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THE INTERACTION OF ADENOSINETRIPHOSPHATE AND INORGANIC PHOSPHATE WITH THE SODIUM PUMP IN RED CELLS

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SUMMARY

1. An increase in the intracellular concentration of inorganic phosphate (P_i) reduces the rate of the Na:K exchange catalysed by the Na pump in red cells. The inhibitory effect of P_i is exerted on the maximum rate of flux, P_i having no appreciable effect on the apparent affinity of the Na pump for either internal Na or external K. The effect of P_i is exerted along a rectangular hyperbola which tends to zero as P_i tends to infinity and is half-maximal at about 17 mM internal P_i.

2. P_i does not modify the rate of Na:Na exchange catalysed by the Na pump.

3. A reduction in the intracellular concentration of ATP reduces the maximum rate of Na:K exchange having no effect on the apparent affinity for either internal Na or external K.

4. The effects of ATP and P_i are mutually independent.

5. The lack of effect of ATP and P_i on the apparent affinity for internal Na is compatible with the idea that the affinity of the inner sites of the Na pump remains constant during a pump cycle.

6. The lack of effect of ATP on the apparent affinity for external K and the independence between the effects of ATP and P_i are difficult to explain if the only effect of ATP were its combination at a phosphorylating site.

7. The apparent affinities for K and phosphate become independent of the concentration of ATP, if it is assumed that in our experimental range the phosphorylating site is fully saturated with ATP, the rate of pumping being controlled by the state of occupation of a second non-phosphorylating site whose affinity for ATP is much lower.

8. The lack of effect of P_i on the apparent affinity for external K seems to indicate that during Na:K exchange the conformations of the pump that predominate are endowed with reactivity towards inorganic

phosphate and have the same high affinity for K in both their phospho and their dephospho states.

9. The kinetic behaviour of the Na pump in regard to its interactions with inner and outer cations, ATP and P_i seems to indicate that, in contrast with what happens with soluble allosteric proteins, in the active transport system ligand-induced changes in reactivity are more important than ligand-induced changes in affinity. In this respect therefore the Na pump behaves as an allosteric 'V system'.

INTRODUCTION

We have shown previously that a relatively simple kinetic scheme provides an accurate description of the interactions of Na and K with the Na pump in red cells (Garay & Garrahan, 1973). The kind of interaction predicted by our kinetic equations places a set of rather stringent restrictions on the possible mode of operation of the molecular machinery of active transport (Garrahan & Garay, 1974).

Caldwell, Hodgkin, Keynes & Shaw (1960) first drew attention to the fact that changes in orthophosphate and ATP might modify the relative proportions of the Na:K and the Na:Na exchanges catalysed by the Na pump. The aim of this paper is to extend our study on the interactions of ligands with the Na pump to cover the effects of ATP and inorganic phosphate (P_i) on the rate of active Na and K transport in red cells. The results show that additional restrictions have to be taken into account in postulating plausible mechanisms for active transport. A preliminary account of some of the experiments reported here has already been published (Garrahan & Garay, 1974).

METHODS

Freshly drawn human blood from haematologically normal adults was always used. The procedures employed for washing the cells, changing their internal cation composition, measuring Na or K fluxes as well as the incubation solutions were essentially similar to those described by Garay & Garrahan (1973). Flux determinations were carried out during incubation periods not exceeding 20 min in order to avoid undue changes in the intracellular concentrations of ATP and P_i.

Phosphate loading. Intracellular phosphate was increased by incubation of red cells during 10 min at 37° C in about 20 volumes of solutions prepared by mixing adequate amounts of 75 mM citric acid titrated to pH 7·4 with KOH and 100 mM-K-phosphate buffer (pH 7·4) (see Glynn & Lew, 1970). The resulting intracellular inorganic phosphate was measured by the method of Weil-Malherbe & Green (1951) after precipitation of the haemolysates with trichloroacetic acid. Control cells were preincubated for 10 min at 37° C in a phosphate-free citrate medium.

