Affinities or Apparent Affinities of the Transport Adenosine Triphosphatase System

I. M. GLYNN and V. L. LEW

From the Physiological Laboratory, the University of Cambridge, Cambridge, England

ABSTRACT The interactions of potassium ions and ATP on transport ATPase activity are discussed, and the interpretation of these interactions is shown to be often ambiguous. Caldwell's (1968) Physiological Review model is discussed with particular reference to the observed kinetics of sodium: sodium exchange in red cells. Recent experimental work on the properties of the ouabain-sensitive component of potassium efflux from red cells is described. This component of efflux occurs only if *either* sodium *or* potassium are present in the external medium, but the effects of external sodium and potassium are not additive. The relation between ouabain-sensitive potassium efflux and the external concentration of sodium (in a potassium-free medium) or of potassium (in low- and high-sodium media) are described. When starved sodium-poor red cells are poisoned with iodoacetamide, loaded with phosphate, and incubated in high-sodium potassium-free media, the ouabain-sensitive efflux of potassium appears to be accompanied by the reversal of the entire ATPase system. About two to three potassium ions leave by the ouabain-sensitive route for each molecule of ATP synthesized. If potassium is present in the external medium, no ouabain-sensitive synthesis of ATP occurs and the ouabain-sensitive efflux of potassium presumably involves the reversal of only the last part of the ATPase system.

Under physiological conditions, cell membranes hydrolyze ATP at the inner surface and use the energy released to pump sodium ions outwards and potassium ions inwards. In red cells, and possibly in other cells too, these changes are accompanied by a small outward movement of potassium through the pump mechanism. I shall say more about this outward movement later.

These are the changes observed under physiological conditions; under other conditions the pump can behave in other ways (1). If there is no potassium in the outside medium, and certain other criteria are met, the pump catalyzes an exchange of sodium ions across the membrane (2–5). If conditions are such that the energy available from ATP is insufficient to drive the uphill movements of sodium and potassium, the pump appears to run backwards, in-

corporating inorganic phosphate into ATP at the expense of downhill movements of the cations (6). I shall say a little more about reversal of the pump at the end of this talk. All these events can be seen without, as it were, looking "inside" the pump. If you look inside the pump, and you can do this by supplying ^{32}P-labeled ATP and studying the incorporation of radioactivity within the membrane, then you see further events of a kind that I imagine Dr. Post and Dr. Hokin will be talking about later this afternoon. All these events may be affected by the concentrations of sodium or potassium ions at the outer surface of the membrane, and also by the concentrations of sodium, potassium, magnesium, ATP, ADP, or inorganic phosphate at the inner surface. By studying the effects of these various substances, at different concentrations, we can arrive at a whole host of apparent affinity constants, or dissociation constants; perhaps it is safest, because it commits one to nothing, to refer to them simply as half-saturation constants. I want to consider one or two of these half-saturation constants and to show that, however tempting it is to interpret them on the basis of hypothetical mechanisms, such interpretation is dangerous because the kinetics are apt to be ambiguous.

THE EFFECT OF POTASSIUM IONS ON THE APPARENT AFFINITY FOR ATP

The first problem is this. If you take a given membrane preparation and study the rate of splitting of ATP by the (Na^+ + K^+)-activated ATPase, under optimal conditions, as a function of the level of MgATP complex, you find that a certain concentration of MgATP is neeeded for half-maximal activity. If, with the same preparation but in the absence of potassium, you use [γ-^{32}P]ATP to study the effect of MgATP concentration on the incorporation of terminal P into the membrane, you get a figure for the concentration necessary for half-maximal incorporation of radioactivity. The striking thing is that these figures may be very different. With a guinea pig kidney preparation, for example, Kinsolving, Post, and Beaver (7) found that the ATPase activity under optimal conditions was half-maximal at about 0.3 mM MgATP. With the same preparation, and in the same laboratory, Post, Sen, and Rosenthal (8) found that phosphate incorporation was half-maximal at about 1 μM. Furthermore, Post et al. (8) found that the half-saturation level for the ATPase activity was reduced if the potassium concentration—and, therefore, the maximal rate of splitting—was reduced. In other words, lowering the potassium concentration not only reduced V_{max} but also increased the apparent affinity of the system for MgATP. As a possible explanation of these interesting findings, they suggested the presence of a step or steps antecedent to the formation of the phosphorylated intermediate. They supposed that the earlier step had a much *higher* K_m (that is to say, a much lower affinity) and that it became rate limiting at high reaction rates.

The problem of the shifting K_m has been studied in much more detail more recently by Robinson (9), who worked with a microsomal preparation from rat brain. Fig. 1, taken from his paper, shows a series of Lineweaver-Burke plots relating the reciprocal of the velocity of the ATPase to the reciprocal of ATP concentration, at a series of potassium levels. There are two things to notice: the lines are quite straight and they are strictly parallel to one another. These features imply that the K_m is varying directly with the V_{max}, and Fig. 2, also taken from Robinson's paper, shows that a plot of K_m against

