THE SODIUM PUMP

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INTRODUCTION

The recent startling growth of the literature on the sodium pump may make a review timely, but it does not make the task of writing it easy. If the great mass of work that has been done had led to the general acceptance, even provisionally and even in outline, of a hypothesis accounting for the working of the pump, we could have described that hypothesis and then considered the evidence for it. Unfortunately, no such hypothesis exists and we must adopt a more piecemeal approach. In the first six sections of this review we discuss, in turn, various ways in which the investigation of the pump has been attempted. In the final seventh section we consider a number of mechanisms that have been suggested to account for the working of the pump in the light of conclusions reached in the earlier sections.

The limited space available means that if we wish to be critical we cannot also be comprehensive. For this reason, we do not discuss recent work on the role of lipids, and we consider only briefly problems connected with the "low K" "high K" dimorphism of certain red cells. We also exclude any consideration of Na transport by systems other than the widespread cardiac-glycoside-sensitive (Na + K)-ATPase.

References 6, 47, 84, 88, 168, 211–213, 241 are to earlier reviews that discuss the Na pump. Another invaluable source of references is the report of the Symposium on (Na + K)-ATPase arranged by the New York Academy of Sciences (222).

STUDIES ON ION MOVEMENTS

It is now clear that by suitable manipulation of the conditions it is possible to make the sodium pump operate in five different modes, namely: 1. Na–K exchange—the normal mode, 2. a reversed mode, 3. Na–Na exchange, 4. K–K exchange, and 5. Na efflux not accompanied by the uptake of Na or K (88). Modes 2 and 4 have been seen only in red cells, but they would be difficult to demonstrate in other cells; there is no reason to think that they cannot occur generally. The belief that all five kinds of behavior are brought about by the same system depends on their sensitivity to

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cardiac glycosides, on similarities in their responses to various physiological ligands, and on the fact that, with the exception of the reversed mode, which has not been tested, all are inhibited by an antiserum to a partially purified preparation of pig kidney (Na + K)-ATPase (89).

Na–K Exchange

Under physiological conditions, internal Na is exchanged for external K at the expense of energy derived from the hydrolysis of ATP at the inner surface of the cell membrane. Evidence for the coupling of the Na and K movements, for the location, external or internal, of the sites at which the ions activate the ATPase, and for the identity of pump and ATPase may be found in earlier reviews (6, 47, 84, 88, 168, 211–213, 241). In human red cells the hydrolysis of each molecule of ATP is accompanied by the outward movement of about three Na ions and the inward movement of about two K ions (76, 198, 240), and there is evidence for a similar ratio of Na ions pumped to ATP molecules hydrolyzed in nerve, muscle, and a variety of other tissues (18, 36, 55). In red cells, at any rate, the number of Na ions expelled for each molecule of ATP hydrolyzed is much the same whether pumping is downhill, on the level, or uphill (76, 198, 240).

The inequality in the active movements of Na and K in the human red cell, and a similar inequality in the squid axon (19), suggests that the pump may be "electrogenic"; that is to say, its action leads directly to a net movement of charge across the membrane. That this is indeed so is shown by the ouabain-sensitive hyperpolarization seen in a number of excitable tissues when the internal Na concentration is raised by repeated stimulation (tetanus), by injection of Na, or by storage in K-free solutions rich in Na (1, 45, 51, 122, 125, 150, 164, 224, 225). It might be suggested that the hyperpolarization comes about merely because the increased activity of the Na pump lowers the K concentration immediately adjacent to the cell membrane, but that possibility has been excluded in two ways: 1. In experiments on unmyelinated nerves, post-tetanic hyperpolarization was greatly reduced in K-free media, but promptly increased when K was added to the medium shortly after a tetanus. Addition of K could not lower the concentration at the cell surface (164). 2. In the crayfish stretch receptor, a single impulse is followed by a short after-potential caused by the persistence, for something of the order of 100 msec, of a high K permeability that displaces the membrane potential in the direction of the K equilibrium potential. If post-tetanic hyperpolarization were the result of K depletion in the region just outside the cell membrane, a single stimulus given during the period of post-tetanic hyperpolarization should cause an action potential with a greatly increased after-potential. This has not been found (150).

In voltage-clamped snail ganglion neurones, Thomas (224) has shown that the outward current generated by Na pumping after iontophoresis of Na into the cell is equivalent to ¼–⅓ of the total charge on the Na ions expelled. This is roughly what would be expected if three Na ions were exchanged for two K ions. On the basis of similar experiments, Kostyuk et al (125) recently claimed that the current generated by the pump may vary with the membrane potential, being very small when the membrane is hyperpolarized. A variable coupling ratio between Na and

K is also suggested by work on frog muscle (1, 45) and on perfused squid axon. In the axon, Mullins & Brinley (37, 147) found that the Na efflux and K influx varied in quite different ways as the internal Na concentration was changed, the Na/K ratio dropping from 3:1 to 1:1 as the internal Na concentration was decreased.

Reversal

By arranging the concentration gradients for Na and K to be steeper than normal, and the ratio $[ATP]/([ADP]\cdot[P_i])$ lower than normal, it is possible to make the red cell sodium pump run backwards and synthesize ATP at the expense of downhill movements of the cations (77, 90, 131, 132, 135). The rate of synthesis is roughly proportional to the external Na concentration (90), and synthesis is inhibited by external K with a $K_{0.5}$ of about 1.3 mM ($[Na]_o = 150$ mM). This is similar to the $K_{0.5}$ for stimulation of ouabain-sensitive K efflux by external K under similar conditions (91), and it is also similar to the $K_{0.5}$ for activation of the forward running of the pump by external K when normal cells are incubated in media containing 150 mM Na (81, 189). It is difficult to obtain accurate figures for the stoichiometry of the pump when it is running backwards, but the synthesis of each molecule of ATP seems to be associated with the loss of something like two or three K ions. The ratio of ATP molecules synthesized to Na ions entering is much lower, but this is almost certainly because most of the ouabain-sensitive Na entry is associated with Na–Na exchange (see below) rather than with reversal of the pump.

Because the free energy of hydrolysis of ATP is so high, one would not expect to observe reversal of the pump in situations in which energy was not available from ion gradients, and in fact the incorporation of P_i into ATP is never seen when fragmented membranes are incubated in solutions of fixed composition. Recently Post et al (163) showed that ^{32}P-ATP was synthesized when ADP + Na was added to membrane particles that had been incubated with $^{32}P_i$ in a (Na + K)-free medium. Here, of course, the energy is provided by the act of changing the concentration in the medium.

Na–Na Exchange

When red cells are incubated in K-free, high-Na media, they show a ouabain-sensitive exchange of internal and external Na ions (32, 73, 75, 184). A similar exchange has been seen in partially poisoned giant axons (19, 41, but cf 146) and probably also in frog muscle (104, 123, 209). Na–Na exchange, in red cells at any rate, is roughly one-for-one, but it shows a very marked asymmetry in the affinity for Na on the two sides of the membrane (69, 75, 184). Internally it is nearly saturated at 10–15 mM Na, but externally the Na activation curve is slightly S-shaped and flattens off only at levels well above 150 mM Na (69). K in the external solution inhibits Na–Na exchange and makes possible Na–K exchange; since the $K_{0.5}$ for the two effects is identical—about 1 mM—it seems likely that the same binding sites are involved (75, 184). Much more surprisingly, internal K has a facilitatory effect on Na–Na exchange (69). The explanation for this is unknown, but the effect is important because it explains the apparent inhibition of Na–Na exchange at high internal Na concentration (75) (the effect is really caused by lack of K) and because it points to a role

for K at the inner face of the pump. Stimulation of Na–K exchange by internal K has also been described recently (69, 124, 188). The intracellular K does not act merely by maintaining the level of ATP through its activating effect on pyruvate kinase (124).

Na–Na exchange is associated with the hydrolysis of little or no ATP (76), but it is not observed in resealed ghosts prepared with no ATP (75). Experiments on resealed ghosts containing a regenerating system to control the ATP and ADP levels have shown that the exchange varies linearly with ADP concentration up to 300 μM, the highest concentration tested, but is independent of ATP concentration over the range 300–1500 μM (86). Unfortunately, the presence of adenylate kinase makes it impossible to obtain ghosts containing sufficient ADP with low ATP levels, so an ATP requirement cannot be proved. In squid axon, too, a dependence of Na–Na exchange on ADP has been demonstrated (50); this probably accounts for the absence of the exchange in fresh axons and its appearance in axons that are partly poisoned.

The fact that ADP is not required for the normal working of the pump, but is required for Na–Na exchange, suggests that the entry of Na is associated directly or indirectly with the rephosphorylation of ADP by the phosphorylated pump protein. That would account, too, for the absence of ATP hydrolysis. The movement of Na must, however, also involve something beyond the transfer of phosphate, since oligomycin inhibits Na–Na exchange (76) but does not inhibit, and may stimulate, the Na-dependent ATP–ADP exchange that presumably reflects the transfer of phosphate to and from the enzyme (33, 34).

K–K Exchange

K–K exchange occurs in human red cells under physiological conditions, about one entering K ion in five being exchanged for an internal K rather than an internal Na (91). It does not depend on the simultaneous occurrence of Na–K exchange, however, and is best studied in resealed ghosts containing little Na (208). Like Na–Na exchange, it is roughly one-for-one, and is not associated with the hydrolysis of ATP (208). The apparent affinities for K on the two sides of the membrane are strikingly asymmetric, and the asymmetry is in the reverse direction to the asymmetry of the affinites for Na in Na–Na exchange (91, 208), suggesting perhaps that the same sites are involved. With 0.7 mM Na inside, the $K_{0.5}$ for K internally is about 10 mM (208), and with no Na outside the $K_{0.5}$ for K externally is about 0.25 mM (91), i.e. the same as for Na–K exchange in a Na-free medium. K–K exchange does not occur unless inorganic phosphate is present inside the cells (91), but ADP is not required (208). If a reversible transfer of phosphate is involved, it is therefore likely to be a transfer between the enzyme and water rather than between the enzyme and ADP. In this connection it is interesting that Dahms & Boyer (48) have shown that (Na + K)-ATPase preparations can catalyze an exchange of ^{18}O between water and P_i, which is presumably the result of the alternate formation and hydrolysis of a phosphorylated intermediate. This exchange is stimulated by K ions.

An interesting feature of the K–K exchange is that it does not occur in the absence of nucleotide (87, 208), though T. J. B. Simons (personal communication) has recently been able to show that the β,γ-imido or methylene analogs of ATP are satisfactory substitutes for ATP. This implies that the role of ATP is not to phosphorylate, though what it is remains obscure. ATP might be necessary to accelerate the release of K (see later, and reference 157).

Uncoupled Na Efflux

A ouabain-sensitive efflux of Na, not associated with the entry of Na or K, may be detected when red cells or resealed ghosts are incubated in choline or Mg media (32, 73, 74, 120, 136). It is not the result of an exchange of Na either for K ions leaking from the cell interior or for ammonium ions contaminating the solution; an exchange with Mg or choline has not been excluded, but is unlikely. A ouabain-sensitive efflux of Na into solutions lacking both Na and K has also been described in crab nerve (17) and frog muscle (31), but in these experiments the observed efflux may have been in exchange for K ions leaking from the cells.

