

Just as potassium ions can be made to enter the occluded-potassium form from either face of the pump, so they can be released quickly to either face: to the *interior*, by the addition of ATP; to the *exterior*, by the addition of inorganic phosphate (and, if not already present, magnesium ions). This release to the exterior shows an interesting and unexpected feature. If the medium contains no potassium (or potassium congener) both occluded ions are released quickly. But if potassium or a congener is present, the first occluded ion comes out quickly but the second is greatly delayed, as if potassium ions from the medium occupy the hole left by the escaping first ion and block the way for the second ion (Glynn & Richards, 1984, 1989; Glynn, Howland & Richards, 1985*a*; Forbush, 1985, 1987, 1988; Glynn & Karlish, 1990). The implication is that the two occluded ions leave in order, and detailed examination of the kinetics suggests that, provided sufficient Mg^{2+} and inorganic phosphate are present, the rate limiting step is not the phosphorylation step but the subsequent escape of the two ions as they pass, in order, through a narrow channel, or a channel only intermittently open (Forbush, 1987).

So much for the occluded-potassium form. What about the occlusion of sodium? You will remember that we had thought that sodium ions might be occluded in the E_1P form of the phosphoenzyme, i.e. the form that is generated first when the enzyme is phosphorylated by ATP. To test this we needed to generate E_1P in the

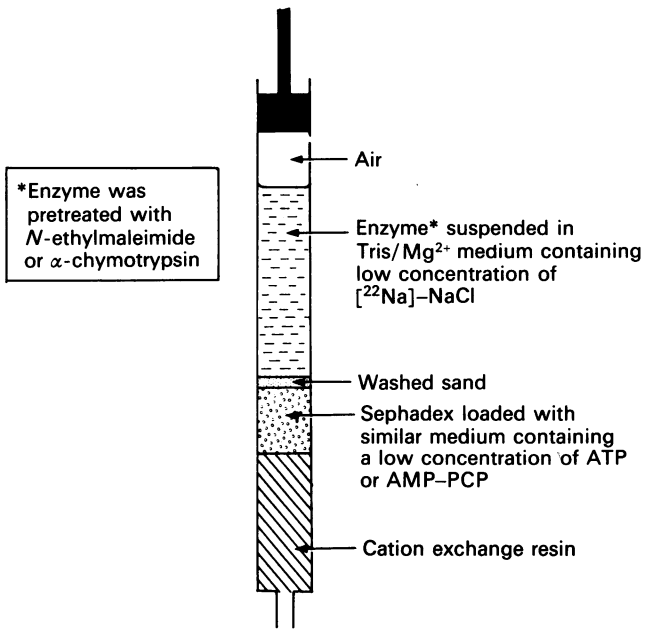

Fig. 11. Method for detecting the occlusion of sodium ions in the E_1P form of the pump. For further details see text.

presence of radioactive sodium, to force the enzyme suspension rapidly down a cation exchange column, and to measure radioactivity in the effluent.

It was, of course, necessary to prevent the spontaneous conversion of E_1P to E_2P since that would have allowed the occluded sodium ions to escape to the exterior; and it was also necessary to prevent E_1P from reacting with ADP, since that would have allowed the occluded ions to escape to the interior. To prevent the conversion of E_1P to E_2P we pretreated the enzyme with *N*-ethyl maleimide or with α-chymotrypsin; to avoid the presence of significant amounts of ADP we worked at 0 °C, and we allowed the enzyme to have only brief access to ATP, by putting the ATP in a thin layer of Sephadex on top of the resin column (see Fig. 11). In the event the enzyme did carry extra sodium through the column, the amount being close to three sodium ions per phosphorylation site (Glynn, Hara & Richards, 1984). It was only after we had done this experiment, that I realized with some chagrin that there was no reason why the experiment with *N*-ethyl-maleimide-treated enzyme could not have been done thirteen years earlier when Joseph Hoffman and I first suspected that sodium ions were occluded in E_1P. We simply never thought of it.

There is a great deal more information that has been obtained by the approaches we have been considering, as well as by approaches that I have not even mentioned.