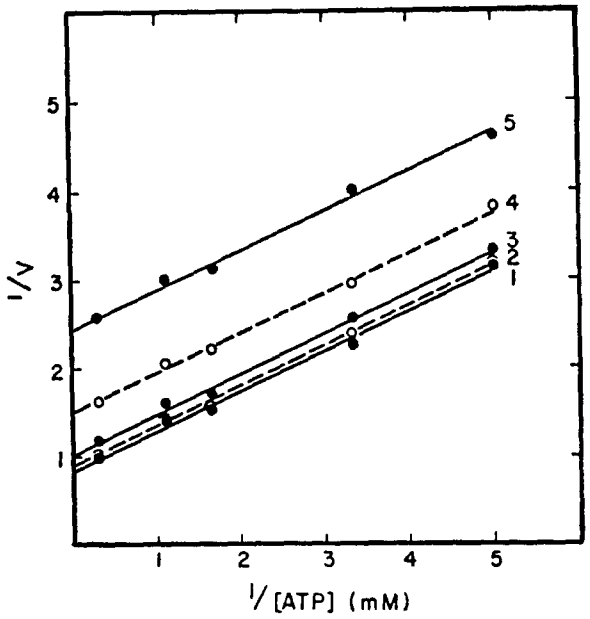

FIGURE 1. Lineweaver-Burke plots relating ATPase activity to ATP concentration in media containing 0.5, 1, 2, 4, and 10 mM KCl. *Figure reprinted by permission from Biochemistry, 1967, 6:3250.*

V_{max} gives a straight line going through the origin. Robinson interprets his results in terms of allosteric effects, but there is some difficulty about this interpretation. In the first place, neither at high nor at low potassium concentrations is there any evidence of "cooperativity" as far as ATP is concerned. The Lineweaver-Burke plots were quite straight and the Hill plot (reference 9, see Fig. 2B) had a slope of 1. Another difficulty is the lack of inhibition by high levels of ATP. According to the most straightforward form of the theory proposed by Monod, Wyman, and Changeux (10), a substance increases the K_m of an enzyme by holding more of it in the form in which its true affinity for the substrate is less; and such substances are, of course, generally inhibitors. Now potassium is an activator of the ATPase, so that, if the effect of potassium

is an allosteric effect, we are dealing with a case of a more unusual type where the form with the higher affinity is catalytically inactive. And, as Monod, Wyman, and Changeux point out, in that situation, excess substrate should inhibit. There is no sign of inhibition of the ATPase by excess substrate in Robinson's experiments, and though slight inhibition has been described in red cell ghosts by Dunham and Glynn (11) and in a nerve preparation by Skou (12), in those experiments ATP was increased at constant magnesium concentration. In the nerve experiments, at least, the inhibition seems to have been related to a decrease in the concentration of free Mg^{2+} ions. The effects of ATP and magnesium concentrations on the ATPase are, however, complicated and probably deserve further investigation.

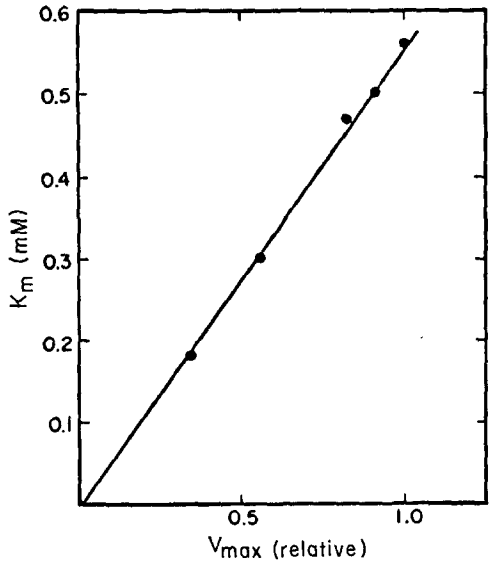

FIGURE 2. Data of Fig. 1 replotted in terms of the K_m for ATP at each KCl concentration against the corresponding V_{max}. *Figure reprinted by permission from Biochemistry, 1967, 6: 3250.*

Perhaps the simplest way of explaining the shift in K_m, without postulating extra steps or allosteric effects, is by the use of Briggs-Haldane kinetics. Classical Michaelis theory assumes that the reaction between enzyme (E) and substrate (S) is so rapid in comparison with the breakdown of the enzyme substrate complex (ES), that ES always remains in equilibrium with E and S during the course of the reaction. In 1925, Briggs and Haldane (13) pointed out that if this assumption was not true the kinetics would be similar but the half-saturation constant, instead of being given by k_{-1}/k_{+1}, would be given by $(k_{-1} + k_{+2})/k_{+1}$. Since the maximal velocity is equal to $k_{+2}e$, where e is the total enzyme concentration, there is a simple relationship between V_{max} and K_m. Anything which increases k_{+2}, and therefore the V_{max}, also increases K_m and so decreases the apparent affinity of the enzyme for the substrate. A plot of apparent K_m against V_{max} gives a straight line intercepting the K_m axis at

the true dissociation constant of the enzyme substrate complex, that is k_{-1}/k_{+1}.

Robinson considers Briggs-Haldane theory but rejects it on the grounds that his graph of K_m against V_{max} goes through the origin. Rejection on these grounds may be unnecessary. In the first place, it is not clear how far from the origin the intercept ought to be. Robinson worked with a rat brain microsomal preparation, and we have been unable to find any published data obtained with that preparation from which one could calculate the K_m for the incorporation of inorganic phosphate into the membrane. If the K_m for the incorporation were anything like that found by Post, Sen, and Rosenthal (8) with the guinea pig kidney preparation, about 1 μM, then the distance of the intercept from the origin would be quite undetectable. Not all preparations that have been investigated give such a low K_m for ^{32}P incorporation, but there is a more powerful argument. At a meeting of the Faraday Society in 1955, Dixon (14), discussing some work of Slater's on notatin, pointed out that Briggs-Haldane theory must be modified slightly if an intermediate is released during the course of the enzyme reaction; and the modified theory predicts a constant ratio between V_{max} and K_m. For the transport ATPase reaction, ADP provides the obvious candidate for the liberated intermediate. We may write the reaction

$$E + ATP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\text{-}ATP$$

$$E\text{-}ATP \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E \sim P + ADP$$

$$E \sim P \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E + P_i$$

where k_{+3} is some monotonically increasing function (f) of the external potassium concentration ($[K]_o$). If, following Dixon, we ignore the reactions represented by the dotted arrows—on the basis that we are interested only in initial reaction velocities—and we make the usual "steady-state" assumption, it is easy to show that

$$v = \frac{\dfrac{k_{+2}\, eS}{1 + k_{+2}/f([K]_o)}}{S + \dfrac{k_{-1} + k_{+2}}{k_{+1} + k_{+1}\, k_{+2}/f([K]_o)}}.$$

$$\text{Hence } \frac{V_{max}}{K_m} = \frac{k_{+1}\, k_{+2}\, e}{k_{-1} + k_{+2}}$$

i.e., the ratio is constant and independent of $[K]_o$.