Like Na–K exchange and Na–Na exchange, the uncoupled efflux in red cells appears to involve the combination of intracellular Na with sites of high affinity, the flux being saturated at 2 mM, which is the lowest concentration that has been tested (120, but cf 56) ($[K]_i$ was 1 mM). Uncoupled efflux is associated with the hydrolysis of ATP, about three Na ions leaving for each molecule of ATP hydrolyzed (unpublished experiments by Karlish & Glynn); this suggests that the flux may be related to the ATPase activity detected in broken cell preparations at low ATP concentrations when Na is present without K (33, 46, 117, 152, 157). Comparison of the uncoupled Na efflux from ghosts containing different nucleoside triphosphates shows that the ratio (Na–K exchange)/(uncoupled Na efflux) decreases with decreasing order of nucleotide-binding affinity (120), just like the ratio (Na + K)-ATPase/Na-ATPase (204, 220); with low enough levels of poorly bound nucleotides, K inhibits both efflux and hydrolysis. There are, however, two puzzling differences between the uncoupled Na efflux and the Na-ATPase activity: 1. The concentration of ATP required for half-maximal efflux is about 70 μM (120), much higher than the concentrations of ATP at which Na-ATPase activity is generally observed. This suggests that both high- and low-affinity ATP binding sites may be involved in the Na efflux and the Na-ATPase activity (see also 118, 142). 2. The uncoupled efflux is inhibited more or less completely by external Na at concentrations as low as 5 mM, yet Na-ATPase is observed with broken membrane preparations when the Na concentration must be high at both faces.

The Association of Ion Movements and Biochemical Events

The general picture that emerges from the study of different fluxes and the accompanying biochemical changes is that the outward and inward movements of Na are associated with the transfer of phosphate from ATP to the enzyme and from the phosphoenzyme to ADP, and that the inward and outward movements of K are associated with the transfer of phosphate from the phosphoenzyme to water and

from P_i to the phosphoenzyme. Whether the phosphoenzyme transfers its phosphate to ADP or to water depends on the composition of the external medium. If K is present the phosphoenzyme transfers its phosphate to water and K enters. If Na is present without K, the phosphoenzyme transfers its phosphate to ADP and Na enters. If neither is present in the external medium, the phosphate is transferred slowly to water, and the slow hydrolysis of ATP is accompanied by an "uncoupled" efflux of Na.

Ouabain-Insensitive Fluxes

It is generally assumed that fluxes observed in the presence of sufficient concentrations of ouabain are not connected with the sodium pump. For the efflux of Na and K, at least from the human red cell, this is probably true, since the antiserum to pig kidney ATPase inhibits the ouabain-sensitive fluxes completely and does not affect the ouabain-insensitive fluxes (89). The argument is not quite watertight, however, because the antiserum does not inhibit all ouabain-sensitive partial reactions of the (Na + K)-ATPase (11, 14, 89).

For influxes the antiserum test is not available, as influx measurements into resealed ghosts are difficult, and with intact cells the antibody cannot reach the inner surface of the cell membrane where it acts (114). When human red cells are incubated in high-Na media containing saturating concentrations of ouabain, the addition of K to the medium causes a small increase in the Na influx (75, 185); the $K_{0.5}$ for this effect is the same as the $K_{0.5}$ for inhibition of Na–Na exchange by external K. There is also a small saturable component of K influx even in the presence of ouabain (82), and a connection between this flux and the K-induced Na entry is likely since the saturable component of K influx is not seen in Na-free media (91). A very puzzling effect of cardiac glycoside has been described by Mullins (146), who found that the addition of strophanthidin to a squid axon perfused with a solution containing almost no ATP caused a tenfold increase in the (uphill) efflux of Na with no change in the Na influx. He suggests that some of the inward Na movement is carrier-mediated and can become coupled to Na efflux in the presence of strophanthidin.

ENZYME AND TRANSPORT KINETICS

A knowledge of the kinetic parameters describing the transport and enzymic functions of the Na pump is useful in restricting the number and type of conceivable reaction mechanisms. In considering ATPase activity, the pump can be regarded as an enzyme, with ATP as substrate and the various cations as cofactors. In transport experiments with intact cells the alkali-metal ions must be regarded as substrates or products—depending upon which side of the membrane they are on—and the movement across the membrane is formally equivalent to the conversion of substrate to product. Na and K activation with fragmented ATPase preparations is complicated by the fact that Na and K, having access to both faces of the membrane, compete at both internal Na- and external K-activation sites (211) and perhaps have other, unknown, effects. Although this "product inhibition" may be minimized by

working over particular ranges of Na and K, it can be eliminated only by the use of intact cells in which the composition of the internal and external media can be controlled.

Cation Activation of Transport and ATPase Activity

Information about the number of Na or K binding sites at each surface, their apparent affinities, and their possible interactions, is obtained from curves showing the activation of fluxes (or of ATPase activity) by Na or K at constant concentrations of the other species. When nerves (19), muscles (209, 210), or red cells (69, 189) are incubated in Na-rich media the curves showing activation of Na–K exchange by external K, or Na–Na exchange by external Na, are both sigmoid. In red cells, two sites of equal apparent affinity for K ($K_K = 0.4$–0.5 mM) are involved in Na–K exchange (189) and three sites of equal apparent affinity for Na ($K_{Na} = 31$ mM) are involved in Na–Na exchange (69). In nerve, however, three sites with different Na affinites seem to be necessary to account for Na activation of Na–Na exchange (19).

At low or zero external Na, the apparent affinity for K activation of Na–K exchange is greatly raised, and the curves are much less sigmoid (19, 74, 209). Although it has sometimes been supposed that Na affects the K affinity only by competition with K, several observations suggest that Na bound to a relatively high-affinity external site has a regulatory effect on the K affinity of the K activation sites. (*a*) In the absence of external Na, the activation of Na–K exchange is best fitted by a two-site model with *unequal* K affinities ($K_K = 5$ and 100 μM) (136). (*b*) Garrahan & Glynn (74) observed that low concentrations of external Na had a large effect on the K affinity of Na–K exchange, but much higher concentrations of Na were necessary to activate Na–Na exchange. (*c*) Independent evidence for the existence of an external high-affinity Na site is provided by the inhibition of the uncoupled Na efflux by low concentrations of external Na (see the section "Studies on Ion Movements"); it is tempting to assume that this is the site at which external Na acts to change the K affinity. Sachs (190) has shown that inhibition of Na–K exchange by external Na requires the binding of only a single Na ion.

The activation of Na–K and Na–Na exchange in red cells by *internal* Na involves multiple binding (69, 184), most probably at three sites of equal apparent affinity ($K_{Na} = 0.19$ mM). Internal K competes with Na (69, 124), probably with a binding affinity that is the same at all three sites ($K_K = 9$ mM). The stimulatory effect of K_i on the V_{max} of Na–K or Na–Na exchange (69, 124, 188, the section "Studies on Ion Movements") indicates the existence of an internal site for K separate from the Na activation sites. Mullins & Brinley (37, 147) made the puzzling observation that when giant axons in K-containing media are perfused with solutions of different Na concentrations, ATP-dependent Na efflux varies linearly with $[Na]_i$ up to 200 mM, whereas ATP-dependent K influx saturates when $[Na]_i$ is about 50 mM. There is much independent evidence from cation activation studies of fragmented membrane ATPase indicating that multiple Na and K binding sites are involved (139, 173, 211).

Although cation activation curves provide useful information, they do not in themselves indicate or exclude any particular kinetic mechanism. A more critical approach, suggested originally by Baker & Stone (20), is to see how the apparent affinities for the cation activation of each flux depend on the cation concentrations at the opposite surface of the membrane. In an important series of experiments, Hoffman & Tosteson (101) and Garay & Garrahan (69, 72) demonstrated that the apparent cation affinities for activation of fluxes from each surface of the membrane are independent of the nature and concentration of the alkali-metal ions at the opposite surface. The relation between the flux and the cation concentrations may, therefore, be described by the product of a constant and two factors that express the saturation of the cation activation sites at the interior and exterior surfaces. The simplest interpretation of these findings is to suppose (*a*) that the apparent affinities for the activating ions at the two surfaces of the membrane are simply related to their true binding affinities, and equal to them if competing ions are absent, and (*b*) that Na and K may bind rapidly, randomly, and independently, at internal and external sites, and that transport occurs only when the pump has bound both Na and K. This conclusion shows that the Na pump is not like the circulating carrier models discussed by Shaw (202) and Caldwell (40), in which Na and K binding sites are formed consecutively and do not exist at the same time. In such models the apparent affinities for ions at each surface of the membrane are complicated functions of the rate constants of the various reactions in the cycle. They are not, except by chance, equal to the true binding constants, and each depends strongly on the ion concentrations at the opposite surface. They would also be expected to vary with the mode of operation of the pump. The finding of Glynn & Lew (90) that in high-Na media K has the same $K_{0.5}$ in activating Na–K exchange and K–K exchange and in inhibiting pump reversal and Na–Na exchange, is also difficult to reconcile with such models.

LK and HK Cells

Before leaving the topic of cation activation, it seems pertinent to mention the curious features of the cation activation of fluxes, or of ATPase activity, in red cells from species showing low red cell K (LK) and high red cell K (HK) dimorphism. There is now evidence that the low rate of active transport in LK cells is the result of competitive inhibition of internal Na sites by K (85, 133, 188). It seems that the internal Na-activation sites in goat LK cells have an affinity for K at least 3 times higher than for Na, which compares with an affinity for K about 50 times lower than for Na in human red cells. It seems also that LK sheep cells have fewer pumps than HK cells, as judged by ouabain binding, although previous estimates (54) have recently been revised (110) and the low K content of LK cells is now attributed more to inhibition of pumping by internal K than to lack of pumps. The specific anti-L antibody, which dramatically increases the rate of pumping in LK cells (60), acts by increasing the relative affinity for Na over K at the internal sites (85, 133, 188), even though it is itself bound externally. Previous claims that the antibody increases the maximal number of ouabain binding sites in LK cells have not been substantiated, although there is an effect on the rate of ouabain binding (110, 187).

Nature of the Nucleotide Substrate of the ATPase. Nucleotide Binding

Although both ATP and Mg are necessary for ATPase activity, it is, perhaps surprisingly, still uncertain which of the species Mg-ATP, free-ATP, and free-Mg combine with the enzyme under physiological conditions. The likely assumption that Mg-ATP is the true substrate was supported by a kinetic test showing that the $K_{0.5}$ for free Mg at a particular total ATP concentration was equal to the $K_{0.5}$ for free ATP at the same total Mg concentration (98, 182). Excess free ATP appeared to inhibit by competing with Mg-ATP, while excess Mg inhibited, uncompetitively in one study (98) and noncompetitively in the other (182) (see ref. 43 for definition of terms). That Mg-ATP is the only substrate has been questioned by Hegyvary & Post (97), who demonstrated that ATP may bind to the enzyme in the total absence of Mg, and once bound may phosphorylate the protein upon subsequent addition of Mg and Na (160, see also 215).

Measurements of equilibrium binding of ATP to microsomal (Na + K)-ATPase preparations have shown that in the absence of Mg and alkali metal ions, ATP binds to a site of very high affinity [$K_d \simeq 0.29$ μM (97) or 0.12 μM (109, 154)] with a hint of binding to a second site of lower affinity (97). In the presence of K, the binding affinity was lowered ($K_d = 30$ μM) but the (calculated) number of molecules bound at infinite ATP concentration was doubled (97). K congeners also antagonized ATP binding with an order of effectiveness: K = Rb > Tl > NH_4 > Cs. Li had no effect; Na slightly enhanced binding when added alone, and counteracted the effect of K with a high affinity ($K_{Na} = 0.9$ mM) (97).

ATP binding was also reduced by competition from other nucleotides, and the following order of affinities was deduced: ATP > ADP > 2'deoxy ATP > β,γ-methylene ATP > CTP > ITP > UTP (97, 109). ATP did not combine with preparations pretreated with ouabain.

The dissociation constant of the high-affinity ATP binding site (0. 12–0.29 μM) is only a little lower than the concentration of ATP that is half maximal for phosphorylation of the enzyme (0.31–3.6 μM) (118, 162) or for Na-dependent ATPase activity (0.1–2 μM) (117, 152). That it is lower is probably due to the absence of Mg in the binding experiments, since the binding of ADP has recently been shown to be reduced by Mg (119). (The effect of Mg on ATP binding cannot be tested because some hydrolysis inevitably occurs if Mg is present.)