Determination of intracellular ATP and ADP. Intracellular ATP and ADP were measured by ion exchange chromatography on a Dowex 1 resin, after deproteinization of a suitably diluted haemolysate. The procedure employed was essentially

similar to that of Garrahan & Glynn (1967*a*); except that deproteinization was achieved with perchloric acid (final concentration 3% v/v). Before chromatography perchloric acid was precipitated out of the solution by adding enough KOH to bring the pH to about 7. The concentration of nucleotides in the effluents of the column was estimated by measuring the absorption at 260 nm.

Sources of materials. ^{42}KCl and ^{22}NaCl were obtained from the Comisión Nacional de Energía Atómica, Argentina. All salts and reagents were A.R. grade. The solutions were prepared in doubly glass-distilled water.

RESULTS

Many of the results to be reported in this paper were obtained from experiments performed in red cells containing different levels of P_i or of K or submitted to partial starvation. It seemed therefore important to know beforehand to what extent these different conditions affected the intra cellullar concentrations of ATP, ADP and P_i. The results of experiments performed with this aim are shown in Table 1. Inspection of the Table makes clear that:

TABLE 1. The content of ATP, ADP and inorganic phosphate in red cells submitted to different experimental conditions

Condition	Intracellular concentration (mmole/l. cells)		
	ATP	ADP	P_i
Cells containing 1·2 m-mole K/l.	1·06	0·35	—
Cells containing 93 m-mole K/l.	1·12	0·35	—
Control cells	0·90	0·63	0·98
P_i-loaded cells	0·99	0·50	24·5
Control cells	1·00	0·61	1·8
Cells incubated for 5 hr in a substrate-free medium	0·29	0·45	1·7

Before measurements all the cells were incubated for a length of time similar to that used in the flux experiments. In the K-poor cells internal K was replaced by choline chloride.

(i) The decrease from 93 to 1·5 m-mole/l. cell in the intracellular concentration of K has no effect on the intracellular concentration of ATP and ADP.

(ii) The intracellular levels of ADP and ATP in cells loaded with 25 m-mole/l. cell of inorganic phosphate are similar to those of control cells.

(iii) A 5 hr long incubation in a glucose-free medium decreases the intracellular concentration of ATP to about a third and has very little effect on the intracellular levels of ADP and P_i.

The lack of effect of intracellular K on the concentrations of ATP and ADP lends support to the view that the effects of K on the turnover rate of the Na pump (Garay & Garrahan, 1973) are due to a direct action of the K on the Na pump, rather than to be the result of changes in the metabolic state of the cells.

The effects of inorganic phosphate on cation fluxes

Na efflux. Fig. 1*a* shows the result of an experiment in which the efflux of Na into a K-rich (10 mM-K) medium (Na:K exchange) was measured as a function of internal Na concentration in control cells and in cells that had been loaded with 43 mM internal P_i. In both cases Na efflux raised