Although Briggs-Haldane kinetics probably provides the simplest explanation of the effect of external potassium concentration on K_m, it would be rash to assume that this explanation is correct. It is possible, for example, to derive similar kinetics from Michaelis-Menten theory in the following way. Suppose there is an inhibitor that can combine with the enzyme-substrate complex, but not with the free enzyme or with the free substrate. If we measure reaction velocity at different concentrations of substrate and inhibitor, then, as we increase the concentration of inhibitor we shall get a series of parallel Lineweaver-Burke plots of just the kind that Robinson obtained. A substance which in some way prevented access of inhibitor would therefore appear to act as potassium acts in the ATPase reaction. There is more than one way in which such a substance could act, but an interesting mechanism has been suggested by Keleti (15), who postulates the existence of a "liberator" which competes with the inhibitor for the enzyme-substrate complex but has itself no effect on the reactivity of this complex. Application of Keleti's idea to the transport ATPase would suggest that the role of K^+ ions was merely to displace Na^+ ions from sites at the external surface of the membrane at which they were inhibitory, and this idea receives some support from the observations of Baker (16), and of Garrahan and Glynn (2, 17), on the efflux of sodium from nerves or red cells immersed in solutions free of both sodium and potassium.

Although both Briggs-Haldane kinetics, and the hypothesis just outlined, could account for a decrease in the apparent affinity for MgATP as the concentration of potassium was raised, neither hypothesis predicts a fall in the rate of hydrolysis of ATP with increasing potassium concentration. It is therefore interesting that Czerwinski, Gitelman, and Welt (18) have recently described a marked inhibitory effect of potassium ions on the Na^+-activated ATPase of rat erythrocyte membranes exposed to very low concentrations of ATP. Similar behavior has not been found in human red cells,[1] and Czerwinski et al. (18) are inclined to attribute it to an enzyme distinct from the usual transport ATPase; but there is no proof of this duality, and it may be that the interactions of K^+ ions and ATP on the transport ATPase are more complicated than has been thought.

ACTIVATION BY POTASSIUM IONS

A problem closely related to the effect of potassium on the apparent affinity for ATP is the effect of ATP on the apparent affinity for potassium; and it is perhaps worth pointing out that the Briggs-Haldane approach does forecast changes in the apparent affinity for potassium with changing substrate concentration. If, for example, we take the straightforward formulation

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES$$

[1] L. G. Welt. Personal communication.

$$ES \xrightarrow{k_{+2}} E + \text{products}$$

and we make the very simple assumption—too simple to be true—that $k_{+2} = \frac{\alpha [K^+]}{\beta + [K^+]}$, where α and β are constants, it is easy to show that the the apparent K_m for potassium is given by β (the real dissociation constant) multiplied by a factor:

$$\frac{k_{-1} + k_{+1} S}{\alpha + k_{-1} + k_{+1} S}.$$

At high substrate concentrations the factor approaches unity, but at low levels of substrate α in the denominator makes the K_m for potassium appear smaller; i.e., the affinity appears greater. It is possible that an effect of this kind contributes to the differences observed between the potassium concentrations necessary for half-activation of the ouabain-sensitive phosphatase activities of cell membrane preparations exposed to different substrates (19–27).

An interesting feature of the effect of external potassium on the pump system is the S shape of the potassium activation curve at low potassium concentrations ([2], 5, 9, 17, 28–30). Although it is possible to account for an S shape without postulating multiple sites in the enzyme system (31), the fact that the hydrolysis of each molecule of ATP is accompanied by the movement of several cations makes the existence of multiple sites very likely. Given the existence of these sites, one can invoke either allosteric reactions or hypotheses involving carriers that move only when a certain number of sites are filled.

SODIUM:SODIUM EXCHANGE

When red cells are incubated in potassium-free media they show a ouabain-sensitive exchange of sodium ions across their membranes. Fig. 3, from the paper in which Dr. Garrahan and I (2) reported this exchange, shows the ouabain-sensitive influx and efflux of sodium as functions of external sodium concentration. The fluxes appear to be roughly equal in the two directions, and the exchange increases almost linearly with external sodium concentration. The simplest explanation for a sodium:sodium exchange would be a shuttle of the Ussing (32) type, but with such a mechanism the rate of exchange should begin to level off once the sodium concentration outside was much above the concentration inside. We mentioned, in the paper, three possible reasons why this leveling off might not occur. One was that, as the shuttle moved outward across the membrane, there was a decrease in real affinity. Another possibility was that there was no change in the real affinity of the carrier but that for some reason—electrical potential or the rigidity of

[2] E. T. Dunham. Personal communication.

interatomic bonds—the loaded carrier had a greater tendency to remain at the outer surface of the membrane. The third possibility, which was mentioned only in an appendix to the paper, was that at the outside surface of the membrane the carrier reacted with something (calcium ion or some component of the membrane) and so was prevented from moving back again; in other words the carrier was "buffered" at the outer surface. We had no strong preference for one of these explanations rather than the others, though we