An important question to which a clear answer cannot yet be given is whether the K antagonism to ATP binding occurs from an external or an internal site. The relatively high K affinity for the effect [$K_K = 0.2$ mM (97)] suggests that an external K-binding site is involved, and a reduction in nucleotide binding caused by K at the outer surface of the membrane would explain the inhibition by external K of Na efflux from energy-depleted cells (75) and from ghosts containing UTP or low concentrations of CTP (120). It could also account for the effect of K on the substrate affinity of the (Na + K)-ATPase of microsomal preparations (170). On the other hand, Skou (215) has shown that in a medium containing a total (Na + K) concentration of 150 mM, the ratio of Na/K at which ATPase activity was half maximal fell as the total ATP level was raised. This indicates that ATP regulates

cation binding to the Na activation sites, increasing the affinity for Na relative to the affinity for K (see page 36). Surprisingly, the effect of ATP on the selectivity of the Na activation sites, was related to the total ATP concentration irrespective of the proportion that was bound to Mg. The order of effectiveness of K and its congeners in inhibiting ATP binding (see above) is different from their order of effectiveness in stimulating dephosphorylation of the phosphoenzyme ($Tl > Rb > K > Cs > NH_4 > Li$) (97), and this too may indicate the involvement of a site other than, or in addition to, the external K-activation site.

Order of Addition of Substrates and Release of Products

The cation transport kinetics and ATP binding data indicate that ATP, Na_i, and K_o do not have to combine with the enzyme in any fixed order. Several kinetic studies of the ATPase have been reported (98, 155, 170), but interpretation of the initial velocity and product inhibition patterns (cf 43, 66) is not always clear, partly because of the complexity of the enzyme and partly because of conflicting results.

A Lineweaver-Burk plot of the activation of ATPase by ATP at different K concentrations shows a parallel pattern (98, 170), indicating that K raises the V_{max} and K_m for ATP equally. In view of the clear effect of K on ATP binding, it now seems reasonable to accept Robinson's suggestion that the effect of K on V_{max} and K_m is allosteric (170). The surprising suggestion of Peter & Wolf (155) that K must bind before ATP implies that at infinite ATP levels maximal activity could be obtained with vanishingly small concentrations of K. Their view is also difficult to reconcile with the ATP binding experiments discussed above.

Lineweaver-Burk plots with both parallel and intersecting patterns have been reported for the activation of ATPase by ATP at different Na concentrations (98, 155). A random order of ATP and Na binding is certainly possible, since Na increases the initial rate of phosphorylation of enzyme by ATP without affecting the apparent affinity for ATP (235).

The product inhibition patterns of ADP (competitive) and phosphate (noncompetitive) were interpreted by Hexum et al (98) to mean that ADP is released from the enzyme after P_i, although this contradicts the evidence from studies of (*a*) the phosphorylated intermediate (see "Phosphorylated Intermediates"), (*b*) ADP-ATP exchange (see 25, 26, and "Phosphorylated Intermediates"), and (*c*) the metabolic requirements for the various transport modes (see "Studies on Ion Movements"). The pattern with ADP may be explained by supposing that under the conditions of the investigation, the ADP-sensitive form of the enzyme (E_1P) is not an intermediate, for reasons that we will discuss in connection with "half-of-the-sites-reactivity" (see page 45). If this suggestion is correct, ADP could not act as a product inhibitor, but would merely compete with ADP at the substrate site.

Noncompetitive inhibition by phosphate could be explained if the conversion of the form of the enzyme that combines with P_i (presumably E_2K—see "Phosphorylated Intermediates") to the form that combines with ATP (? E_1 or E_1Na) were not readily reversible under the conditions of the experiment. This interpretation is, however, difficult to reconcile with the view that in the presence of K, ATP reacts with E_2K, releasing K from an occluded form (157).

PHOSPHORYLATED INTERMEDIATES

It has always seemed likely that hydrolysis of ATP by the Na pump involves the transfer of phosphate to a group or groups in the enzyme before its ultimate transfer to water. An obvious way to investigate the existence of possible phosphorylated intermediates was to expose membrane fragments to γ^{32}P-ATP and then to look for incorporation of radioactivity into the membrane. It soon became apparent that in the presence of Na + Mg, ^{32}P was incorporated into the membrane lipoprotein and could be released as inorganic phosphate by K ions. For this release neither Na nor Mg was necessary. (See earlier reviews for references.)

The properties of the bound phosphate were those of an acyl phosphate, and there is now good evidence that it is a β-aspartyl phosphate with a serine or threonine on one side of it and a lysine on the other (49, 102, 158, 161). Since acyl phosphates have a high free energy of hydrolysis, it was reasonable to expect the phosphorylation of the protein by ATP to be reversible, but Fahn et al (63, 64) found that they could detect a Na-dependent ATP–ADP exchange only if they either used very low Mg concentrations (but cf 217) or pretreated their preparation with *N*-ethylmaleimide (NEM). It later turned out that a number of other conditions also promote Na-dependent ATP–ADP exchange, or a decrease in membrane bound ^{32}P on the addition of ADP. These include (*a*) substitution of Ca for Mg (226 , 227), (*b*) the presence of oligomycin (34, 64), and (*c*) the presence in equimolar amounts of 2,3-dimercaptopropanol and arsenite (BAL-arsenite) (203). To explain the need for low Mg or NEM etc, Albers and his colleagues (63, 64, 203) suggested that the phosphorylated compound first formed, which they called E_1P, was converted to a lower energy form, E_2P, by a reaction that needed Mg ions and that was blocked by the inhibitors.

The overall hydrolysis of ATP would therefore be represented by

$$E_1 \xrightarrow[(Mg,Na)]{(ATP)} E_1P \xrightarrow[(blocked\ by\ NEM)]{(Mg)} E_2P \xrightarrow{(K)} E_2 \longrightarrow E_1$$

The original justification for assuming the existence of two stable forms of the unphosphorylated enzyme (E_1 and E_2) was that BAL-arsenite caused a big drop in the $K_{0.5}$ for Na activation of the phosphorylation step (203). This implied an action on the unphosphorylated enzyme, and the economical assumption was that BAL-arsenite stabilized a form of the enzyme E_1 with a higher affinity for Na. The existence of more than one form of the dephosphoenzyme is also suggested by observations on the binding of ouabain and of other ligands (see "Inhibitor Studies").

Just as Na in low concentrations appears to shift the equilibrium $E_1 \rightleftharpoons E_2$ to the left, so in high concentrations it appears to shift the equilibrium $E_1P \rightleftharpoons E_2P$ to the left. Tobin et al (226) have recently shown that, in rat brain preparations, phosphorylation in the absence of inhibitors and with 1 mM Mg yields a product of which a fraction is sensitive to ADP; the size of this fraction is increased by high concentrations of Na (see also 159, 163).

Although E_1P and E_2P have different qualities—E_1P being sensitive to ADP but not to K, and E_2P being sensitive to K but not to ADP—treatment with proteolytic enzymes yields peptides with similar electrophoretic behavior (160, 206), suggesting that the difference is a matter of configuration rather than of basic chemical structure. The free energy of hydrolysis of E_1P has been estimated from measurements of the fraction of enzyme phosphorylated after incubation with different ATP/ADP mixtures in the presence of oligomycin (159). It is about 1 kcal/mole less than the free energy of hydrolysis of ATP under the conditions of the experiment.

Since the overall (Na + K)-ATPase reaction is reversible, phosphorylated intermediates should also be formed from inorganic phosphate under appropriate conditions, and it seems that they are. Incorporation of P_i was first detected in experiments in which membrane fragments that had been exposed to ouabain were incubated with P_i + Mg (9) or P_i + Mg + K (137). Na was inhibitory. The properties of the phosphoenzyme obtained in this way, including the results of electrophoresis of proteolytic fragments, suggested that, chemically, the phosphoenzyme was similar to that formed from ATP + Mg in the presence of Na (160, 206). Since acyl phosphates do not normally exchange their phosphate groups with inorganic phosphate, it is likely that energy provided by the combination of the protein with ouabain stabilizes the acyl phosphate. The acyl phosphate must also be able to exist in a fairly low-energy form (? E_2P) without ouabain, however, since some phosphorylation is found even in the absence of ouabain (159, 163).

There are interesting differences in the yields and properties of the phosphoenzyme formed by the incorporation of P_i under different conditions, although all seem to be chemically similar (163). The highest yield was obtained with ouabain and was equal to the maximum yield obtained by incubation with ATP + Na + Mg (9). Incorporation of P_i in the presence of K or its congeners (excluding Na) gave small yields, and the product was sensitive to K but not to ADP, i.e. it was not distinguishable from the phosphoenzyme (E_2P) formed in the presence of ATP + Na + Mg (159, 163). When no alkali metal ions were present, yields were larger and the product was insensitive both to K and to ADP. The addition of Na in high concentration, together with the Mg chelator (1,2-cyclohexylenedinitrilo) tetraacetic acid (CDTA) to prevent further incorporation of P_i, now had the remarkable effect of making the phosphoenzyme sensitive to both K and ADP, as though the Na were converting the "insensitive" form into a mixture of E_2P and E_1P (159, 163). The effect occurred slowly over many seconds, and very high concentrations of Na were required, reminiscent of those required extracellularly for Na–Na exchange or pump reversal in intact red cells. The membrane fragments used in these experiments were vesicular, but it is not known whether the existence of a Na concentration gradient across the membrane played any part.

Phosphoenzyme similar to that formed from ATP can also be formed from other nucleoside triphosphates and from *p*-nitrophenylphosphate (*p*NPP) and acetyl phosphate (24, 35, 52, 106, 107, 175, 195, 204, 205, 220). Mg is always essential. Na is essential for phosphorylation by nucleoside triphosphates, and was found to increase phosphorylation by acetyl phosphate unless K was also present (35). Phos-

phorylation by *p*NPP and by acetyl phosphate will be discussed further in "Phosphatase Activity of the Sodium Pump."

The Role of Phosphorylated Intermediates in the Normal Working of the Pump

Since, in the presence of ATP, Na causes phosphorylation of a membrane protein and K causes the transfer of the phosphate group from the protein to water, it is tempting to suppose that the occurrence of these events, in sequence, accounts for the hydrolysis of ATP, and that the chemical changes catalyzed by each kind of ion somehow result in the transfer of ions of that kind across the membrane. A long time ago, however, Skou (211, 220) pointed out that the formation of phosphoenzyme might be a side reaction that occurred only in the absence of K, and that under certain conditions (low temperature, use of ITP) K decreased the level of phosphoenzyme at the same time as it decreased, or had no effect on, the overall rate of hydrolysis. K ions have been shown to inhibit the ouabain-sensitive Na-dependent hydrolysis of UTP and CTP (24, 204, 205), and there have now been several reports that with low enough concentrations of ATP the ouabain-sensitive Na-dependent ATPase activity is inhibited by K ions at concentrations that would be stimulatory at normal levels of substrate (34, 46, 117, 152, 157). These observations imply that if the phosphorylated form of the enzyme is indeed an intermediate in the normal working of the pump, K ions must interfere with its formation as well as its breakdown. Competition with Na ions at the Na-loading sites would be negligible at the low concentration of K ions used in these experiments, and some other inhibitory action must be sought.

What this action may be is suggested by a series of ingenious experiments by Post et al (157). They first exposed membrane preparations to Mg + γ^{32}P-ATP + Na at 0°, and by trial and error found concentrations of Li and Rb, which, when added with CDTA to stop further phosphorylation, gave equal rates of breakdown of the phosphoenzyme. They then showed that at these concentrations in the absence of CDTA, the two ions gave quite different rates of hydrolysis and quite different steady state levels of phosphoenzyme. With Rb, the more strongly bound ion, hydrolysis was slower and the steady state of level of phosphoenzyme was lower. This experiment showed that Rb inhibited synthesis more than Li, but did not show at which step in the sequence the inhibition occurred. A further experiment showed that if Rb and Na were present in the medium initially, and phosphorylation was started by the addition of Mg + γ^{32}P-ATP, the level of phosphoenzyme rose sharply and then fell to a steady level; at all times the level was the same as that observed at equal times after the addition of Rb to enzyme fully phosphorylated by Mg + γ^{32}P-ATP + Na already in the medium. This shows that Rb did not inhibit phosphorylation until after the enzyme had been phosphorylated the first time, which is what would be expected if dissociation of Rb from recently dephosphorylated enzyme were rate-limiting.