There is good evidence, for example, that the release of occluded sodium ions to the exterior takes place in two stages, one ion being lost first and then the remaining two (Lee & Fortes, 1985; Yoda & Yoda, 1987*a*, *b*; Jørgensen, 1991; see also pp. 181–182 in the review by Glynn & Karlish, 1990). But I want to finish by trying to relate the schematic cycle that I have been talking about to the actual structure of the pump.

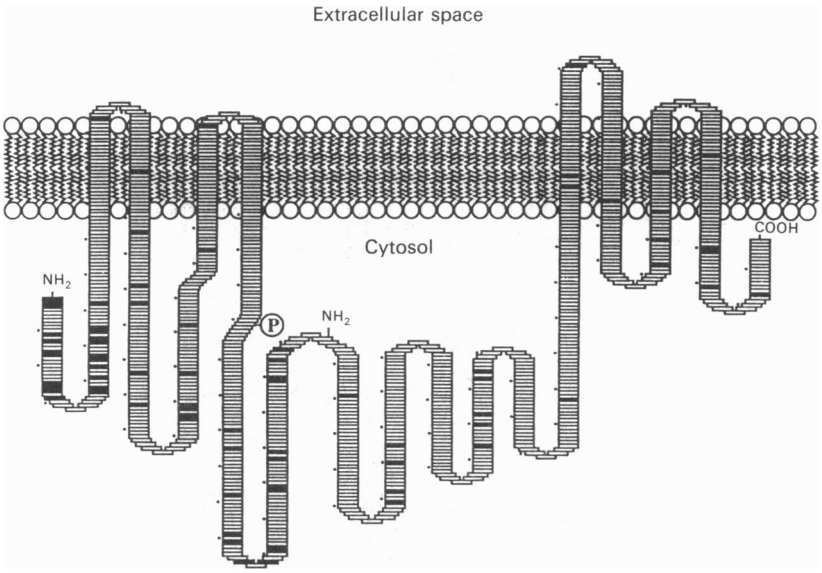

Fig. 12. Cartoon of the α-subunit of the chicken Na^+,K^+-ATPase, drawn with the transmembrane topology suggested by Shull, Schwartz & Lingrel (1985). Reproduced with permission from Fambrough *et al.* 1991. In the large cytoplasmic loop, an encircled 'P' indicates the phosphorylation site, and 'NH_2' a lysine required for ATP binding. Each amino acid is indicated by a rectangle (some of the rectangles are marked in black to indicate homologies but these are irrelevant to the present context).

Relation of mechanism to structure

Although the pump exists in the membrane as a diprotomer, $(\alpha\beta)_2$, and there is some evidence suggesting that the two halves interact (Scheiner-Bobis, Fahlbusch & Schoner, 1987; Plesner, 1987; Askari, 1988; Reynolds, 1988; Nørby & Jensen, 1991), it is possible, with suitable detergents, to get solubilized preparations consisting solely of single $\alpha\beta$-units, which seem to be able to catalyse all the reactions and partial reactions that can be catalysed by the intact pump – though, of course, it is impossible to test for transport in a solubilized preparation. The problem is, then, to relate the pump cycle to the structure of the single $\alpha\beta$-unit.

Figure 12 is a cartoon showing how the α-chain is supposed to be related to the lipid bilayer. The chain is thought to contain eight (or possibly ten) transmembrane helices, which in reality are bunched together, though precisely how is not known. Between the fourth and fifth transmembrane segments there is a large cytoplasmic loop, which accounts for nearly half of the total length of the chain. This loop includes both the aspartyl group that provides the phosphorylation site, and

various amino acids that make up the ATP-binding site. There is probably only one ATP-binding site, which changes affinity in the course of the cycle, since modification of a single lysine abolishes both high-affinity and low-affinity binding (Ellis-Davies & Kaplan, 1990; Kaplan 1991). The ouabain-binding site is on the outside of the