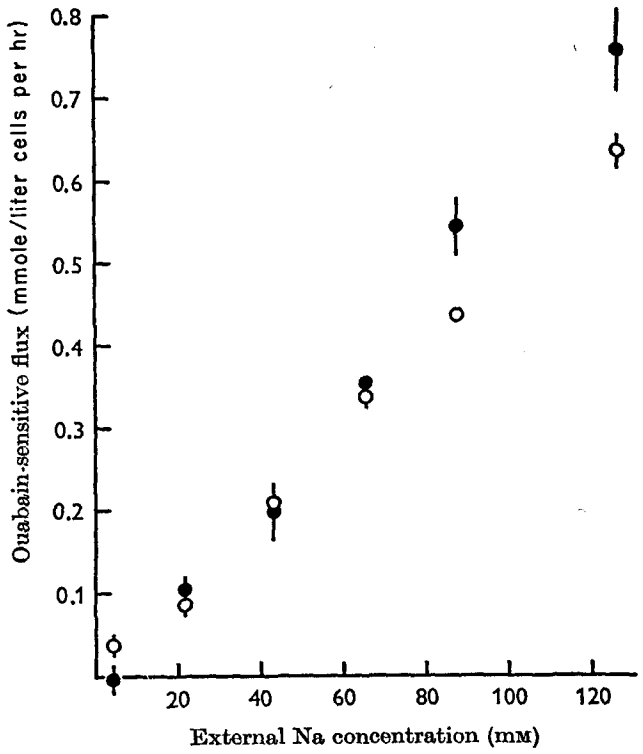

FIGURE 3. A comparison of the ouabain-sensitive inward and outward movements of sodium when red cells were incubated in potassium-free choline media containing different levels of sodium. ●, influx; ○, efflux. The vertical lines show ±1 SE. *Figure reprinted by permission from the J. Physiol. (London), 1967, 192:159.*

were rather attracted by the notion of a change in affinity as the carrier moved across the membrane, feeling that a change in affinity between sodium and potassium had to occur somewhere and it might as well occur there; and we were not much attracted by the idea of buffering, because calcium ions—the obvious choice for the role of buffer—seemed not to be involved. More recently, however, Caldwell (33) has made the interesting suggestion that the reaction responsible for buffering the carrier at the outer surface of the membrane might be the reaction which is responsible for converting the sodium

carrier into the potassium carrier. Caldwell's scheme is reproduced in Fig. 4. It is very similar to the classical circulating carrier hypothesis, but the energy is supposed to be fed in at the outer surface of the membrane, converting the sodium form to the potassium form. If there is no potassium outside, the sodium carrier can still shuttle back and forth; but because the equilibrium on the outside is held further over to the left than the equilibrium on the inside, the apparent affinity of the shuttle for sodium outside is less than inside. Furthermore, if energy is fed in outside, a decrease in the level of ATP or increase in the levels of ADP or phosphate, ought to displace the equilibrium outside to the right, and this could explain the observed effect of such changes

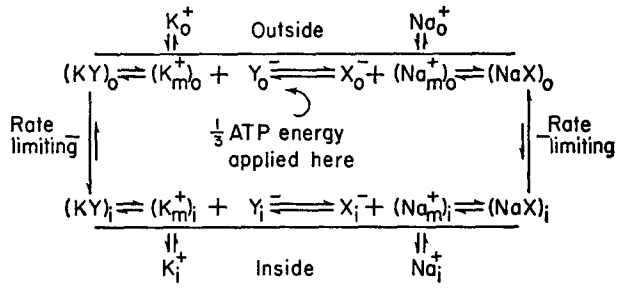

FIGURE 4. Diagram illustrating Caldwell's model for the transport of sodium and potassium. "Normally all stages except diffusion of the ion carrier complexes across the membrane are regarded as being at equilibrium. Free energy made available by splitting of ATP is regarded as being split into three subunits and applied to the conversion of sodium carrier (X^-) to potassium carrier (Y^-) on the outside of the membrane. This could be brought about by an initial splitting of ATP on the inside of the membrane to form a high-energy intermediate in the membrane that would eventually give rise to three molecules of another intermediate on the outside of the membrane. The breakdown of the latter would then be coupled to the conversion of X^- to Y^- on the outside of the membrane." *Figure reprinted by permission from Physiol. Rev., 1968, 48:1.*

in increasing the sodium:sodium exchange. In this way, Caldwell avoids having to make the subsidiary hypotheses that Garrahan and I had to make to explain the effects of internal phosphate and of the internal level of ATP (6). On the other hand his model does not explain why some ATP should be required if sodium:sodium exchange is to take place.

Another interesting feature of Caldwell's model is that the true affinities of the sodium and potassium carriers for the carried ions are supposed to be extremely low; in other words, the carriers are not subject to saturation. In spite of this, the behavior predicted by the model shows apparent saturation at quite low levels of sodium and potassium. On the left of Fig. 5 is a graph showing the observed effects of external potassium concentration on the rates of sodium:sodium exchange and sodium:potassium exchange. As external potassium is increased sodium:sodium exchange switches off and sodium:po-

tassium exchange switches on, and the concentration of potassium to produce the 50 % effect is the same for both. On the right hand side of the figure are the curves predicted by Caldwell's model when suitable values are assigned to the constants. The fit is not very good, but it is interesting that these quite low apparent dissociation constants, quite high apparent affinities, come out of a model where the real dissociation constants are extremely high, that is to say where there is no effective saturation.