Further evidence that the release of cation from dephosphoenzyme is slow came from experiments in which the rate of rephosphorylation was studied. In the presence of 200 mM Na (which competes effectively with the low concentrations of Rb

or Li used in these experiments) and oligomycin (which blocks the conversion of E_1P to E_2P) any available *free* dehosphoenzyme should be promptly rephosphorylated and trapped as E_1P. The rate of increase in phosphoenzyme when 200 mM Na + oligomycin was added to a preparation of enzyme exposed to Mg + γ^{32}P-ATP + Na in the presence of Li or Rb, gave a measure of the rate of release of Rb or Li from the newly dephosphorylated enzyme. Rb was released more slowly than Li. Furthermore, the difference in rates was unchanged even if the addition of Na was combined with an addition of Li to the Rb samples, or Rb to the Li samples, so that during the rephosphorylation the composition of the solutions was identical. This provides clear evidence that the dephosphoenzyme can "remember" which ion promoted dephosphorylation; much the simplest way to explain the "memory" is to suppose that the ion is still bound to the enzyme. Extra Mg + γ^{32}P-ATP, added with the Na, was found to accelerate rephosphorylation, presumably by accelerating the release of the bound ion; it is lack of this accelerating effect that is thought to account for the inhibitory effect of K on ATPase activity at very low concentrations of ATP.

A quite separate argument for the existence of a rate-limiting step subsequent to dephosphorylation can be based on the findings of Mårdh & Zetterqvist (140), who used a rapid-mixing technique to study rates of phosphorylation. At 21° and with 5 μM ATP, 3 mM Mg, and 120 mM Na, they found that in the absence of K the rate of phosphorylation was about nine times the rate of overall hydrolysis. If 20 mM K was also present in the medium the rate of hydrolysis was unchanged and the steady state level of phosphoenzyme was reduced by a factor of about 5. Yet the initial rate of phosphorylation, when ATP was first added, was at least a quarter of the rate in the absence of K and could not have been rate-limiting. By exclusion, the rate-limiting step must have been subsequent to dephosphorylation. (See references 141 and 159 for further evidence supporting this interpretation of Mårdh & Zetterqvist's results.)

Positive evidence that the phosphoenzyme is an intermediate in the normal working of the pump comes from (*a*) experiments looking at rates of hydrolysis and levels of phosphorylation in the steady state, under various conditions, and (*b*) experiments using rapid-mixing techniques to measure rates of phosphorylation and dephosphorylation.

STEADY STATE EXPERIMENTS At temperatures and ATP levels not too far from physiological, steady state experiments (117, 153) show that under conditions in which phosphorylation should be rate-limiting—Na and K high and fixed, ATP variable or ATP and K high and fixed, Na variable—the rate of hydrolysis is, as might be expected, proportional to the level of phosphoenzyme. Under conditions in which dephosphorylation should be rate-limiting—ATP and Na high and fixed, K variable—the rate of hydrolysis is proportional to the level of dephosphoenzyme. This too is to be expected, though less obviously. In the steady state, the level of phosphoenzyme will be that at which its rate of formation is equal to its rate of breakdown, and both rates will be equal to the overall rate of hydrolysis. But, with

ATP and Na levels both high, the rate of formation will be proportional to the level of dephosphoenzyme, and so the rate of hydrolysis will be also. (Release of K from the dephosphoenzyme will not be rate-limiting because of the high ATP concentration.)

RAPID-MIXING TECHNIQUES Experiments with rapid-mixing techniques have been reported by Tonomura and his colleagues (68, 118, 235) and by Mårdh & Zetterqvist (140–143), and it is convenient to consider the work by the two groups in turn.

One of the most striking pieces of evidence demonstrating that hydrolysis proceeds via the phosphoenzyme is provided by an experiment in which Kanazawa et al (118) measured the time course of EP formation and P_i liberation for one second immediately after the mixing of enzyme with 1 μM ATP in a medium containing 3 mM Mg, 140 mM Na, and 0.6 mM K. The concentration of EP increased without any lag, and approached a steady value within 1 sec. The rate of P_i liberation showed a pronounced lag and did not reach a steady value until the concentration of EP had stopped rising. At any instant, the observed rate of liberation of P_i was close to that calculated from the momentary level of EP, assuming a turnover calculated from the ratio v/EP found during the steady state.

Experiments by Kanazawa et al (118) and by Fukushima & Tonomura (68) have also given information about the steps leading to the formation and breakdown of the phosphoenzyme: 1. The rate constant for the breakdown of phosphoenzyme can be estimated either from the ratio v/EP in the steady state or by measuring the rate constant (k_D) describing the fall of E^{32}P when rephosphorylation by γ^{32}P-ATP is stopped by the addition of EDTA or an excess of unlabelled ATP. In the absence of K the two methods agree well, but with K present v/Ep exceeds k_D and is about double k_D when K is 0.6 mM. 2. In the absence of K, the rate of loss of E^{32}P when resynthesis is stopped is roughly equal to the rate of liberation of $^{32}P_i$. In the presence of K, the liberation of $^{32}P_i$ is faster than the loss of E^{32}P, and with 0.6 mM K the amount of $^{32}P_i$ formed is about double the amount of the E^{32}P lost. 3. When K + EDTA was added to enzyme phosphorylated by γ^{32}P-ATP + Mg + Na, there was an initial rapid fall in EP followed by a slower exponential loss. The rapid fall in EP was accompanied by the formation of an equivalent amount of ATP. 4. With NEM-treated enzymes, the effects of adding ADP at different times after phosphorylation had been started with γ^{32}P-ATP showed that the newly formed phosphoenzyme would not lose ^{32}P to ADP, but the greater part of the phosphoenzyme became sensitive to ADP within a second or two.

The authors suggest that newly formed phosphoenzyme is resistant to added ADP because ADP is still bound to it. The results described in points 1–3 can be explained by supposing (*a*) that there is an equilibrium between a form of the enzyme with tightly bound ATP (E_2S) and the form of the phosphoenzyme with bound ADP (EP•ADP), (*b*) that this equilibrium is normally heavily in favor of EP•ADP, but is displaced toward E_2S by K, 0.6 mM K giving an equilibrium constant near unity, (*c*) that the interconversion of E_2S and EP•ADP does not require Mg,

(*d*) that the formation of E_2S from E_1S,[1] the first enzyme–substrate complex to be formed, is not readily reversible and is blocked by EDTA, and (*e*) that the tightly bound ATP is released from the enzyme during extraction with trichloracetic acid.

If these assumptions are correct, it is clear that when phosphorylation by γ^{32}P-ATP is stopped, either by EDTA or by an excess of unlabelled ATP, the formation of EP•ADP from E_2S will slow the disappearance of bound ^{32}P, therefore reducing k_D, and the breakdown of that EP•ADP will contribute the extra P_i. By increasing the quantity of E_2S, K ions will increase these effects. Some support for this role for K comes from the stimulation of Na-dependent ATP–ADP exchange under certain conditions (26).

Because the hypothetical EP•ADP would presumably not react with added ADP, Tonomura and his colleagues identified it with the classical E_2P; and since the experiments of Fukushima & Tonomura (68) showed that the newly formed phosphoenzyme is insensitive to ADP and becomes sensitive with time, they have to assume that E_2P is the precursor of the ADP-sensitive form (E_1P). This hypothesis is difficult to reconcile with an experiment of Post et al (160). When membrane fragments were incubated with Mg and γ^{32}P-ATP in the absence of Na, the γ^{32}P-ATP became bound to the enzyme sites but no phosphorylation occurred. When a "chasing" solution containing Na, Mg, and unlabelled ATP was then added, there was a rapid rise in membrane-bound ^{32}P followed by a slow fall as the phosphoenzyme broke down. The crucial point was that when ADP was present in the chasing solution, the initial rise in membrane-bound ^{32}P was slower, but the rate of fall was the same. This strongly suggests that the ADP-sensitive form of phosphoenzyme (E_1P) precedes the ADP-insensitve form. [It is just possible that ADP acted not by combining with E_1P but by displacing bound ATP by an allosteric effect from another site. Since ADP was more effective than unlabelled ATP this is unlikely (160).]

To accommodate the findings of Post et al (160) together with those of Kanazawa et al (118) and of Fukushima & Tonomura (68) it seems to be necessary to suppose (*a*) that the phosphoenzyme first formed is resistant to ADP because it has ADP still bound to it, (*b*) that the ADP is then lost, giving an ADP-sensitive form, E_1 P, and (*c*) that this is transformed to an ADP-sensitive, K-sensitive form, E_2P.

Quite a different explanation for the results of Kanazawa et al (118) has been suggested by Skou (217). He supposed that when both internal and external sites of the pump carry Na, hydrolysis of ATP is via a phosphorylated intermediate, EP, but that when the internal site carries Na and the external site carries K, hydrolysis occurs by a parallel pathway without any phosphorylated intermediate. If ATP bound to the enzyme can continue to be hydrolyzed after the addition of EDTA, it is possible to explain the discrepancy between the liberation of P_i and the disappearance of EP, and also the discrepancy between v/EP and k_D.

[1]In describing the results of Tonomura et al (68, 118) we have used their nomenclature, but the forms of the enzyme-substrate complex E_1S and E_2S are not related to the E_1 and E_2 forms of the enzyme first postulated by Siegel & Albers (203).

Mårdh & Zetterqvist (140, 142, 143) used a more elaborate rapid-mixing technique, which allowed them to reduce the total reaction time (including time for stopping the reaction with TCA) to less than 3 msec. They were therefore able to follow phosphorylation with much higher levels of ATP and at 21°.

With 100 μM γ^{32}-ATP, Na-dependent phosphorylation was apparently first order, with a rate constant of 11,000 min^{-1}. Addition of KCl to a final concentration of 10 mM caused dephosphorylation with a first-order rate constant of at least 14,000 min^{-1} (independent of ATP concentration in the range 5–100 μM). Both rates were, therefore, far more than adequate to account for the overall rate of hydrolysis in the steady state. The rapid disappearance of phosphoenzyme on the addition of K was accompanied by an equally rapid liberation of P_i. Knowing the amount of phosphoenzyme present in the steady state, they calculated the expected hydrolysis rate if all of the phosphoenzyme broke down with a rate constant of 14,000 min^{-1}. The answer turned out to be ten times the observed rate of hydrolysis; this discrepancy suggests that in the steady state 90% of the phosphoenzyme must be in a more stable form.

When 10 mM KCl and excess unlabelled ATP were added simultaneously to enzyme previously phosphorylated by 100 μM γ^{32}P-ATP + Na + Mg, there were two phases of dephosphorylation: an immediate fast phase with a rate constant of at least 14,000 min^{-1}, and a much slower phase. If the fast phase represented the breakdown of E^2P and the slow phase the breakdown of E_1P, then there must have been about three times as much E_2P as E_1P. If KCl was present before the addition of γ^{32}P-ATP, only the slow phase was seen, suggesting that under steady state conditions with 10 mM K and 120 mM Na nearly all the phosphoenzyme is E_1P. The rate constant for the slow phase in this experiment was about 4600 min^{-1}, and the product of this and the amount of phosphoenzyme present agreed reasonably well with the rate of overall (Na + K)-stimulated hydrolysis at saturating levels of ATP.

The addition of 10 mM KCl + CDTA to phosphoenzyme also led to two phases of dephosphorylation, but the slow phase was slower than that seen after the addition of KCl with unlabelled ATP. The difference suggests that Mg is required for the conversion of E_1P to E_2P.