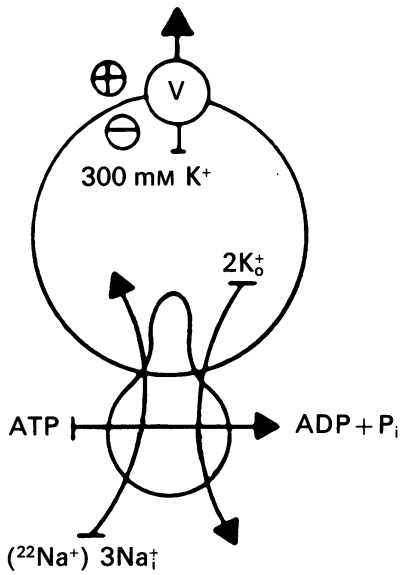

Fig. 13. One of the techniques developed for looking at the movements of electric charge associated with different steps in the pump cycle. A molecule of Na^+,K^+-ATPase is shown (not to scale) inserted into the lipid bilayer that forms the wall of an artificial lipid vesicle. The potential across the lipid bilayer is controlled by using suitable combinations of ion gradients and ionophores, K^+ and valinomycin, or Li^+ and the Li^+ ionophore AS701. Modified from Goldshlegger *et al.* 1987. The technique can be used to look at the effect of membrane potential on ion fluxes, or, if the pump is labelled with a suitable fluorescent probe, on the rates of conformational changes. (The subscripts 'o' and 'i' refer to the original orientation of the pump.)

membrane, and it is known that the affinity for ouabain is very sensitive to substitutions in the extracellular region of the α-chain separating the first two transmembrane helices (Lingrel *et al.* 1991). The conspicuous gap in our knowledge is the nature and position of the sites that bind and occlude cations, and the nature of the path taken by the cations through the pump.

Because phosphorylation leads to occlusion of sodium ions, one might guess that the transferred phospho group acts as a counter-ion to the occluded sodium ions; but that is certainly wrong. It turns out that, in the presence of oligomycin, unphosphorylated enzyme can also occlude three sodium ions (Esmann & Skou, 1985) (perhaps because both phosphorylation and the binding of oligomycin yield similar conformations, or because oligomycin blocks the escape of sodium ions that have already bound).

Some idea of the likely nature of sites occluding cations comes from studies of cation binding sites in other proteins and in ionophores (for discussion and references

see pp. 188–189 of Glynn & Karlish, 1990). Such direct information as we have about the occlusion sites in the sodium pump comes partly from electrical and partly from chemical studies.

In the last few years, a number of ingenious techniques have been developed for looking at the movement of electrical charge associated with the different steps in the pump cycle. You can study the effect of membrane potential on the current generated by the pump in intact cells (Gadsby, Kimura & Noma, 1985; Nakao & Gadsby, 1986; Bahinski, Nakao & Gadsby, 1988; De Weer, Gadsby & Rakowski, 1988; Läuger & Apell, 1988; Gadsby, Nakao & Bahinski, 1991; Läuger, 1991; Rakowski, 1991; Schwarz & Vasilets, 1991; Vasilets & Schwarz, 1991); you can label sodium pumps with fluorescent probes, incorporate them into the walls of artificial lipid vesicles (see Fig. 13) and then look at the effect on fluxes or on fluorescence of changing the membrane potential by suitable combinations of ion gradients and ionophores (Rephaeli, Richards & Karlish, 1986*a*, *b*; Goldshlegger, Karlish, Rephaeli & Stein, 1987); you can apply planar fragments of membranes rich in sodium pumps to a black lipid membrane (so that they are capacitatively coupled), and then look at the electrical transients following voltage jumps or the sudden release of ATP from caged ATP (see Fig. 14; Fendler, Grell, Haubs & Bamberg, 1985; Apell, Borlinghaus & Läuger, 1987; Borlinghaus, Apell & Läuger, 1987; Fendler, Grell & Bamberg, 1987; Eisenrauch, Grell & Bamberg, 1991); and you can use lipid soluble electrochromic dyes that sit in the lipid layer oriented parallel to the hydrocarbon chains and report on the local field strength (Grinvald, Hildesheim, Farber & Anglister, 1982; Klodos & Forbush, 1988; Bühler, Stürmer, Apell & Läuger, 1991; Stürmer, Bühler, Apell & Läuger, 1991).