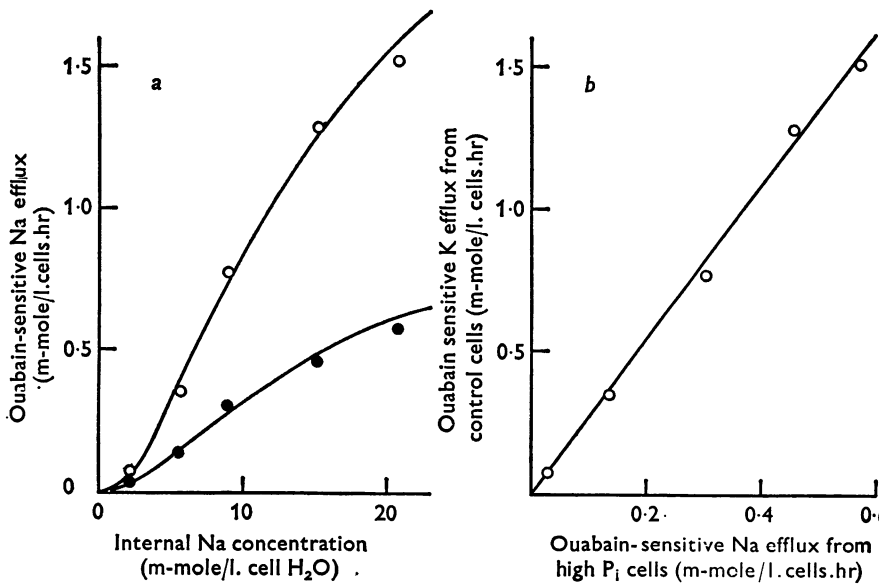

Fig. 1. *a.* The effect of internal Na concentration on the ouabain-sensitive efflux of Na into a K-rich medium (10 mM-K) from control cells (○) and from cells loaded with 43 m-mole/l. H_2O inorganic phosphate (●). The continuous lines are theoretical curves for a three identical site kinetics taking maximum flux 3·33 m-mole/l. cells hr for control cells and 1·28 m-mole/l. cell hr for high P_i cells. For both curves the apparent dissociation constant for internal Na is 5·85 mM. *b*, a plot of the ouabain-sensitive efflux from control cells against the ouabain-sensitive efflux from high P_i cells. External P_i was kept constant at 2·5 mM.

with internal Na following an S-shaped curve, the efflux from the high-P_i cells being always lower than the efflux from the control cells. The mechanism of the inhibitory effect of P_i was analysed by plotting, for each of the internal Na concentrations tested, the efflux from the control cells against the efflux from the high P_i cells. Fig. 1*b* shows that when this

is done the experimental points can be fitted by a straight line of zero intercept, indicating that the inhibitory effect of P_i is independent of the intracellular concentration of Na. The increase in internal P_i therefore decreases the maximum rate of Na efflux, leaving unaffected the apparent affinity for Na of the inner sites of the Na pump. This assertion is further confirmed by the fact that the efflux curves of both control and high P_i cells can be fitted by a three identical sites kinetic equation (Gary & Garrahan, 1973) having the same value for the apparent dissociation constant for internal Na (continuous lines in Fig. 1*a*).

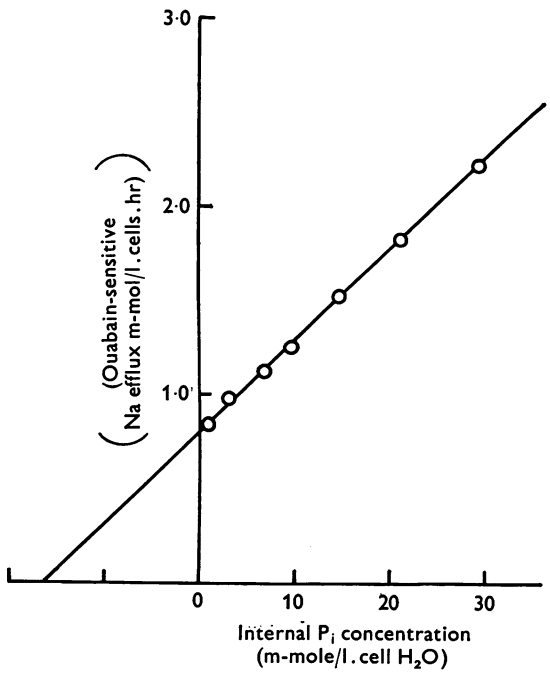

Fig. 2. A plot of the reciprocal of ouabain-sensitive Na efflux into a K-rich medium against the intracellular concentration of inorganic phosphate. External P_i was kept constant at 2·5 mM.

Since P_i acts only on the maximum rate of Na efflux, the relation between inhibition and P_i concentration can be studied by measurement of the effects of different concentrations of P_i on Na efflux at any fixed internal Na concentration.

The results of an experiment of this kind are shown in Fig. 2. It can be observed that when the reciprocal of Na efflux is plotted against the internal concentration of P_i a straight line of positive intercept is obtained.

The Na efflux *vs.* internal P_i curve can therefore be adjusted to an equation of the form

$$M = M^\circ/(1+(P_i)/K_I), \quad (1)$$

where M° is the efflux in the absence of P_i and K_I is the apparent dissociation constant for the P_i-pump complex. Equation (1) predicts that as P_i concentration is raised Na efflux will tend to zero along a rectangular hyperbola. The value for K_I obtained from the intercept at the abscissa of the plot of Fig. 2 is 16·6 mM.