Mårdh & Zetterqvist (142) also drew attention to the existence of bound ATP in their acid-washed precipitates. Acid-resistant ATP binding was first described by Shamoo & Brodsky (38, 201), who believed that the binding involved the (Na + K)-ATPase since it was reduced by ouabain, provided that both Na and K were present. Because, in the absence of ouabain, Na and K did not alter the level of acid resistant binding, any such binding should not affect measurements of Na-dependent ^{32}P incorporation.

Taken together, the results discussed in this section provide strong evidence for an intermediary role of phosphoenzyme in the normal working of the pump, and, more specifically, for the classical Albers scheme. So far, nearly all forms of the phosphoenzyme that have been studied seem to be similar chemically, though Robinson (176) reported that phosphorylation by ^{32}P-*p*NPP at pH 5 led to K-dependent incorporation of ^{32}P into a serine phosphate. In any event, the possibility

that chemically different forms, perhaps with acid-labile phosphate groups, also exist and play a part in the pump cycle cannot be excluded (39, 180).

PHOSPHATASE ACTIVITY OF THE SODIUM PUMP

Identity of (Na + K)-ATPase and K-Dependent Phosphatase

The suggestion of Judah et al (2, 116) that (Na + K)-ATPase is responsible for the K-dependent ouabain-sensitive hydrolysis of *p*NPP is now generally accepted. The ATPase and *p*NPPase activities are similarly distributed (2, 15, 67, 238, 244); both activities increase in parallel when membrane fractions are purified (115, 148), and both decrease in parallel when membrane preparations are subjected to heat or to treatment with trypsin or a wide variety of inhibitors (16, 67, 106, 148, 243). Resemblances in the responses of the two activities to various ligands (see below), and the "sidedness" of the action of these ligands, provide further evidence of identity (78, 80, 166).

The results of radiation inactivation show that the target area for inhibition of *p*NPPase activity is only about half that for inhibition of ATPase activity, suggesting that only a part of the (Na + K)-ATPase system is involved in the hydrolysis of "phosphatase substrates" (121). That makes it easier to understand how dimethyl sulfoxide (DMSO) and phlorizin can inhibit (Na + K)-ATPase activity at the same time as they stimulate *p*NPPase activity (7, 145, 177, 180; see also 44, 67, 172, 239), and may also help to account for the different effects of "anti-Na pump" antisera on the two activities (11, 14, 89).

Substrate Specificity

Although loosely called phosphatase activity, the K-dependent hydrolytic activity of (Na + K)-ATPase preparations is in fact limited to substrates with phosphate groups with a moderate or high free energy of hydrolysis. Acetyl phosphate (AcP), carbamyl phosphate, *p*NPP, and umbelliferone phosphate are hydrolyzed, whereas phenyl phosphate, glucose phosphate, α and β glycerophosphates, and phosphoryl serine are not (67, 156, 243). Nucleoside triphosphates are hydrolyzed only if Na ions are present.

Effects of Physiological Ligands on K-Dependent Phosphatase Activity

The interaction of different ligands is extremely complicated, and, because this was not always appreciated, some of the early work can mislead by suggesting effects on V_{max} that are really effects on the $K_{0.5}$ for some other ligand present in less than saturating concentrations. A second source of confusion is the inhibitory effect of high ionic strength (44). A third, in work on red cells, is that there is evidence that the K-independent hydrolysis of *p*NPP in red cell membranes may not be the action of a separate enzyme, so that estimates of K-dependent activity can be misleading (78). Fortunately, most of the work on tissues other than red cells has been done

with preparations in which the K-dependent phosphatase activity accounts for most of the activity.

EFFECTS OF K AND Mg At constant substrate concentrations and Mg concentrations, K activates in sigmoid fashion (but cf 78) with a $K_{0.5}$ of a few millimolar, i.e. much greater than the $K_{0.5}$ for activation of the (Na + K)-ATPase at zero external Na concentrations (8, 78, 216). The activating effect is partly on the V_{max} and partly the result of an increase in the apparent affinity for substrate (8, 78, but cf 216). Even where K ions appear to increase the affinity for *p*NPP, an increase in *p*NPP concentration does not affect the affinity for K ions (78), so at least one of the apparent affinities cannot be an equilibrium binding constant. Inhibition by high K concentrations is probably at least partly an ionic strength effect, since it was not seen in experiments in which choline was used to maintain constant ionic strength (216).

Although the K-dependent dephosphorylation step in (Na + K)-ATPase activity can occur in the absence of Mg, Mg is necessary for phosphatase activity. Ca cannot substitute for Mg (16), and appears to compete with it (148). The optimal level of Mg is not affected by the concentration of substrate (78), but there is a complex relation between the levels of Mg and K required for maximal activity, the optimal level of each being higher at high concentrations of the other (16, 67, 148, 216).

EFFECT OF Na IN THE ABSENCE OF ATP Without K, Na ions promote a minimal phosphatase activity (8, 107, 174) with $K_{0.5}$ for Na of about 3.5 mM (8). At high K concentrations Na inhibits, but only if the Na concentration is also high (67, 148, 216). Of much greater interest is the discovery by Nagai et al (148) that at low K concentrations Na stimulates the hydrolysis of *p*NPP. This has been confirmed by more detailed recent studies (8, 216), which, however, differ from each other in a puzzling way. With K fixed at 2.5 mM, Albers & Koval (8) found that increasing Na gave a large increase in the apparent affinity for substrate, whereas Skou (216) found that the apparent affinity for substrate was little affected. Albers & Koval claimed that with high enough Na concentrations the total enzyme displayed "high" affinity for K, which was not true of Skou's preparation. It is not known if the cause of these discrepancies is related to the use of a DMSO-treated preparation by Albers & Koval.

EFFECT OF ATP There is general agreement that, in the absence of Na, ATP inhibits the hydrolysis of phosphatase substrates (67, 79, 107, 156), but there is disagreement about whether this inhibition is competitive (79, 107) or noncompetitive (67). In any event, as Israel & Titus (107) point out, competitive inhibition does not imply that the ATP and substrate necessarily combine at the same site. ATP might act by keeping the enzyme in a configuration in which it did not combine with *p*NPP. As well as decreasing the apparent affinity for substrate, ATP decreases the apparent affinity for K (79, 148). Nucleoside triphosphates other than ATP are also inhibitory, but only at much higher concentrations (107, 149, 216).

In the presence of Na the effects of nucleoside triphosphates are quite different. Except at high K concentrations, they cause a great increase in the rate of hydrolysis of phosphatase substrates, and the hydrolysis becomes much more like the hydrolysis of ATP in its sensitivity to K and to ouabain (79, 149, 165, 171, 174, 244). The stimulatory effect is prevented by oligomycin (12, 79, 107, 174) and *N*-ethylmaleimide (NEM) (174), although these substances do not inhibit K-dependent phosphatase activity in the absence of both Na and ATP.

Skou (216) recently made a careful study of the effects of 0.1 mM ATP on the rate of *p*NPPase hydrolysis at K concentrations between 0 and 100 mM, using either Na or choline or a mixture of both to keep the ionic strength constant. In the absence of ATP, and with a high Na concentration, the K activation curve showed two steps: a small step that seemed to represent a process with a high affinity for K, and a large step that seemed to represent a process with a low affinity for K. The main effect of 0.1 mM ATP was to cause a very large increase in the height of the first step without altering the apparent affinity for K of the process responsible for that step.

Skou supposed that the activity at low K/Na ratios represents a sluggish activity of the enzyme when the outside (high K-affinity) sites are loaded with K and the inside (low K-affinity) sites are loaded with Na, and that the activity at high K/Na ratios represents a more vigorous activity of the enzyme when both inside and outside sites are loaded with K. The effect of ATP is, he supposed, to increase the activity of the pump in the K_o/Na_i form, so that it becomes comparable to the activity of the K_o/K_i form. A secondary effect of ATP observed by Skou is that in the presence of saturating levels of K the pump was made more sensitive to inhibition by low concentrations of Na. This fits the hypothesis well, since experiments on protection agains NEM inhibition (see "Inhibitor Studies") suggest that ATP increases the preference of the inside sites for Na.

The main weakness of Skou's hypothesis is that it demands a stimulatory effect of internal K and an inhibitory effect of internal Na, yet the evidence from experiments on red cells (166) is that only external K is relevant, and that internal Na is not inhibitory. The hypothesis also gives no clue about the way in which ATP might cause the postulated increase in the activity of the K_o/Na_i form; nor is it clear why, in the presence of ATP, the K_o/Na_i and K_o/K_i forms should give the same V_{max}.

Before we consider alternative hypotheses to explain the stimulatory effect of Na + ATP, we must try to settle a narrower problem, i.e. is phosphorylation of the enzyme an essential step in the stimulation. The following considerations suggest that it is: 1. Acetyl phosphate (AcP), which we know can phosphorylate the enzyme (15, 52, 107), stimulates *p*NPP hydrolysis in the presence of Na (174). 2. Although, in the presence of Na, high concentrations of CTP, ITP, and GTP act like ATP (149, 171, 174, 216, 244), β,γ-methylene ATP, a nonphosphorylating analog, was ineffective (126). Unfortunately, this evidence is weakened by the fact that the α,β-methylene analog, which can be a substrate for (Na + K)-ATPase (unpublished work of J. D. Cavieres and I. M. Glynn), was ineffective. There is conflicting evidence about the effectiveness of ADP (cf 79, 244). 3. Oligomycin and NEM, which are thought to block the conversion of E_1P to E_2P, have no action on

phosphatase activity in the absence of Na, but prevent the stimulation of *p*NPPase by Na + nucleotide or Na + AcP (12, 107, 174). The finding that low concentrations of oligomycin can *stimulate* phosphatase activity in the presence of Na but without nucleotide (12) suggests, however, that the action of oligomycin may be more complicated than has been supposed. 4. The parallelism between (*a*) the actions of Na + nucleotide or Na + AcP on *p*NPP hydrolysis and (*b*) the actions of Na + nucleotide or Na + *p*NPP on (Na + K)-ATPase inhibition by Be^{2+} or F^- (178, 179, 181) is so close that a common mechanism seems likely. As shown in the section "Inhibitor Studies," there is now good evidence that phosphorylation is involved in the effects of Na + nucleotide or Na + *p*NPP on inhibition by Be^{2+} or F^-. 5. The fact that Na alone acts to some extent like Na + nucleotide does not prove that phosphorylation is not involved, since we know that phosphatase substrates can phosphorylate the enzyme, yielding a product chemically similar to that formed from ATP (35, 52, 106, 107, 191).

If we accept that Na + ATP acts by phosphorylating the enzyme, we must then ask how phosphorylation brings about the changed behavior; this question cannot be separated from the general question of the nature of the phosphatase activity.

An early idea that substrates like *p*NPP and AcP compete with *E*P for the K-activated dephosphorylating mechanism now seems very unlikely in view of (*a*) the known capacity of these substances to phosphorylate, (*b*) the fact that the formation of *E*P from Na + ATP stimulates phosphatase activity, and (*c*) the fact that AcP interferes with the formation of $E^{32}P$ from $\gamma^{32}P$-ATP, but does not affect the breakdown of $E^{32}P$ present before the addition of AcP (35). We shall therefore assume that the hydrolysis of substances like *p*NPP takes place through the formation and breakdown of phosphoenzyme.

Robinson (174) has suggested that in the absence of Na and ATP, the E_1 form of the enzyme is phosphorylated by the substrate in a reaction that is catalyzed by Mg + K. The need for moderately high concentrations of K reflects, he supposed, the low K affinity of E_1. In the presence of Na, Robinson again supposed that E_1 is phosphorylated by the substrate, but now the phosphorylation may be catalyzed by K or Na, the relative effectiveness of each depending on the substrate. To the extent that catalysis of this step is by Na, K will be required only for the hydrolysis, and the overall reaction will reflect the (presumably) high K affinity of E_2P. In the presence of ATP + Na, Robinson supposed that E_1 is phosphorylated exclusively by ATP, and that the phosphatase substrate phosphorylates E_2P yielding a doubly phosphorylated form of the enzyme. Under these conditions there is no K-activated phosphorylation of E_1 and the low affinity of E_1 for K is therefore not reflected in the K-activation curve of the overall reaction.