There is general agreement that the phosphorylation step that leads to the occlusion of sodium ions

$$E_1.3Na^+ + ATP \rightarrow E_1P(3Na^+) + ADP$$

is insensitive to membrane potential, and so is the conformational change of the unphosphorylated enzyme that leads to the release of the occluded potassium ions

$$E_2(2K^+) \rightarrow E_1 + 2K^+.$$

On the other hand, the conformational change of the phosphoenzyme that leads to the release of occluded sodium ions

$$E_1P(3Na^+) \rightarrow E_2P + 3Na^+$$

is accelerated by making the cytoplasmic face of the pump positive, and is therefore believed to be accompanied by an outward movement of positive charge.

The fact that changing the membrane potential does not affect the rate of the reaction:

$$E_2(2K^+) \rightarrow E_1 + 2K^+,$$

but does affect the rate of the reaction:

$$E_1P(3Na^+) \rightarrow E_2P + 3Na^+,$$

is often used as an argument that the cation binding sites within the pump molecule supply two negative charges, so that when they are loaded with two potassium ions

the complex has no net charge, but when they are loaded with three sodium ions the complex has a net positive charge (Nakao & Gadsby, 1986; Goldshlegger *et al.* 1987; De Weer *et al.* 1988; Glynn & Karlish, 1990; Läuger, 1991). However, although this argument is obviously valid if, during transport, the cation-binding sites with the

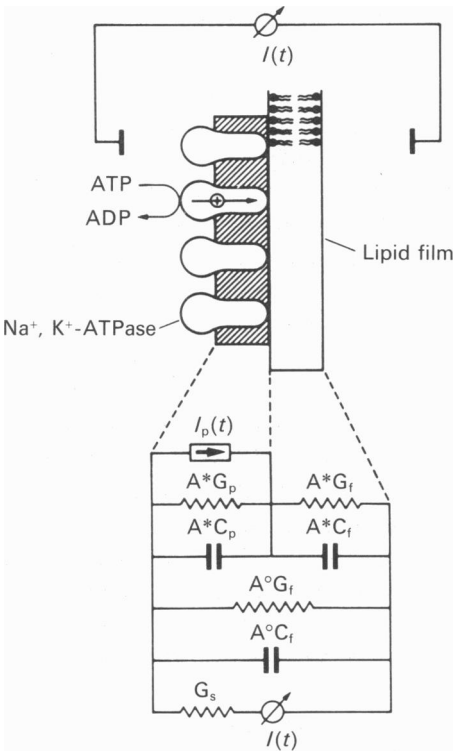

Fig. 14. Another of the techniques developed for looking at the movements of electric charge associated with different steps in the pump cycle. The diagram shows the equivalent circuit of the compound membrane system consisting of a black lipid bilayer with applied planar fragments of Na^+,K^+-ATPase-rich membrane. Release of ATP from caged ATP leads to a transient pump current $I_p(t)$. In the external measuring circuit a time-dependent current $I(t)$ is recorded. For further details see Borlinghaus *et al.* (1987), from whose paper the figure is reproduced with permission.

cations bound to them move a substantial distance with or against the electric field (see for example Fig. 11 of Läuger, 1991), the interpretation is less clear if the cation binding sites do not move in this way.

Occlusion seems to need a relatively small fraction of the entire pump molecule. Experiments using radiation inactivation showed that the target size of the mechanism that occludes rubidium by the direct route, i.e. the route not involving phosphorylation, was only about 40 kDa (Richards, Ellory & Glynn, 1981). Recently, Karlish and his colleagues (Karlish, Goldshleger & Stein, 1990; Karlish, Goldshleger, Tal & Stein, 1991) showed that occlusion was not prevented by a prolonged treatment with trypsin that removed nearly all of the cytoplasmic loop

(with the phosphorylation and ATP-binding sites) and fragmented the remaining parts of the chain, embedded in the lipid, so that the largest fragment had a molecular mass of only 19 kDa (see Fig. 15). In other experiments, digestion of the β-chain did not prevent occlusion (Capasso, Hoving, Tal, Goldshleger & Karlish (1992).