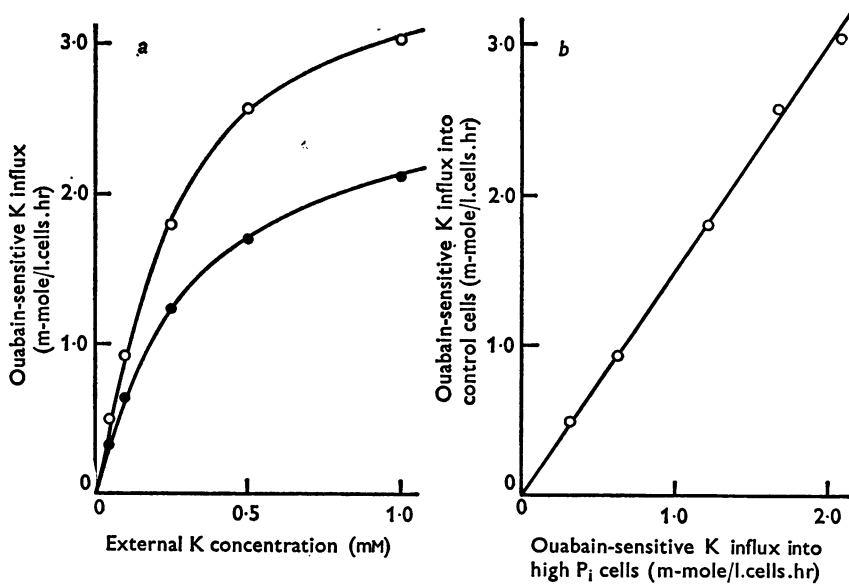

Fig. 3. *a*. The effect of the external concentration of K on the ouabain-sensitive influx of K into control cells (○) and into cells loaded with 25 m-mole/l. H_2O inorganic phosphate (●). The cells were suspended in a Na-free choline medium. *b*, a plot of the ouabain-sensitive K influx into control cells against the ouabain-sensitive K influx into high P_i cells. External P was kept constant at 2·5 mM.

K influx. In the conditions used in the experiments of Figs. 1 and 2, Na efflux is coupled to K influx. It was therefore of interest to analyse the effects of P_i on the rate of K influx. Fig. 3*a* and *b* show the result of an experiment in which the influx of K into both control and high P_i cells was measured as a function of external K concentration. It is clear that at any of the K concentrations tested P_i acted as an inhibitor of K influx. The plot of the influx into high P_i cells against the influx into low P_i cells yields a straight line of zero intercept (Fig. 3*b*). Phosphate therefore inhibits K influx by lowering its maximum rate and leaves unaffected the apparent affinity for K or the external sites of the Na pump.

Essentially similar results to those in Fig. 3 were obtained measuring the K influx curves in a 0·01–0·2 mM external K range. The inhibitory effect of P_i on K influx is less than that expected from the results in Fig. 2 probably because the inhibition of Na:K exchange is partially offset by the stimulation by P_i of the K:K exchange catalysed by the Na pump (see Glynn, Lew & Luthi, 1970).

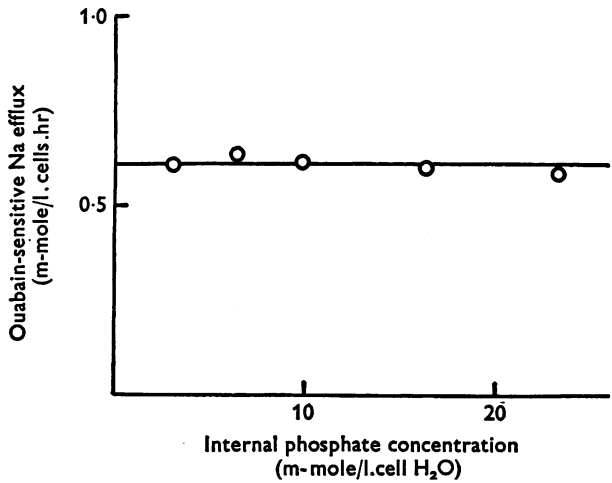

Fig. 4. The effect of the intracellular concentration of inorganic phosphate on the ouabain-sensitive Na efflux into a K-free Na medium. External P_i was kept constant at 2·5 mM.