An awkwardness of this otherwise attractive theory, as it is stated, is that we have to suppose that phosphorylation of the E_1 configuration of the enzyme can be catalyzed either by Na, presumably acting from the inside surface, or by K, presumably acting from the outside surface in view of the results of Rega et al (166).

Albers & Koval (8) proposed a more general scheme in which sites that must be occupied by K for catalysis to occur are unmasked only if *either* one set of regulatory sites is occupied by K *or* a different set is occupied by Na. If the K catalytic

sites have a higher affinity than the K regulatory sites, the kind of stepped K-activation curve that is seen in the presence of Na can be explained. This hypothesis does not explain the effect of ATP or take account of the probable role of phosphorylation.

If Na + ATP acts by phosphorylating the enzyme, and if phosphatase substrates are hydrolyzed via phosphoenzyme, then *either* there must be a doubly phosphorylated enzyme *or* the enzyme must be phosphorylated consecutively by ATP and by the phosphatase substrate. At present, neither possibility can be excluded. There is no evidence for a doubly phosphorylated enzyme, but it might not be easy to isolate and the idea is not unattractive. There is no evidence for consecutive phosphorylation, but a plausible way in which it could account for the observed stimulation of phosphatase activity has been suggested by Post et al (157). They proposed that it is the transient E_2K form of the enzyme that is most readily phosphorylated by substrates like *p*NPP, and that Na + ATP accelerates hydrolysis of such substrates by forming first E_2P and then E_2K. Since *p*NPP itself phosphorylates the enzyme, its own hydrolysis should presumably also lead to the formation of E_2K, but if *p*NPP reacts much more slowly with E_1Na than with E_2K, then ATP should help by "repriming" the system whenever a *p*NPP molecule fails to phosphorylate before E_2K has been converted to E_1 Na. In the presence of Na + ATP, K would be needed only to convert E_2P to E_2K, and the affinity of K should therefore be the same as for (Na + K)-ATPase activity. In the absence of Na and ATP, K in high concentrations is needed to enable *p*NPP to phosphorylate, perhaps because E_2K is formed directly from unphosphorylated enzyme. In the presence of Na without ATP, the K-activation curve for the hydrolysis of a phosphatase substrate would show more or less of the high affinity component, depending on how well the substrate was able to phosphorylate E_1Na.

Is Phosphatase Activity Associated with Ion Transport?

The resemblance between K-dependent phosphatase activity and the dephosphorylation step in the normal pump cycle makes it natural to ask whether the hydrolysis of phosphatase substrates is accompanied by an inward movement of K. Brinley & Mullins (147) were unable to detect any transport in squid axons perfused with AcP. Garrahan & Rega (80) showed that *p*NPP did not support a ouabain-sensitive Rb influx or Na efflux in red cells depleted of ATP by starvation, though it did reduce both fluxes in cells containing ATP, particularly at subnormal concentrations. Na–K exchange and Na–Na exchange were equally sensitive to inhibition by *p*NPP; this is interesting because it implies that even if *p*NPP phosphorylates the E_2K form of the enzyme most readily, it can block reactions with ATP equally well in the absence of K.

Why no ion movements accompany the hydrolysis of *p*NPP is uncertain. An explanation suggested by the need for ATP or its nonphosphorylating analogs in K–K exchange (see "Studies on Ion Movements") is that nucleotide is required in a nonphosphorylating role. This might be the release of K from an occluded form of the enzyme, as postulated by Post et al (157). We may suppose that *p*NPP and P_i phosphorylate the same form of the enzyme (? E_2 or E_2K). In the absence of

nucleotide, K may bring about dephosphorylation, and hence *p*NPP hydrolysis or $P_i/H_2{}^{18}O$ exchange (48), but may remain in an occluded form. The puzzling findings of Askari & Rao (13) suggesting a role for nucleotides at the outer surface of the cell remain unexplained.

INHIBITOR STUDIES

In this section we discuss two groups of inhibitors which have provided useful information on the working of the pump: 1. irreversible inhibitors, and 2. cardiac glycosides. The effects of oligomycin have been mentioned in the sections "Studies on Ion Movements" and "Phosphorylated Intermediates."

Use of Irreversible Inhibitors to Measure the Affinities of the (Na + K)-ATPase for Physiological Ligands

If the initial rate of inhibition by an irreversible inhibitor is affected by the concentration of a ligand, then measurement of the rate of inactivation at different ligand concentrations gives a measure of the affinity of the ligand for the site at which it exerts its effect on the inhibition. Binding affinities can be measured under conditions in which ATP is not being hydrolyzed, and the method is particularly useful for ligands where low affinity or lack of specificity makes methods like equilibrium dialysis inapplicable (181). For the results to be interesting it is, of course, necessary to provide evidence that the site at which the ligands act to slow or hasten inhibition is the same as that at which they exert their physiological effects.

A number of inhibitors have been employed in this way, chiefly to measure the affinities of the (Na + K)-ATPase for Na and K.

N-ETHYLMALEIMIDE (NEM) The effects of NEM on (Na + K)-ATPase include: (*a*) inhibition of the hydrolysis of ATP (27, 63, 214, 219); (*b*) alteration of the enzyme so that the phosphoenzyme formed in the presence of Mg + Na + ATP reacts with ADP (160) but its hydrolysis is no longer stimulated by K (27, 63, 65, 160) (on the "Albers scheme" this is thought to be the result of inhibiting the conversion of E_1P to E_2P); (*c*) prevention of the effect of Na + ATP on the K affinity of *p*NPPase (174) (this too, is probably because NEM blocks the conversion of E_1P to E_2P); (*d*) stimulation and, at higher concentrations or after longer exposure, inhibition of Na-dependent ATP–ADP exchange (28, 63, 65); (*e*) prevention of K-stimulation of Na-dependent ATP–ADP exchange (25, 26); (*f*) inhibition of phosphorylation of the enzyme by ATP under certain conditions (27, 28, 64); (*g*) inhibition of the incorporation of inorganic phosphate into the ouabain-treated enzyme (206).

Measurements of (Na + K)-ATPase activity under standard conditions, following exposure to NEM in the absence of Mg and in the presence of different concentrations of ATP, Na, and K, have shown (*a*) that ATP protects against inhibition by NEM, (*b*) that K or ouabain abolishes this protection, and (*c*) that Na reduces it (219). In the presence of ATP and K, the addition of Na restores the protection; in the presence of ATP and high concentrations of Na, the addition of small

amounts of K restores full protection (219). CTP, GTP, UTP, and ITP have effects similar to those of ATP but much smaller (27, 214), probably in line with the much lower affinities of the enzyme for these nucleotides (97, 154). ADP has effects similar to those of ATP, but roughly double the concentration is required, and, with ADP, Na does not reduce the protection though it does reverse the "antiprotective" action of K. Skou has made a careful comparison of the effects of various levels of ATP, Na, and K on inactivation by NEM on the one hand, and on ATPase activity of the same preparation untreated with NEM on the other (214). The parallelism between the effects of Na and K on the susceptibility to NEM inhibition and on ATPase activity, at different ATP concentrations, suggested that binding of ATP by the enzyme raised the affinity of the internal Na-activation sites for Na relative to their affinity for K. The ratio (Na affinity)/(K affinity) increased from 0.4:1 to 3:1. (See also "Enzyme and Transport Kinetics.") This effect of nucleotide on the selectivity of the Na activation sites appears not to involve phosphorylation since ADP had an effect similar to that of ATP.

Skou also examined the concentration of K necessary to overcome the antagonistic effect of Na on protection by ATP. The effective range of K concentration was the same as that for activation of ATP hydrolysis in the presence of high concentrations of Na. Since the enzyme was preincubated with NEM and ligands in the presence of EDTA, the results appear to suggest that external K-activation sites with a high affinity for K exist even before the enzyme is phosphorylated. This conclusion is probably not justified. There are reasons to suspect that even in the presence of EDTA some phosphorylation occurred when ATP + Na was present. In the first place, Skou found that extremely low levels of free Mg (3 mM EDTA + 1 mM Mg) were sufficient to make the enzyme susceptible to NEM. Secondly, Na alone did not reduce the protective effect of ADP, suggesting that the effect of Na with ATP depends on phosphorylation.

The presence of physiological ligands can affect not only the speed of inhibition by NEM but also the nature of that inhibition. Banerjee (27, 28) and his colleagues showed that under many conditions exposure to NEM led to inhibition of dephosphorylation of the phosphoenzyme, a parallel fall in overall ATPase activity, and a stimulation of ATP–ADP exchange. Unless very high concentrations of NEM were used, phosphorylation was not affected. When the enzyme was preincubated with NEM in the presence of Mg, Mg + P_i, or Mg + ATP + Na, the main effect was inhibition of phosphorylation. Since the conditions leading to NEM inhibition of phosphorylation are also those that promote ouabain binding (see below), Banerjee et al suggested that phosphorylation is inhibited if NEM alkylates an E_2 form of the enzyme, and that dephosphorylation is prevented if NEM alkylates an E_1 form of the enzyme.

To account for the different results of NEM inhibition, Banerjee et al (27, 28) supposed that in different states of the enzyme different groups were alkylated by NEM. This view is supported by measurements of NEM binding by Hart & Titus (95, 96), who used ^{14}C- and ^{3}H-labelled NEM to compare the binding in the presence and absence of different ligands. All effects of the ligands were confined to the binding of NEM to the 98,000 Dalton peptide, separated by acrylamide gel electrophoresis (see "Purification of the Sodium Pump"). In the presence of ATP + Na,

about 2 molecules of NEM were bound per molecule of peptide. With Mg + ATP + Na (? E_2P) about 4 molecules were bound, and with Mg + ATP + Na + oligomycin (? E_1P) about 6 molecules were bound. The effects of selectively blocking inward facing or outward facing sulfhydryl groups (100) also show that the blocking of different groups has different effects.

BERYLLIUM (Na + K)-ATPase activity is inhibited by Be ions, provided that both Mg and K are present (178, 234). The onset of inhibition is first order, the dependence of the rate constant on the concentration of K, at a fixed concentration of Mg and Be, is independent of the Mg and Be concentrations and gives a measure of the affinity for K ions at the sites controlling Be inhibition (178). Robinson found that the dissociation constant for K was 1.4 mM in the absence of Na, and was increased by Na (acting either by direct competition or indirectly from its own site) with a K_I of 7 mM. CTP alone caused a slight decrease in the affinity for K, but CTP and Na together caused a 20-fold increase in K affinity, strongly reminiscent of their combined effect on *p*NPPase activity (178). Inhibition of umbelliferone phosphatase activity by Be showed a similar dependence on the concentration of K and Na (178). The presence of umbelliferone phosphate during the exposure to Be had no effect if Na was absent, but greatly increased the affinity for K if Na was present. Be inactivation and *p*NPPase activity showed a similar dependence on K concentration, both in the presence and absence of Na, suggesting that the same K binding sites are involved in both processes.

That the increase in K affinity caused by Na + nucleotide, or Na + phosphatase substrate, depends on phosphorylation of the enzyme is shown by further experiments in which Robinson (179) compared the effects on K affinity, measured by the Be inhibition technique, of Na + *p*NPP and of Na + a nonphosphorylating analog, *p*-nitrobenzyl phosphonate. Na + *p*NPP increased the K affinity, but Na added with the nonphosphorylating analog appeared merely to compete with K. The analog was shown to be a powerful competitive inhibitor of *p*NPPase activity and must therefore have been able to reach the substrate sites.