From knowledge of ion binding in ionophores or other proteins, one might expect the occluded cations to be largely dehydrated, and ligated with six to eight oxygen-containing groups, carboxyl, hydroxyl or phenolic. Many years ago, Robinson (1974), on the basis of studies with the hydrophobic carboxyl reagent dicyclohexylcarbodiimide (DCCD), suggested that carboxyls were included in the ion-binding sites. This approach has recently been taken further by Kaplan and his colleagues in the University of Pennsylvania (Arguello & Kaplan, 1991) and by Karlish and his colleagues in the Weizmann Institute (Shani-Sekler, Goldshleger, Tal & Karlish 1988; Goldshleger, Tal, Moorman, Stein & Karlish, 1992). A particularly striking finding of Shani-Sekler *et al.* (1988) is that, in certain conditions, DCCD inhibited the occlusion of sodium and the occlusion of rubidium *at identical rates, and with the same kinetics*; what is more, *each ion protected against inhibition of the occlusion of either ion.* These results are most simply explained by supposing that DCCD reacts with a small number of carboxyl residues in a non-aqueous cation-binding domain, and that *rubidium ions and sodium ions are occluded in the same sites, and are transported through the same channels at different stages of the enzyme cycle.* Very recently, using labelled DCCD and their heavily trypsinized enzyme, Karlish and his colleagues have shown that two particular carboxyl groups are likely to form part of, or at least be close to, the ion occlusion sites. One (Glu 953) is in the 19 kDa fragment; the other (?Glu 327) is in a smaller unidentified fragment, probably from a transmembrane segment on the amino-terminal side of the cytoplasmic loop (Goldshleger *et al.* 1992).

The picture that emerges from this work (see Fig. 16) is that there is a channel between some of the bunched transmembrane helices which, somewhere along its length, in the neighbourhood of two carboxyl groups (from different helices), can occlude either two potassium ions or three sodium ions, which are, of course, smaller than potassium when unhydrated. Access from the occlusion sites to the inner and outer faces of the pump is controlled by two gates, and the opening and closing of these gates, as well as the relative affinities of the sites for sodium and potassium, are controlled by conformational changes that depend on events in the cytoplasmic loop, phosphorylation, dephosphorylation, and the binding of ATP. It is possible that the coelenterate toxin, palytoxin, which converts sodium pumps reversibly into massive, ouabain-sensitive, univalent-cation leaks (Moore & Scheuer, 1971; Habermann & Chhatwal, 1982; Ishida, Takagi, Takahashi, Satake & Shibata, 1983; Ozaki, Nagase & Urakawa, 1985; Grell, Lewitzi & Uemura, 1988; Habermann, 1989; Tosteson, Halperin, Kishi & Tosteson, 1991), acts by causing both gates to be wide open at the same time.

You may wonder why, in Fig. 16, the occlusion sites are drawn so far from the outer surface of the pump. The answer is that there is some evidence that the binding sites are at the inner end of a narrow access channel or 'ion well' (see discussion by Läuger & Apell, 1988; Läuger, 1991). First, in several tissues (*Xenopus* oocytes,