Na:Na exchange. The fluxes we have analysed up to this point are coupled to the hydrolysis of ATP with the release of inorganic phosphate. It seemed worth while to compare the effect of P_i on these fluxes with the effect of P_i on the exchange of internal for external Na which the pump catalyses in the absence of external K. Na:Na exchange requires ATP but in contrast with Na:K exchange is not coupled to the net release of P_i from ATP (Garrahan & Glynn, 1967*b*, *c*).

In the experiment shown in Fig. 4 the rate of Na:Na exchange was measured in cells containing from 3·0 to 23·4 mM internal P_i. It is evident that, in sharp contrast with the effects it has on Na:K exchange, internal phosphate leaves unaltered the rate of Na:Na exchange. The lack of effect of P_i was further confirmed by studying the dependence of Na:Na exchange on the internal concentration of Na in control cells and in cells containing 25 m-mole/l. P_i. The results (not shown) demonstrated that neither the apparent affinity for internal Na nor the maximum velocity of Na:Na exchange were affected by P_i.

The effects of ATP on cation fluxes

Na efflux. Fig. 5*a* compares the relation between the efflux of Na and the internal concentration of Na in cells that had been preincubated for 5 hr in media with and without glucose. It is clear that at each of the concentrations of Na tested starvation reduced the rate of Na efflux. The plot of the efflux of control cells against the efflux from starved cells yields a straight line of zero intercept (Fig. 5*b*). Changes in ATP concentration (see Table 1) therefore alter the maximum velocity of Na efflux leaving unmodified the apparent affinity for Na of the inner sites of the Na pump.

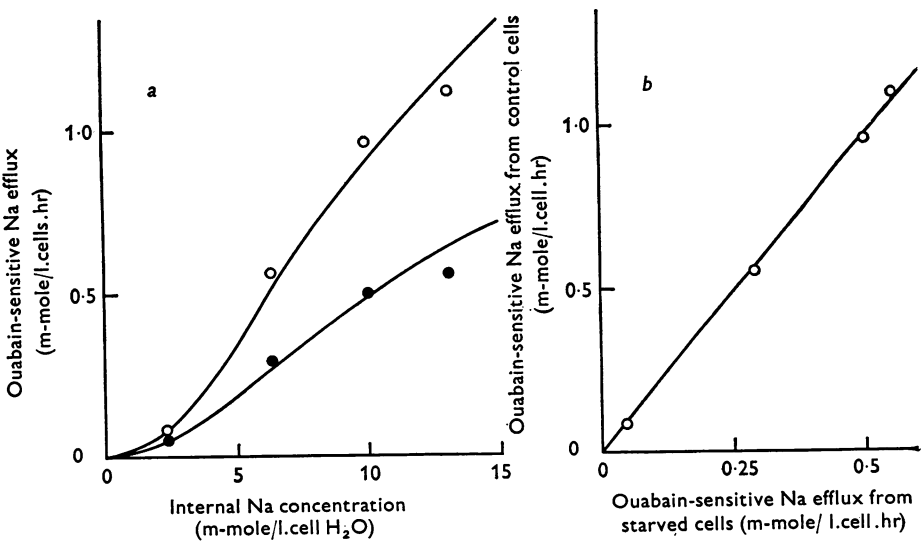

Fig. 5. *a.* The effect of the internal Na concentration on the ouabain-sensitive efflux of Na into K-rich media from red cells that had been preincubated for 5 hr in media with (○) and without (●) glucose. The continuous lines are theoretical curves for a three identical site kinetics drawn taking maximum flux: 3·57 m-mole/l. cell hr for control cells and 1·89 m-mole/l. cell hr for starved cells. For both kinds of cells the apparent dissociation constant for internal Na was taken as 5·85 mM. *b*, a plot of the ouabain-sensitive efflux of Na from control cells aganst the ouabain-sensitive Na efflux from starved cells.

K influx. Fig. 6*a* and *b* compare the relation between the external concentration of K and the influx of K into control cells and into cells whose ATP content had been reduced by a 5 hr long preincubation in a substrate-free medium. It is evident that also in this case the decrease in the concentration of ATP decreased the maximum rate of K influx leaving unchanged the apparent affinity for K of the external sites of the Na pump.