The effects of phlorizin and of dimethyl sulfoxide (DMSO) provide further evidence that K binds at the same external sites in promoting Be inhibition and ATPase and phosphatase activity. There is a parallelism—closer for phlorizin than for DMSO—between the effects of these substances on the sensitivity to K and Na of (*a*) the rate of inhibition by Be, (*b*) the rate of hydrolysis of ATP, and (*c*) the rate of hydrolysis of *p*NPP (145, 172, 177–181). The relative effectiveness of Tl, K, and ammonium ions in the three processes is also similar (178).

If the identity of the K binding sites involved in these three processes is accepted, the lack of effect of Na + *p*-nitrobenzyl phosphonate on K affinity measured by the Be inhibition technique leads to the extremely important conclusion that phosphorylation is necessary for the appearance of the external high-affinity K-binding sites (181). (See also experiments with fluoride described below.)

FLUORIDE Because nucleotides bind Be ions, their effects on the rate of onset of Be inhibition may be complicated by binding. Fortunately, fluoride ions resemble Be in causing an irreversible inhibition provided that Mg and K ions are present.

Robinson (181) has investigated the effects of nucleotides, and of nucleotides + Na, on the K dependence of the rate of onset of inhibition by LiF. Na and ATP together caused a 12-fold increase in K affinity, and Na + CTP had an even greater effect. On the other hand, Na with EDTA, or with the nonphosphorylating β,γ-methylene analog of ATP, seemed merely to compete with K. Again the implication is that phosphorylation is necessary for the appearance of high-affinity K-binding sites. Remarkably, oligomycin did not prevent the effect of Na + CTP, suggesting that the formation of E_1P is sufficient to give the effect on K affinity. The fact that oligomycin does prevent the (Na + CTP)-stimulation of K-dependent *p*NPPase activity (12, 174) is understandable if stimulation of phosphatase activity requires not merely phosphorylation but also the subsequent formation of E_2K.

DICYCLOHEXYLCARBODIIMIDE (DCCD) Robinson (183) has taken advantage of the protective action of Na against inactivation of (Na + K)-ATPase by DCCD (194) to measure the Na affinity of the enzyme. The site at which Na exerts its protective action is thought to be the internal Na-activation site because the $K_{0.5}$ for protection in the absence of other ligands was similar to the $K_{0.5}$ for Na activation of ATPase at low K concentrations, and because the $K_{0.5}$ for both effects was similarly increased by phlorizin. The apparent affinity for Na, measured by its protective action, was roughly halved by Mg alone, perhaps because Mg stabilizes the E_2 form of the enzyme. ATP in the absence of Mg roughly doubled the affinity, and Mg and ATP together had little effect on the affinity. This is an important finding because, as Robinson points out, it implies that phosphorylation, which, as we saw above, causes a 12- to 20-fold increase in the affinity of the external K-activation sites, has little effect on the affinity of the internal Na-activation sites.

Interaction of Cardiac Glycosides with the (Na + K)-ATPase

Since the discovery by Schatzmann (192) of the inhibitory effect of cardiac glycosides on the Na pump, a voluminous literature on the subject has appeared. Early work establishing the molecular features necessary for inhibition, the external site of action, the parallel inhibition of transport and ATPase activity, and the protective effect of external K, is described in reference 83 and the reviews mentioned in the Introduction. It is now generally agreed that the glycosides act by inhibiting dephosphorylation of the phosphoenzyme or phosphorylation of the dephosphoenzyme (199).

The introduction of tritiated glycosides has facilitated measurements of glycoside uptake, made either for the purpose of counting sites (22, 59, 70, 99) or in experiments using glycoside binding as a tool for investigating the pump mechanism (9, 10, 59, 99, 144, 197, 233). It has become clear that the enzyme–ouabain interaction is described by a single reversible equilibrium: $E + Ou \rightleftharpoons E - Ou$ (21, 92, 233). The association rate has been demonstrated to be first order with respect to both enzyme and ouabain concentration (29, 61, 138), the dissociation follows exponential kinetics (61, 231, 232), and the measured equilibrium constant is not significantly different from the quotient of dissociation and association rate constants (61,

231). The different binding constants of ouabain to ATPase preparations vary, reflecting mainly differences in the rates of dissociation of the enzyme–ouabain complex (231, 232).

The rate of glycoside binding was observed to depend on the presence of various physiological ligands, and the rate of onset of inhibition always varied in parallel with the rate of glycoside binding (9, 10, 29, 82, 94, 99, 105, 144, 197, 207, 218). The picture that has emerged is that ligands, or combinations of ligands, that stabilize the E_2 or E_2P conformation of the enzyme—Mg, Mg + P_i, Mg + Na + nucleoside triphosphates, Mg + Na + phosphorylating ATP analogs, Mg + Na + phosphatase substrates—all increase the rate of binding and lower the dissociation constant, whereas ligands or combinations of ligands that stabilize E_1 or E_1P—Na, nonphosphorylating ATP analogs, Na + nonphosphorylating ATP analogs—decrease the rate of binding and raise the dissociation constant (62, 229). Previous reports, claiming that stimulation of binding by CTP, UTP, ITP, and ADP implies that the nucleotide action does not involve phosphorylation, are now regarded as mistaken since these nucleotides do phosphorylate (204, 220, 230). With ADP, the phosphorylation is probably due to traces of ATP produced by adenylate kinase present in some membrane preparations. In the presence of added ATP, ADP inhibits binding (93). Nonphosphorylating ATP analogs reduce the effect of ATP on binding and cause a release of glycoside previously bound in the presence of Mg + P_i (62, 228, 229).

Although physiological ligands alter the rate of binding and the dissociation constant, they do not affect the amount of binding at infinite glycoside concentrations, which presumably gives a measure of the number of enzyme sites present (94, 102).

A great deal of attention has been given to the effects of K and its congeners (excluding Na) on glycoside inhibition of transport (22, 82) and ATP hydrolysis (53, 193). At low glycoside concentrations inhibition can be completely prevented, but at high concentrations there seem to be mixed competitive and noncompetitive interactions between glycoside and K (53, 82, 128, 193, 242).

K reduces inhibition by cardiac glycosides by affecting their binding, and not by conferring activity on the enzyme–glycoside complex. There is clear evidence from experiments with ATPase preparations that the rate of ouabain binding in the presence of Mg, Mg + P_i, or Mg + Na + ATP is reduced by K (3, 4, 9, 21, 138, 144, 231–233, 237). Surprisingly perhaps, under some conditions K has also been observed to lower the rate of dissociation of ouabain from the enzyme–ouabain complex (4, 5), but under all conditions K decreases the binding affinity (62, 94, 233). The binding capacity is not affected.

In red cells and HeLa cells it is clear that it is external K that counteracts the effects of low concentrations of ouabain (21, 82). If choline is taken as a standard, K and Rb decrease, and Na, and to a lesser extent Cs and Li, increase the binding affinity (70, 71). Part of this increase may be the result of competition with K ions leaking from the cells, but the effect is too big to be accounted for entirely in this way, and there is other evidence in red cells (30) and squid nerve (23) suggesting a genuine Na–ouabain interaction (see also 138).

Although the K congeners seem to affect ouabain binding with the same order of affinity in both whole cells and microsomal preparations (i.e. Rb > K > NH_4 > Cs > Li) (21, 22, 62, 70, 71, 196), certain anomalies suggest that the cation effects in the microsomal preparations may not be restricted to the external face of the membrane. The effects of K on the dissociation constant in Erdman & Schoner's brain preparation were not saturated by up to 10 mM K (62), although in other preparations (94, 138) the K concentrations for half-maximal effects were 0.2–0.3 mM, as expected for an effect at the external sites. Cs and Li appear to increase the binding affinity in red cells (71), but decrease it in brain (Na + K)-ATPase (62, 196). Recent measurements of the initial rates of ouabain binding to brain ATPase (105, 138) indicate that K and Na compete for an external site (K_K 0.2 mM; K_{Na} 13.7 mM) from which K inhibits and Na stimulates the rate, and that in addition Na stimulates the rate of binding at a high affinity site (K_{Na} 0.63 mM) on the inner surface.

The kinetics of the K–ouabain interaction in whole cells (21), and in microsomal ATPase preparations (138), suggest that only one cation binding site interacts with each glycoside binding site, although at least two cation sites are involved in activation of transport and ATPase activity (see "Enzyme and Transport Kinetics").

Scatchard plots of equilibrium binding data suggest that at low glycoside concentrations there is only a single type of binding site in red cells and in most (Na + K)-ATPase preparations (61, 70), but in brain preparations a number of investigators have reported complex binding (61, 231). Taniguchi & Iida (223), using a medium containing Mg, ATP, Na, and K, observed equal numbers of sites of low affinity ($K_{0.5}$ = 20 μM) and high affinity ($K_{0.5}$ = 0.18 μM); these results suggest that there may be negative cooperativity between two ouabain binding sites. Reports (99, 187) that Cs or K reduce the amount of ouabain bound to the Na pump in red cells in addition to that required to inhibit transport suggest that there are at least two binding sites per pump, and that binding of ouabain to only one of them is sufficient to inhibit. The view that one glycoside molecule per pump is sufficient to inhibit activity is supported by experiments relating rates of ATP hydrolysis (128, 242) or rates of transport (186) to glycoside concentration. The idea that the pump contains two ouabain binding sites, only one of which needs to be occupied to block transport or ATPase activity, fits in well with a recent observation by J. C. Ellory & S. R. Levinson (personal communication). In experiments on llama red cell ghosts, they found that the "molecular weight" of the ouabain binding unit determined by radiation inactivation was about 140,000, whereas the "molecular weight" of the ATPase was about 250,000. (See also "Purification of the Sodium Pump.")

PURIFICATION OF THE SODIUM PUMP

Since P. L. Jorgensen is preparing a detailed review of work on the purification of the (Na + K)-ATPase, we give only a very brief survey.

All attempts at purification have started with membrane preparations from tissues rich in (Na + K)-ATPase activity—brain (151, 236), outer medulla of kidney

(111–113, 127, 129, 130), *Electrophorus* electric organ, or dogfish rectal gland (102, 103)—and have then employed two alternative strategies. One is to extract irrelevant material from the membrane by treatment with NaI or detergents, leaving the (Na + K)-ATPase in situ. The other is to solubilize a large fraction of the membrane, including the (Na + K)-ATPase, by more vigorous treatment with detergents, and then to fractionate the mixture by selective precipitation or by gel or ion-exchange chromatography. Each method of attack may be used alone (102, 103, 112) or the first may be followed by the second (102, 127, 129, 130, 151). ATP may be used to protect the enzyme during treatment with detergents (112).

The purification may be followed by measuring the hydrolytic activity of the preparation, but since the various treatments alter the turnover rate, it is desirable also to measure the ability of the preparation to incorporate ^{32}P from ATP or to bind ATP or ouabain. Finally, the purified preparation may be solubilized with sodium dodecyl sulfate (SDS) and subjected to electrophoresis on polyacrylamide gels to determine the molecular weight of the component polypeptides.

Preparations with purities approaching 100% have now been obtained from several sources (102, 112, 113, 127, 129, 130, 151). Most investigators find that SDS treatment yields two polypeptides, a large one of 89,000–135,000 Daltons containing the phosphorylation site, and a smaller glycopeptide (35,000–57,000 Daltons). There is no direct evidence that the glycopeptide is connected with the pump, but the fact that both peptides are generally found together, that their yields increase in parallel on purification (236), and that they can be cross-linked in a 1:1 fashion by treatment of the native enzyme with dimethyl suberimidate (129), makes the connection extremely likely.

Partly because of uncertainties in the molecular weight measurements (129), there is disagreement about the ratio of large to small peptides; estimates of 2:1 (102, 103), 1:1 (113, 127, 130), and 1:2 (129) have all been obtained. Radiation inactivation (121) gives a figure of about 250,000 Daltons for the molecular weight, so that if the ratio of large to small peptides is 1:1 the enzyme would be a dimer of which each half consists of one large and one small peptide.