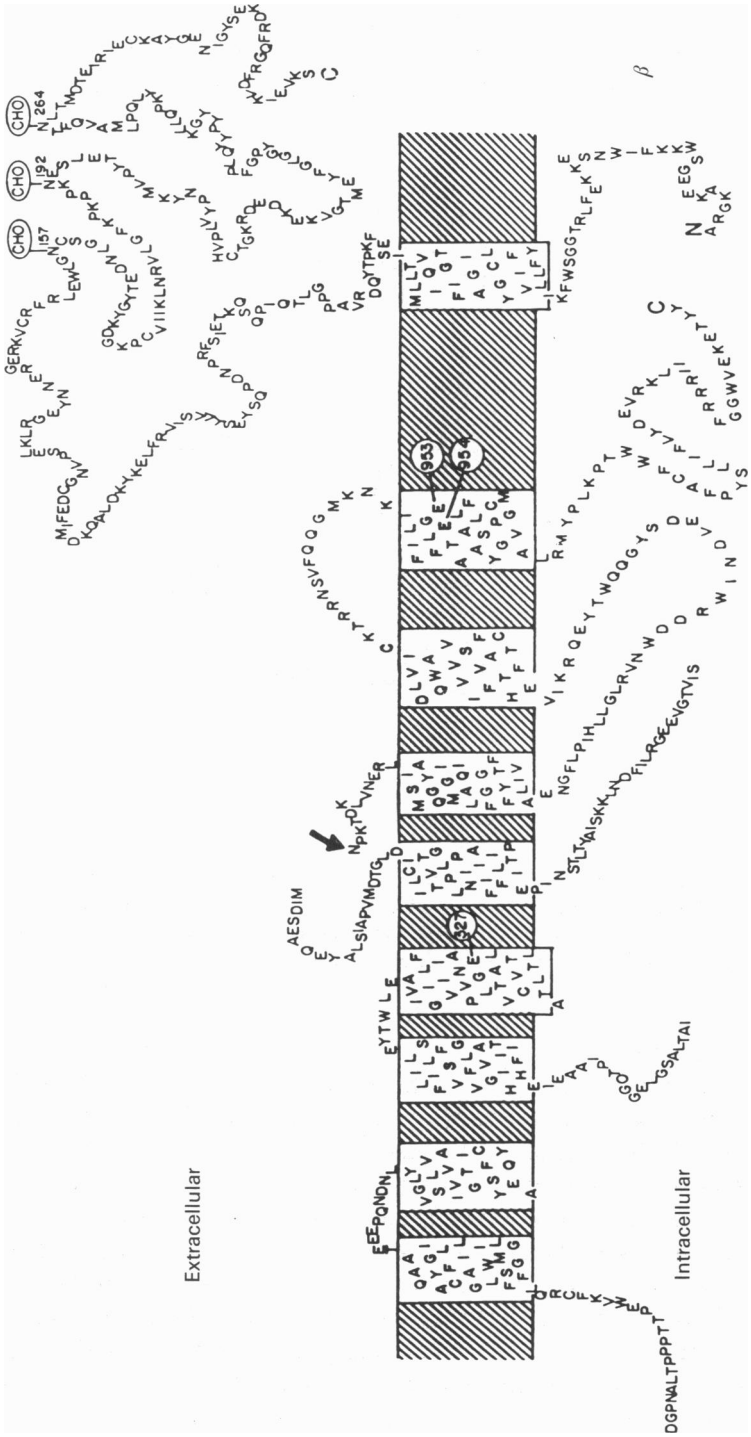

Fig. 15. Hypothetical arrangement of peptides in Na^+,K^+-ATPase that had been subjected to a prolonged treatment with trypsin. The treatment removed most of the cytoplasmic loop, and fragmented the remaining parts of the α-chain, but did not prevent occlusion of rubidium. The arrow indicates the N-terminal end of the largest (19 kDa) fragment. Glutamyl residues 953 and 954 in the 19 kDa fragment, and 327 in a smaller fragment, are indicated by encircled figures. For further details see text. Reproduced with permission from Karlish *et al.* 1991.

Torpedo electric organ, and squid giant axons, but not guinea-pig ventricular myocytes) the stimulation of sodium–potassium exchange by external potassium, or the stimulation of sodium–sodium exchange by external sodium, is voltage dependent. Making the outside more positive increases the apparent affinity for the

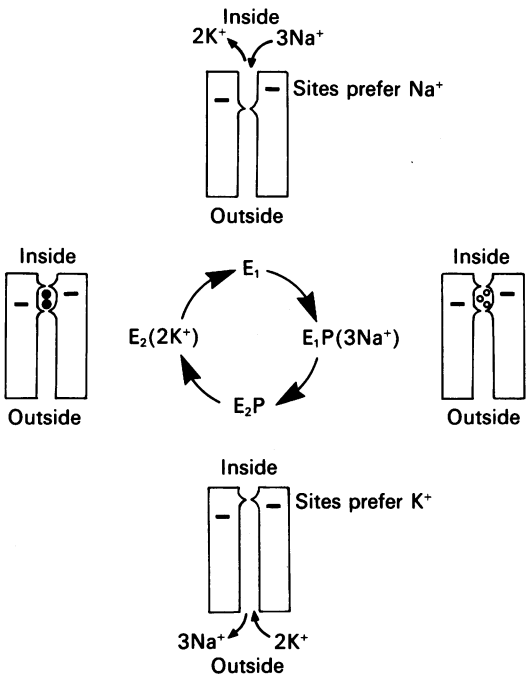

Fig. 16. Diagram showing how the four main stages in the pump cycle could be related to different states of a doubly-gated channel between some of the transmembrane helices. Although the gates are conveniently shown as localized constrictions, this is, of course, neither necessary nor likely. For further explanation see text.