A minor fault of Caldwell's model is that, because the carriers pick up only single ions, the model does not produce the S-shaped potassium activation curves that are found experimentally. A more serious problem is that the model does not provide an adequate explanation of the properties of the

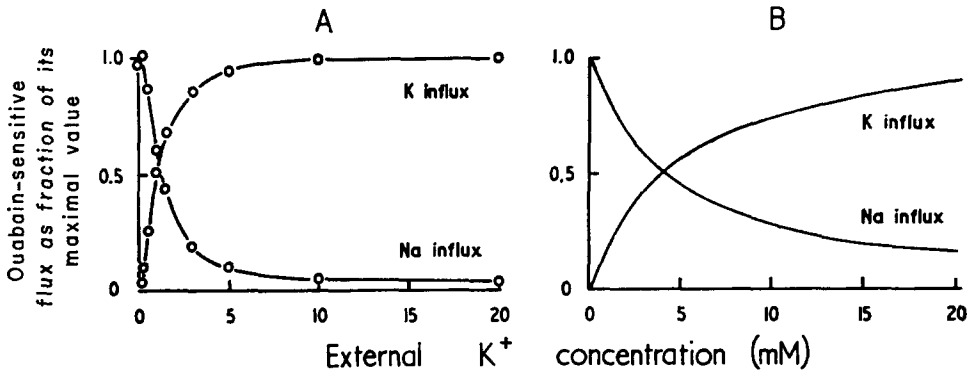

FIGURE 5. The left hand side shows the observed effects of external potassium concentration on the rates of sodium:sodium exchange and sodium:potassium exchange. (Data from Garrahan and Glynn (37); maximal fluxes arbitrarily set at 1.) The right hand side shows the curves predicted by Caldwell's model when suitable values are assigned to the constants. *Figure reprinted by permission from Current Topics in Bioenergetics, III. Academic Press, Inc., New York, 1969.*

ouabain-sensitive potassium efflux. It is this efflux that I now want to talk about.

THE OUABAIN-SENSITIVE EFFLUX OF POTASSIUM

The outward movement of potassium from red cells is of course a downhill movement, but a small part of it is inhibited by ouabain and presumably takes place through the pump mechanism. There seem to be two alternative ways of regarding this ouabain-sensitive efflux—one of them much more interesting than the other. The less interesting interpretation is to suppose that the pump is not perfect at distinguishing between sodium and potassium ions, and that, along with the sodium that it is pumping out, it pumps out a little potassium. The more interesting interpretation is that the ouabain-sensitive potassium loss represents a reversal of the processes involved in

bringing potassium in. In one theory, the potassium is simply going out as a substitute for sodium, the pump running in the forward direction; in the other theory, potassium is going out by a backward movement of, at least, the last part of the pump mechanism. A couple of years ago, Dr. Lüthi and I (35, 36) showed that this efflux of potassium was not seen unless *either* sodium *or* potassium was present in the outside solution, as though one or other ion was needed to bring the carriers back. This observation would fit in with either theory. We also showed that the ouabain-sensitive potassium loss was greatly reduced by inosine. Inosine, we thought, probably acted by reducing the internal phosphate, and since the entry of potassium by the pump is thought to be associated with a dephosphorylation step an apparent need for phosphate was rather in favor of the hypothesis that the potassium loss represented a reversal of the last stage of the pump. During the past year, Dr. Lew and I have carried out a number of experiments which confirm this view.

First we showed that the effects of external sodium and of external potassium were not additive. The ouabain-sensitive potassium efflux into a 4 mM K, high-sodium medium was not significantly greater than the efflux into a 4 mM K choline medium. If sodium or potassium are needed outside simply to get the carriers back, this result is to be expected, because we know that with sufficient potassium outside there is almost no entry of sodium by the pump mechanism, and therefore external sodium ought not to have much effect.

If there is no potassium in the medium, we know that the entry of sodium by the ouabain-sensitive mechanism varies roughly linearly with external sodium concentration; it does not appear to saturate. Experiment showed that the relation between external sodium concentration and the ouabain-sensitive loss of potassium into a potassium-free medium was similarly linear.

Next, we investigated the effects of external potassium concentration. If external potassium facilitates ouabain-sensitive potassium efflux by taking part in a potassium:potassium exchange, we might expect the ouabain-sensitive potassium efflux to vary with external potassium concentration in much the same way as the ouabain-sensitive potassium *influx*. That influx, we know, is half-maximal at very low potassium concentrations—perhaps 0.2 mM—if there is little or no sodium outside, and is half maximal at rather over 1 mM in a high-sodium medium (17). The effects of external potassium on potassium efflux into a low-sodium medium are shown in Fig. 6. In the presence of ouabain, potassium had a very small effect; in the absence of ouabain, half-saturation was at about 0.25 mM K. In a sodium-rich medium the ouabain-sensitive efflux was half-maximal at about 1 mM K, though, surprisingly, external potassium also caused an increase in potassium efflux in the presence of ouabain. Whatever the significance of the effect in the presence of ouabain may be, the ouabain-sensitive efflux behaved precisely as predicted. Unfortunately, we would predict identical effects of external potassium

concentration on potassium efflux, if potassium ions were simply substituting for sodium ions in the forward running of the pump. Fig. 7 shows the effect of external potassium on the sodium efflux, again in high- and low-sodium media. For this flux, too, the K_m in the low sodium medium was about 0.25 mM K, and in the high sodium medium it was about 1.4 mM K. The effects of external potassium on the various fluxes fit nicely together but they do not help to solve our problem.