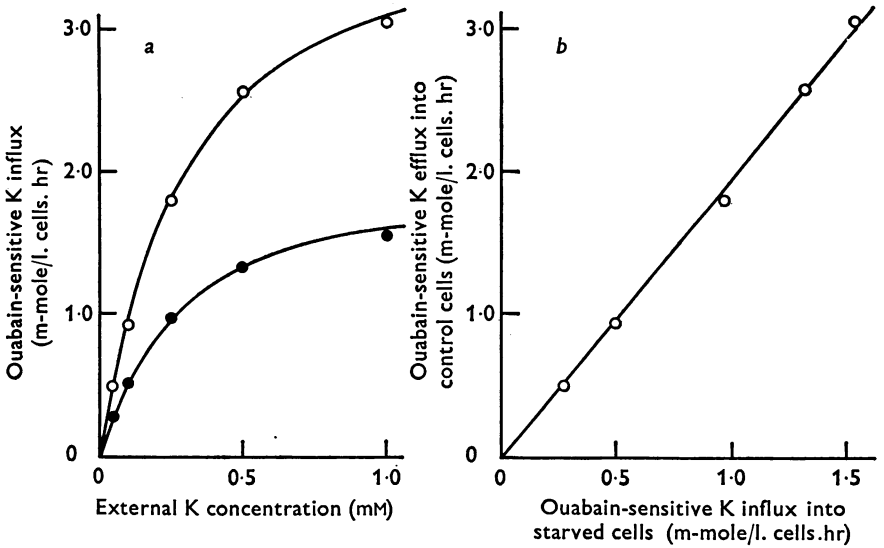

Fig. 6*a*. The effect of the external concentration of K on the ouabain-sensitive influx of K into cells that had been preincubated for 5 hr in media with (○) and without (●) glucose. The cells were suspended in a Na-free choline medium. *b*, a plot of the ouabain-sensitive K influx into control cells against the ouabain-sensitive K influx into starved cells.

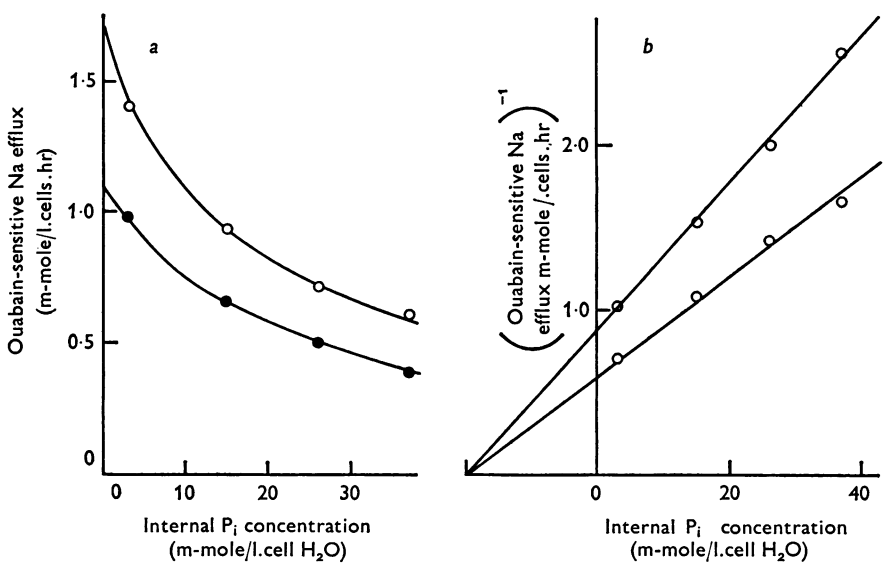

Fig. 7*a* and *b*. The effects of the intracellular concentration of inorganic phosphate on the ouabain-sensitive efflux of Na from cells that had been preincubated for 4 hours in media with (○) and without (●) glucose.

External P_i was kept constant at 2·5 mM.

Essentially similar results to those in Fig. 6 were obtained by measurement of the K influx curves in a 0·01–0·2 mM external K range. These results, which were obtained using low concentrations of K and a Na-free medium, confirm previous findings by Glynn (1956) who showed that a similar invariance to starvation of the apparent affinity for K holds for the K influx curve measured in Na-rich media.