activating cations, as if the electrical potential drives them down the well increasing their concentration at the bottom (Rakowski, 1991; Rakowski, Vasilets, LaTona & Schwarz, 1991; Schwarz & Vasilets, 1991; but cf. Nakao & Gadsby, 1989). Secondly, there are a number of observations with electrochromic dyes that can be readily interpreted on the basis of an outward facing ion well (for discussion and references see Läuger, 1991). And, of course, the notion of a narrow access channel to the outside fits in with the observations I referred to earlier, which suggested that the release of the two occluded potassium ions to the exterior was an ordered release through a narrow channel. Very recently, Vasilets & Schwarz (1992) have shown that the voltage dependence of stimulation of the pump by external potassium ions can be increased by a diacylglycerol analogue which stimulates protein kinase C, and reduced by cAMP which stimulates protein kinase A. The interpretation of these effects is uncertain, and is complicated by accompanying changes in V_{max} (maximum velocity) and in the number of pump molecules active, but a possible hypothesis is

that phosphorylation by the respective kinases increases or decreases the field strength in the ion well by changing its geometry (cf. Fig. 17). Variations in the geometry of the well might also account for the big differences in voltage sensitivity between Na^+,K^+-ATPases from different sources (Rakowski, 1991; Vasilets & Schwarz, 1992).

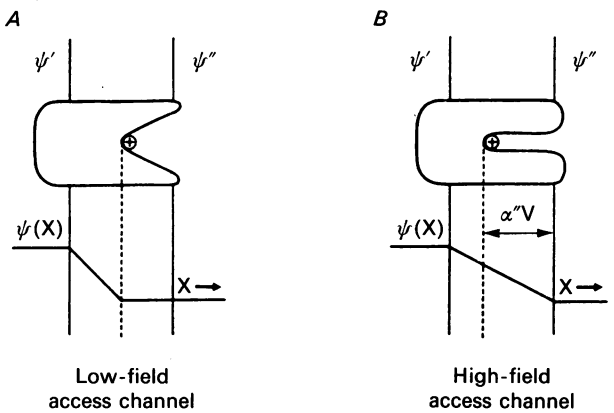

Fig. 17. Two limiting cases for the structure of the access channel connecting an ion-binding site to the external medium. *A*, low-field access channel, consisting of a wide opening into which water and ions can enter freely. *B*, high-field access channel (ion well) consisting of a narrow and ion-selective tube. Note the difference between the two cases in the fraction of the overall membrane potential that is seen as a potential difference between the inner and outer ends of the channel. Reproduced with permission from Läuger, 1991.

And that is about as far as the story goes. For further progress, what is needed is, first, a method for getting three-dimensional crystals of the pump for X-ray analysis. So far only two-dimensional crystals have been made, and though it is possible to do X-ray crystallography on stacks of these the resolution is much too poor to show atomic details (Maunsbach, Skriver & Hebert, 1991). Secondly, it would be a great help to have a better expression system, so that it became possible to study the effects of deliberate point mutations on the various partial reactions. At present, the yield is too small to study occlusion for example.

Reviewing work on the sodium pump five years ago, at a meeting of the Society of General Physiologists at Woods Hole, I started by pointing out that, though those who worked on the structure of the pump, and those who worked on function, were aware of each other's activities and happy that they should be going on, there wasn't much interaction. And I compared the situation to what the child psychologists call 'parallel play'. With hindsight, I think that that was a little harsh; but, in any event, there is no doubt that the 'parallel play' has now developed into a more interesting and more creative game. How that game turns out, we shall have to wait to see.

I am grateful to Dr S. J. D. Karlish for many helpful discussions.

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