One way of solving the problem is to look more closely at the effects of inosine. If we compare the effects of inosine on the ouabain-sensitive potassium efflux and on the ouabain-sensitive sodium efflux, measured on cells from the same batch and under identical conditions, then if potassium is

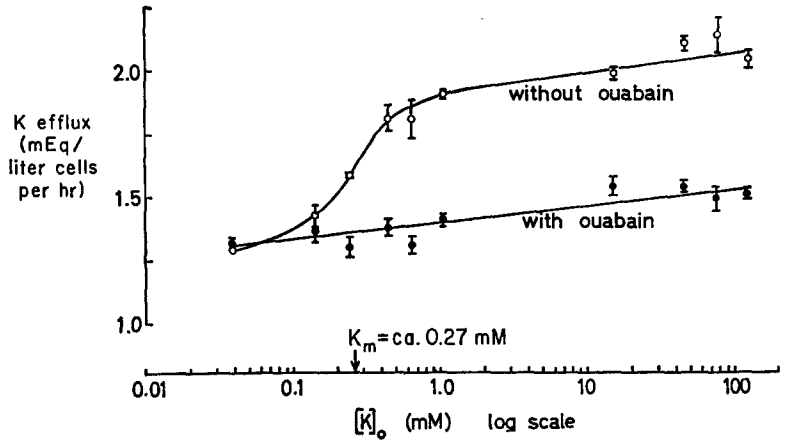

FIGURE 6. Efflux of potassium from red cells incubated in 5 mM Na (choline) media containing different concentrations of potassium, with and without ouabain.

behaving as a substitute for sodium the effects are likely to be similar; if it is not they might be different. The results turn out to be quite clearly different. Fig. 8 shows the results of two experiments of this kind, one on starved cells and one on fed cells. In both experiments inosine reduced the potassium efflux but increased the sodium efflux. Another difference between the effects on the sodium and potassium movements is that glucose was quite a good substitute for inosine in its effect on the sodium efflux, but was relatively ineffective in reducing the potassium efflux. This is understandable if inosine acts on the potassium efflux by removing inorganic phosphate, but acts on the sodium efflux by increasing the supply of ATP.

Another way of deciding between the two possibilities is to take advantage of the fact that, when red cells are sufficiently depleted of energy, the ouabain-sensitive loss of sodium into a medium containing potassium is *less* than into a medium lacking potassium—presumably because the very feeble pump can carry on sodium:sodium exchange, which consumes little or no energy, more

rapidly than it can carry on an exchange of sodium for potassium. If we have cells in such a state, and add potassium to the outside medium, then, if the potassium is going out as a substitute for sodium, the ouabain-sensitive loss of potassium should be decreased; on the other hand if it can go out as part of a potassium:potassium exchange, the ouabain-sensitive loss of potassium might be increased. We have done only one experiment of this kind but it seemed to give a clear cut answer. The cells were depleted of energy by incubating them for several hours with 2-deoxyglucose, so that they used up their

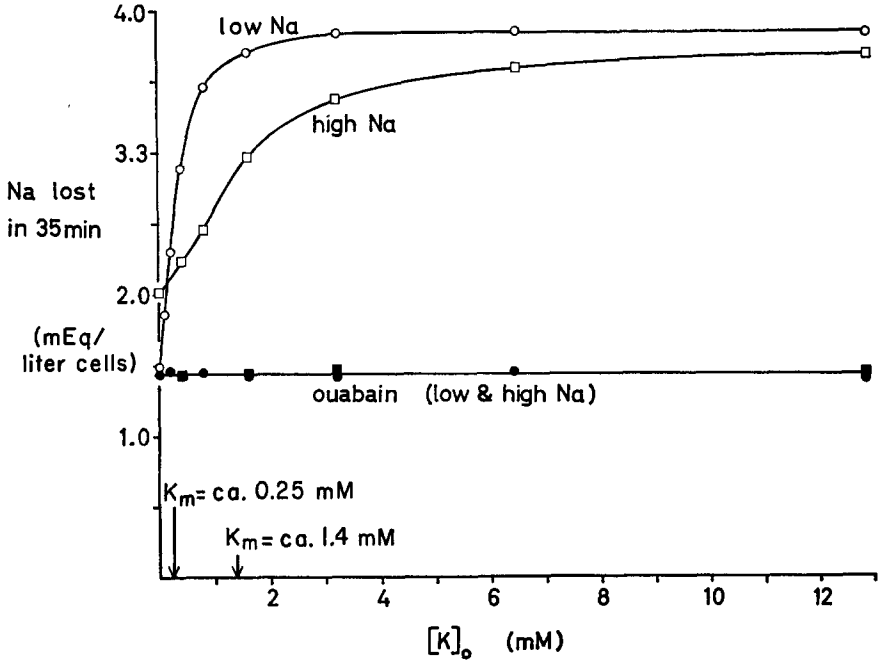

FIGURE 7. The effect of external potassium concentration on the loss of sodium from red cells incubated in high (135 mM) or low (5 mM) sodium media, with and without ouabain.

ATP producing 2-deoxyglucose phosphate which they then could not handle. The addition of potassium reduced the sodium efflux by 38 % but increased the potassium efflux by 32 %—a result in favor of the notion that potassium efflux represents a reversal of the potassium entry mechanism.