The interaction between the effects of ATP and P_i. Fig. 7*a* and *b*, summarizes the results of an experiment in which the efflux of Na was measured as a function of the intracellular concentration of inorganic phosphate in cells that had been preincubated during 4 hr in media with and without glucose. It is evident that for both control and starved cells the plot of the reciprocal of the flux against the concentration of P_i yields straight lines which intersect the same point on the horizontal axis. The apparent affinity for the inhibitory effect of P_i is therefore independent of the intracellular concentration of ATP.

The results of the experiment in Fig. 7 were further confirmed measuring the inhibitory effect of 35 m-mole/l. P_i cell H_2O in control cells and in cells in which starvation was prolonged for a sufficient length of time as to get an inhibition of Na efflux twice as large as that in the experiment of Fig. 7. In this case also the percent inhibition by P_i in starved cells was similar to that of control cells.

DISCUSSION

The results presented in this paper show that both a reduction in the intracellular concentration of ATP and an increase in the intracellular concentration of inorganic phosphate inhibit the rate of Na:K exchange catalysed by the Na pump. The first of these effects is almost certainly due to a decrease in the number of pump units having an adequate degree of occupation by ATP to elicit ion transport. With respect to the mechanism of the inhibitory effect of P_i our results rule out the possibility of a competitive displacement of ATP by P_i, a fact which agrees with the demonstrated uneffectiveness of P_i in displacing ATP bound to the (Na + K) ATPase (Hegywary & Post, 1971). The most likely mechanism for the inhibitory effect of phosphate is the reduction of the net rate of dephosphorylation of the Na pump. It is known that when the phosphorylated intermediate formed by interaction of ATP with the Na pump becomes able to react with water its phosphate moiety exchanges freely with inorganic phosphate (Post, Toda & Rogers, 1975). In this state of the pump, therefore, the rate of rephosphorylation by P_i may attain a sufficiently large value to have a significant effect on the net rate of

dephosphorylation. Additional evidence in favour of the view that phosphate acts as the dephosphorylation stage of the pump comes from our demonstration that the rate of Na:Na exchange is independent of the intracellular concentration of P_i. Na:Na exchange probably requires phosphorylation but in contrast with Na:K exchange it is certainly not associated with the dephosphorylation of the Na pump (Garrahan & Glynn, 1967*b*, *c*).

The independence of the apparent affinities for cations, ATP and P_i. We have shown previously (Garay & Garrahan, 1973) that the apparent affinities for cations of the Na pump at one of the surfaces of the cell membrane is invariant to changes in the state of occupation of sites at the opposite surface of the cell membrane. The experiments reported in this paper show that such indpendence also holds for the effects of ATP and P_i on Na:K exchange in the sense that: (i) the apparent affinities for cations at either surface of the cell membrane are not affected by ATP or P_i and, (ii) there is no interaction between the effects of ATP and P_i.

It seems, therefore, that at least within our experimental range the overall rate equation for Na:K exchange as a function of inner and of outer cations, ATP and P_i is expressible as a product of functions each of them containing one of the variables that affect the fluxes, i.e.

$$\frac{\text{Flux}}{\text{Maximum flux}} = W(\text{inner cations})\,X(\text{outer cations})\,Y(\text{ATP})Z(P_i). \quad (2)$$

The function containing inner cations can be made equal to the probability of finding a pump unit with its inner sites with the adequate degree of saturation to elicit ion translocation (Garay & Garrahan, 1973) and the term containing P_i can be made equal to the probability of finding a pump unit with a phosphate binding site unoccupied by P_i (see eqn. (1) in Results). If the other terms in eqn. (2) could also be equated to probabilities, their combination into a product would mean that Na:K exchange is linearly dependent on the number of pump units having their inner and outer sites occupied by the relevant cations, an ATP-binding site occupied by ATP and a P_i-binding site or sites free of phosphate. The fact that the affinities of each set of sites are independent of the rest places some restrictions on the possible mechanism of the interaction of ligands with the Na pump. We have already discussed these restrictions in connexion with the independence between inner and outer cation binding sites (Garrahan & Garay, 1974). In order to perform a similar analysis for the effects of ATP and P_i it seems convenient to discuss separately the interaction of ATP and P_i with the inner and with the outer cation binding sites.