There is also disagreement about the ratio of ATP binding sites, or ouabain binding sites, to sites capable of being phosphorylated. Jorgensen found the maximum molecular weight was 137,000 per phosphorylation site, 250,000 per ATP binding site, and 278,000 per ouabain binding site. This supports the idea that the enzyme contains two large peptides, and suggests that under the conditions of Jorgensen's experiments (113) both can be phosphorylated but only one at a time can bind ouabain or ATP. Lane et al (130) also found 1 mole of ouabain bound per 250,000–330,000 g of protein. Using radiation inactivation J. C. Ellory and S. R. Levinson (personal communication) showed that the molecular weight of the ouabain binding unit was 140,000, presumably because inactivation of half of the dimer does not prevent binding to the other half. A ratio of phosphorylation sites to ouabain binding sites of 2:1 was also found by Albers et al (9) in electric organ ATPase, though in a preparation from cat brain they found a ratio of 1:1. Kyte (128) found 0.8–1.2 glycoside binding sites per large polypeptide chain, but at maximum

phosphorylation only about 0.36 moles of ^{32}P were bound to the protein per mole of glycoside binding site. The cause of these discrepancies is not clear, but they are of the kind that might be expected if the enzyme consisted of two identical subunits, and if the behavior of each affected the other in a way that depended critically on the conditions. Less interesting explanations cannot be excluded however.

THE MECHANISM OF THE PUMP

Any satisfactory model for the working of the pump must be based on the pump's structure, as well as on its behavior under different conditions. Knowledge of structure is still too limited to give any detailed understanding of the mechanism, but the strong suggestion that the pump may be a dimer—each monomer containing a large and a small peptide—has important implications. The dimeric structure is supported by the existence of two phosphorylation sites (9, 113) and, under appropriate conditions, two ATP binding sites (97) and two ouabain binding sites (223). The fact that under other conditions it is possible to see only one ATP binding site or one ouabain binding site (and in other preparations only one phosphorylation site) suggests that the two halves of the dimer interact in such a way that binding of ATP or ouabain to one half reduces the affinity of the other half.

The phosphorylation site on the large polypeptide must make contact with the inside surface of the membrane, at least at some stage in the cycle. Whether other parts of the peptide make contact with the outside surface is not clear, though with a molecular weight of 100,000 the peptide would certainly be big enough to bridge the membrane. The glycopeptides are generally thought to make contact with the outer surface of the membrane on the grounds that it is the outer surfaces of cell membranes that are rich in glycoproteins. The argument is not altogether convincing, but if this view is correct it ought to be possible to get immunological evidence for the accessibility of these peptides from the outside of the cell.

We do not know which peptide, if either, carries the ion binding sites, and, apart from their selectivity, we know little about the nature of these sites. The relative effectiveness of Rb, Cs, Li, Tl, and NH_4 as K substitutes can probably be explained in terms of relative affinities of the binding sites (see Eisenman 57, 58), but Na is irreplaceable in a way that is not paralleled by the behavior of inanimate systems. It is clear that the pump makes use of differences in reactivity of pump–ion complexes, as well as of differences in affinity (85). The recent extraction from membranes of ionophoric material able to distinguish between Na and K is obviously extremely interesting (200).

The kinds of mechanism that have been suggested, from time to time, to explain the movement of ions from one side of the membrane to the other, can be classified loosely as (*a*) carriers, (*b*) gated channels, and (*c*) internal transfer mechanisms. By "carriers" we mean any system in which a group binds an ion at one side of the membrane, moves through the membrane carrying the bound ion with it, and discharges the ion at the opposite side of the membrane. The carrier may be a mobile binding site on a large molecule or it may be a small molecule. "Gated channels" are self-explanatory. By "internal transfer mechanism" we mean a mechanism in

which ions originating from each surface exchange binding groups in an occluded region within the membrane and then pass on to the opposite surface.

This classification cuts across that of Skou (212, 213), who divides models into "one-site" models, in which the groups responsible for moving Na and K are interconvertible and only one form exists at any time, and "two-site" models, in which separate groups are responsible for moving Na and K. The two-site models may involve carriers that move right across the membrane, or there may be an interchange in an occluded region within the membrane. Skou's classification is obviously closely related to the division of mechanisms into "sequential" and "simultaneous" (20, 69, 72, 101), depending on whether the inward movement of K follows or accompanies the outward movement of Na. It is unfortunate that in enzyme kinetics a "sequential mechanism" is generally understood to mean a mechanism in which all reactants combine with the enzyme before any products are released (43). This behavior is specifically excluded in the "sequential" model for the pump, and to avoid confusion we suggest that the term "consecutive" be used instead.

Until recently, the transport of ions by the pump was generally explained by some kind of circulating carrier model (40, 202), in which a carrier responsible for moving Na outwards was converted at the outer face of the membrane into a carrier that moved K inwards. Energy was supposed to be fed into the sytem by driving the conversion of one form of the carrier into the other at one face of the membrane, the reversion at the other face being thermodynamically downhill. This one-site, consecutive, carrier system provided a ready explanation of the coupling between Na and K movements, and it was able to account for the various fluxes observed under unphysiological conditions and most of the biochemical changes accompanying them. It has had to be abandoned, however, because it is not compatible with the observations on the constancy of ion affinities discussed in the section "Enzyme and Transport Kinetics," nor with the evidence from inhibitor studies (see "Inhibitor Studies") showing that external sites with a moderately high affinity for K and internal sites with a moderately high affinity for Na exist before the enzyme is phosphorylated. It is important, however to remember that, although external K-binding sites with a moderately high affinity for K may exist before phosphorylation, their affinity is much increased following phosphorylation (see "Inhibitor Studies").

Rejecting the circulating carrier model still leaves open the question of the kind of transfer mechanism involved. A simple way to account for the finding that the rate of exchange is proportional to the product of the probabilities of finding the binding sites at each surface appropriately filled is to suppose (*a*) that there is a molecule bridging the membrane with binding sites at each end, (*b*) that if the binding sites are filled by the proper ions, this molecule is actively rotated, and (*c*) that this rotation is accompanied by a change in affinity of the binding sites so that the outward facing sites always prefer K and the inward facing sites always prefer Na. This model would account for the coexistence of Na and K sites, and would accommodate the observed independence of the affinities; but it is not likely on structural grounds.

It is more plausible, in view of the probable dimeric structure of the enzyme, to imagine two carriers (in the sense defined above) coupled together so that their movements are always 180° out of phase. Repke & Schön's (167, 169) model is basically of this type, though the main interest of that model is in the interactions of the two transporting units.

It is not necessary, however, that the cation binding sites move all the way across the membrane, and models with internal transfer have been proposed on the basis of structural and kinetic evidence (72, 213, 221). Internal transfer models are attractive because their occluded form can provide an obvious explanation of the very slow dissociation of K from E_2K (157), and they can also account for the inhibition of the uncoupled Na efflux by low concentrations of external Na (221). Both features could, however, have alternative explanations. Garrahan & Garay (72) have shown that internal transfer models are compatible with their observations on the independence of ion affinities of conditions at the opposite surface only if the lifetime of the occluded state is short compared with the time during which exchange with the medium is allowed. Hints that the independence of affinities does not hold in Na-free media (42, 186) perhaps suggest that in these conditions the lifetime of occluded states is prolonged.

Gated channel systems are plausible since we know that selective gated channels exist in excitable membranes. But in order to accommodate the various features of the pump—coupling between Na and K movements, coupling between ion movements and ATP hydrolysis, constancy of affinities—the channel would need to be so sophisticated that, in the absence of any specific information, it is not profitable to speculate further. The simple system proposed by Jardetsky (108), in which a channel is opened alternately to the inner and outer surfaces of the membrane, and the affinity of sites within the channel is determined by which end is open, does not give affinities independent of conditions on the opposite side.

In considering the ways in which cations move across the membrane during the pump cycle it is important to remember that not all the effects of cations are associated with a movement of the ions across the membrane. Examples of effects thought not to be associated with cation movements are (*a*) the K-dependent hydrolysis of phosphatase substrates, (*b*) the Na-stimulated ATP–ADP exchange seen in the presence of oligomycin, and (*c*) the K-stimulated exchange of ^{18}O between water and P_i when this exchange occurs in the absence of ATP.

Where Na or K ions are cofactors in biochemical events but do not themselves cross the membrane, we must suppose either that the ions exert their effects while bound at the surface of the membrane, or, if the ions leave the surface, that they never pass beyond an occluded region of the membrane to reach the opposite face. Even if the movement of K is blocked by lack of ATP, or the movement of Na is blocked by oligomycin, it is likely that the nature of the cations "on" or "in" the enzyme determine the fate of the phosphoenzyme. K favors the transfer of phosphate to water, and Na favors the transfer to ADP.

Half-of-the-Sites-Reactivity

An interesting suggestion made by Stein et al (221) is that the Na pump may show *half-of-the-sites-reactivity,* as defined by Lazdunski and his colleagues (134) in con-

nection with the behavior of the alkaline phosphatase of *E. coli.* The *E. coli* enzyme is dimeric, and the characteristic feature of its behavior is that, because of conformational changes associated with phosphorylation or with binding of substrate, only one of the subunits can bind phosphate covalently, or ATP noncovalently, at any time. The phosphorylation of one subunit by ATP is accompanied by dephosphorylation of the other, so that the two subunits go through the reaction cycle 180° out of phase. If the Na pump behaves analogously, its cycle must include the forms E_1ATP:E_2P and E_2P:E_1ATP, though at any instant these forms can account for only a fraction of the total enzyme since we know that in the presence of Na + K most of the enzyme is not phosphorylated.

The scheme suggested by Stein et al (221) combines half-of-the-sites-reactivity with internal transfer, and is consistent with the known structure of the enzyme. Half-of-the-sites-reactivity in connection with the Na pump has been discussed more recently by Siegel et al (205) and by Robinson (182). Repke et al (167, 169) have proposed a "flip-flop" scheme that has the surprising feature that during part of the normal cycle both halves of the enzyme exist in a phosphorylated state. A further feature of their scheme is that the steps leading to changes in affinity for the ions are separate from the steps leading to translocation, only the former being associated with the conversion of the "R/T" form of the enzyme to the "T/R" form.

An advantage of models assuming half-of-the-sites-reactivity that has not been commented on is that they provide a simple explanation for the discrepancies between v/EP and k_D, and between loss of phosphoenzyme and appearance of P_i, seen by Kanazawa et al (118). (See "Phosphorylated Intermediates.") These discrepancies can be explained, as they point out, by the existence of γ^{32}P-ATP bound in such a way that it can still form phosphoenzyme after further reaction with γ^{32}P-ATP in the medium has been blocked with EDTA or by an excess of unlabelled ATP. In a half-of-the-sites-reactive enzyme this bound ATP could represent ATP carried by the unphosphorylated half of the enzyme, i.e. ATP in the form E_1ATP:E_2P. In the absence of K, Kanazawa et al found no discrepancies; this could be *either* because the lower rate of dephosphorylation allowed ATP to dissociate from the enzyme before it could phosphorylate, *or* because, in the absence of K, both halves of the enzyme were phosphorylated (9, 113).

Half-of-the-sites-reactivity also provides a ready explanation of the observation that ADP does not behave as a noncompetitive product inhibitor of the ATPase, as might be expected if ADP is released from the enzyme before P_i (see "Enzyme and Transport Kinetics"). If binding of ATP to the form E_2:E_1P • ADP converts it to the form E_1ATP:E_2P + ADP without intervention of the ADP-sensitive form E_2:E_1P, ADP could not act as a product inhibitor. Under conditions in which this conversion cannot occur (NEM, oligomycin) the phosphoenzyme would become ADP-sensitive following the release of ADP from E_2:E_1P • ADP (cf 68).

It is crucial to explanations of this kind that forms of the enzyme binding ATP to one half and ~ P to the other half should exist, and the finding of Tobin et al (229) that nonphosphorylating ATP analogs accelerate the release of ouabain from enzyme phosphorylated by Mg + P_i is therefore important since it suggests that ATP can react with phosphoenzyme.

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