The success of the reversal hypothesis in predicting the results of these two different experiments prompted us to apply a more stringent but much more troublesome test. In potassium-containing media, the ouabain-sensitive potassium efflux is presumably just part of a potassium:potassium exchange. In potassium-free media, one has to assume that the whole pump is running backwards. The question is, can we prove that this is so by detecting a

ouabain-sensitive incorporation of inorganic phosphate into ATP, of a magnitude in line with what might be expected from the size of the ouabain-sensitive potassium efflux? There are a number of technical difficulties about this kind of experiment, which I shall not go into, but we have now done

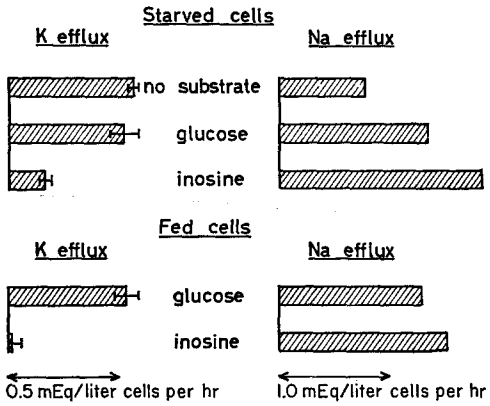

FIGURE 8. The effects of inosine and of glucose on the ouabain-sensitive effluxes of potassium and of sodium from red cells incubated in a high-sodium, 5 mM potassium medium.

TABLE I

STOICHIOMETRY OF THE REVERSED PUMP

Experiment	Ouabain-sensitive K efflux	Ouabain-sensitive $^{32}P_i$ incorporation	Ratio	Corrected for ATP breakdown
	mmoles/liter cells	*mmoles/liter cells*		
1	0.22±0.01	0.061	3.56	−
2	0.20±0.02	0.055±0.003	3.66±0.44	−
		0.070±0.003	2.77±0.44	+

Comparison of ouabain-sensitive K efflux and ouabain-sensitive $^{32}P_i$ incorporation in starved low-Na red cells incubated in a K-free medium The correction for ATP breakdown was based on the decrease in labeled ATP during a second period of incubation after the specific activity of intracellular P_i had been reduced.

three experiments, and we do get about the right amount of ouabain-sensitive incorporation.

In the first experiment, we measured incorporation without attempting to measure potassium efflux simultaneously. Intact cells were starved in a high-potassium medium, poisoned with iodoacetamide—to minimize glycolytic incorporation of inorganic phosphate into ATP—and loaded with radiophosphate ($^{32}P_i$). The loaded cells were washed and then incubated for 30 min in a high-sodium medium with and without ouabain. At the end of this period 79 μmoles of inorganic phosphate had been incorporated into ATP per liter of cells in the ouabain medium, and 143 μmoles/liter of cells in the

control medium. The addition of potassium to the medium, which presumably allows potassium efflux to be part of a potassium:potassium exchange instead of requiring the reversal of the whole pump cycle, reduced the incorporation of inorganic phosphate to the level found in the presence of ouabain. In two further experiments (Table I), simultaneous measurement of $^{32}P_i$ incorporation and potassium efflux allowed us to get some sort of figure for the stoichiometry. Because estimates of both incorporation and efflux depend on the measurement of relatively small differences, the results are not very accurate, and the correction for ATP breakdown adds further uncertainty. Nevertheless, it is clear that the stoichiometry is roughly in the range that one might expect from what is known of the stoichiometry of the pump running forwards.

I have left myself one loose end; I must justify my earlier statement that the properties of the ouabain-sensitive potassium efflux are not easily compatible with Caldwell's model. The point is this. In the model, the sodium and potassium forms of the carrier are supposed to be in equilibrium at the inner surface of the membrane, and the sodium form is supposed to be converted to the potassium form with an input of energy at the outer surface. Now, if the two forms are in equilibrium at the inner surface, it is difficult to see how inosine can at the same time increase the sodium efflux and decrease the potassium efflux. We can get over this difficulty by supposing that inorganic phosphate displaces the equilibrium at the inner surface of the membrane to the left, that is to say, towards the potassium form; but if it did that, it should reduce sodium:sodium exchange, whereas it is clear from earlier experiments (37), that with resealed high-sodium ghosts, high internal phosphate greatly increases sodium:sodium exchange. These difficulties can be circumvented with further subsidiary hypotheses, but by then the theory has lost most of its attractive simplicity.

We are grateful to Dr. P. F. Baker and Dr. K. F. Tipton for helpful discussion.
V. L. L. holds a Fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. The lecture was given by I. M. G.

REFERENCES

1. GLYNN, I. M. 1968. Membrane adenosine triphosphatase and cation transport. *Brit. Med. Bull.* **24**:165.
2. GARRAHAN, P. J., and I. M. GLYNN. 1967. The behaviour of the sodium pump in red cells in the absence of external potassium. *J. Physiol. (London).* **192**:159.
3. CALDWELL, P. C., A. L. HODGKIN, R. D. KEYNES, and T. I. SHAW. 1960. Partial inhibition of the active transport of cations in the giant axons of *Loligo*. *J. Physiol. (London).* **152**:591.
4. KEYNES, R. D., and R. A. STEINHARDT. 1968. The components of the sodium efflux in frog muscle. *J. Physiol. (London).* **198**:581.
5. BAKER, P. F., M. P. BLAUSTEIN, R. D. KEYNES, J. MANIL, T. I. SHAW, and R. A. STEINHARDT. 1969. The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol. (London).* **200**:459.

6. GARRAHAN, P. J., and I. M. GLYNN. 1967. The incorporation of inorganic phosphate into adenosine triphosphate by reversal of the sodium pump. *J. Physiol.* (*London*). **192**:237.
7. KINSOLVING, C. R., R. L. POST, and D. L. BEAVER. 1963. Sodium plus potassium transport adenosine triphosphatase activity in kidney. *J. Cell. Comp. Physiol.* **62**:85.
8. POST, R. L., A. K. SEN, and A. S. ROSENTHAL. 1965. A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes. *J. Biol. Chem.* **240**:1437.
9. ROBINSON, J. D. 1967. Kinetic studies on a brain microsomal adenosine triphosphatase. Evidence suggesting conformational changes. *Biochemistry.* **6**:3250.
10. MONOD, J., J. WYMAN, and J. P. CHANGEUX. 1965. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* **12**:88.
11. DUNHAM, E. T., and I. M. GLYNN. 1961. Adenosinetriphosphatase activity and the active movements of alkali metal ions. *J. Physiol.* (*London*). **156**:274.
12. SKOU, J. C. 1960. Further investigations on a Mg^{++} + Na^{+}-activated adenosinetriphosphatase possibly related to the active linked transport of Na^{+} and K^{+} across the nerve membrane. *Biochim. Biophys. Acta.* **42**:6.
13. BRIGGS, G. E., and J. B. S. HALDANE. 1925. A note on the kinetics of enzyme action. *Biochem. J.* **19**:338.
14. DIXON, M. 1955. *In* The Physical Chemistry of Enzymes. *Faraday Soc. Disc.* **20**:301.
15. KELETI, T. 1967. The liberator. *J. Theor. Biol.* **16**:337.
16. BAKER, P. F. 1964. An efflux of ninhydrin-positive material associated with the operation of the Na^{+} pump in intact crab nerve immersed in Na^{+}-free solutions. *Biochim. Biophys. Acta.* **88**:458.
17. GARRAHAN, P. J., and I. M. GLYNN. 1967. The sensitivity of the sodium pump to *external* sodium. *J. Physiol.* (*London*). **192**:175.
18. CZERWINSKI, A., H. J. GITELMAN, and L. G. WELT. 1967. A new member of the ATPase family. *Amer. J. Physiol.* **213**:786.
19. JUDAH, J. D., K. AHMED, and A. E. M. MCLEAN. 1962. Ion transport and phosphoproteins of human red cells. *Biochim. Biophys. Acta.* **65**:472.
20. ALBERS, R. W., and G. J. KOVAL. 1966. Sodium-potassium-activated adenosine triphosphatase of *Electrophorus* electric organ. III. An associated potassium-activated neutral phosphatase. *J. Biol. Chem.* **241**:1896.
21. BADER, H., and A. K. SEN. 1966. (K^{+})-dependent acyl phosphatase as part of the (Na^{+} + K^{+})-dependent ATPase of cell membranes. *Biochim. Biophys. Acta.* **118**:116.
22. EMMELOT, P., and C. J. BOS. 1966. Studies on plasma membranes. II. K^{+}-dependent *p*-nitrophenyl phosphatase activity of plasma membrane isolated from rat liver. *Biochim. Biophys. Acta.* **121**:375.
23. FUJITA, M., T. NAKAO, Y. TASHIMA, N. MIZUNO, K. NAGANO, and M. NAKAO. 1966. Potassium-ion stimulated *p*-nitrophenyl phosphatase activity occurring in a highly specific adenosine triphosphatase preparation from rabbit brain. *Biochim. Biophys. Acta.* **117**:42.
24. NAGAI, K., F. IZUMI, and H. YOSHIDA. 1966. Studies on potassium-dependent phosphatase; its distribution and properties. *J. Biochem.* (*Tokyo*). **59**:295.
25. NAGAI, K., and H. YOSHIDA. 1966. Biphasic effects of nucleotides on potassium-dependent phosphatase. *Biochim. Biophys. Acta.* **128**:410.
26. YOSHIDA, H., F. IZUMI, and K. NAGAI. 1966. Carbamyl phosphate, a preferred substrate of K^{+}-dependent phosphatase. *Biochim. Biophys. Acta.* **120**:183.
27. ISRAEL, Y., and E. TITUS. 1967. A comparison of microsomal (Na^{+} + K^{+})-ATPase with K^{+}-acetyl phosphatase. *Biochim. Biophys. Acta.* **139**:450.
28. HOFFMAN, J. F. 1966. The red cell membrane and the transport of sodium and potassium. *Ameri. J. Med.* **41**:666.
29. SACHS, J. R., and L. G. WELT. 1967. The concentration dependence of active potassium transport in the human red blood cell. *J. Clin. Invest.* **46**:65.
30. BEAUGÉ, L. A., and R. A. SJODIN. 1968. The dual effect of lithium ions on sodium efflux in skeletal muscle. *J. Gen. Physiol.* **52**:408.

31. SWEENY, J. R., and J. R. FISHER. 1968. An alternative to allosterism and cooperativity in the interpretation of enzyme kinetic data. *Biochemistry.* **7**:561.
32. USSING, H. H. 1949. Transport of ions across cellular membranes. *Physiol. Rev.* **29**:127.
33. CALDWELL, P. C. Energy relationships in the active transport of ions. *In* Current Topics in Bioenergetics, III. R. Sanadi, editor. Academic Press, Inc., New York. In Press.
34. CALDWELL, P. C. 1968. Factors governing movement and distribution of inorganic ions in nerve and muscle. *Physiol. Rev.* **48**:1.
35. GLYNN, I. M., and U. LÜTHI. 1967. Can the later stages of the 'transport ATPase' system be reversed independently of the earlier stages? *J. Physiol. (London).* **191**:104*P*.
36. GLYNN, I. M., and U. LÜTHI. 1968. The relation between ouabain-sensitive potassium efflux and the hypothetical dephosphorylation step in the "transport ATPase" system. *J. Gen. Physiol.* **51**:385s.
37. GARRAHAN, P. J., and I. M. GLYNN. 1967. Factors affecting the relative magnitudes of the sodium:potassium and sodium:sodium exchanges catalysed by the sodium pump. *J. Physiol. (London).* **192**:189.