The effects of ATP and P_i on the properties of the inner sites of the Na pump. We have shown previously (Garrahan & Garay, 1974) that the

independence between the apparent affinities of the inner and outer cation binding sites of the Na pump (see eqn. (2)) requires that the affinity for cations remains constant during a pump cycle in at least one of the surfaces of the cell membrane.

It is easy to demonstrate that if the affinity for internal Na is invariant to the changes induced in the Na pump by ATP and P_i, the apparent affinity of the inner sites of the pump will be independent of the concentrations of ATP and P_i. In this respect therefore our results are consistent with the idea that the invariance in cation affinity demanded by the kinetics of cation fluxes holds at least for the inner sites of the Na pump. This conclusion moreover is in agreement with the findings that: (i) the binding constant for ATP of the (Na + K)-ATPase is unaffected by Na ions (Hegyvary & Post, 1971), and (ii) the apparent affinity for the protective effect of Na on the dicyclohexylcarbodiimide-induced inactivation of the (Na + K)-ATPase is independent of the ligand state of the pump (Robinson, 1974).

The lack of interaction between ATP, P_i and the external sites of the Na pump. If the only action of ATP were the phosphorylation of the pump it is difficult to see how the effects of ATP and P_i could be exerted independently. Any simple kinetic scheme involving phosphorylation and dephosphorylation of the pump will predict interactions between ATP and P_i if, as it is usually done, the ATP-containing and the phosphorylated forms are taken as mutually exclusive states of the Na pump. In addition it seems that, in contrast with what happens with the internal sites, the affinity of the external sites *does* depend on the ligand state of the pump. In fact experimental evidence indicates that as a consequence of phosphorylation the external sites of the pump go from a state of moderate affinity to a state of high affinity for K (Garrahan, Pouchan & Rega, 1970; Robinson, 1973). A kinetic equation describing a cycle of phosphorylation and dephosphorylation will therefore predict not only interactions between ATP and P_i but also effects of these two ligands on the apparent affinity for external K.

This statement can be illustrated in a more quantitative way by working out the kinetic equation of one of the current hypotheses on the mechanism of ATP hydrolysis by the Na pump (see for references Post, Kume, Tobin, Orcutt & Sen, 1969).

$$E_1 + ATP \rightleftharpoons E_1 - P + ADP$$
$$E_1 - P \rightleftharpoons E_2 - P$$
$$E_2 - P + H_2O \rightleftharpoons E_2 + P_i$$
$$E_2 \rightleftharpoons E_1$$

where E_1 and E_2 are two conformational states of the pump. If we assume that (i) the system is saturated with internal Na, and the concentration of

ADP is constant. (ii) K sites exist throughout the cycle their affinity being given by the state of phosphorylation of the pump. (iii) Equilibrium holds for the addition of ligands and steady state for the transitions between the ion-pump complexes. (iv) The over-all rate is proportional to the concentration of the fully saturated E_2 stage of the pump, i.e. $V = k(E_2 - K)$ (Post, Hegyvary & Kume, 1972). The following equation will describe either Na efflux or K influx as a function of ATP, P_i and K concentrations (K^+):

$$\text{Flux} = \frac{k\,E_t}{ABC(1+\alpha_1/(K^+))(1+\beta_1/(\text{ATP})) + C(1+\alpha_2/(K^+))(1+B) + (1+\alpha_1/(K^+))}, \quad (3)$$

where k is a rate constant whose value will depend on whether Na efflux or K influx is being measured; E_t the total amount of pump;

$$A = (E_1\text{ATPK})/(E_1 - \text{PK}); \; B = (E_1 - \text{PK})/(E_2 - \text{PK});$$
$$C = \text{H}(P_i) = (E_2 - \text{PK})/(E_2\text{K}); \; \alpha_1 \text{ and } \alpha_2$$

the equilibrium constants for the dissociation of K from the desphospho and phospho forms of the enzyme respectively and β_1 the equilibrium constant for the dissociation of ATP from the phosphorylating site.

An equation of identical form is obtained if more than a single K-binding site is taken into account.

Inspection of eqn. (2) makes clear that the terms containing ATP and P_i can be factored out from the terms containing K only if the dissociation constants for K were the same throughout the transport cycle, which is at variance with the experimental evidence. Moreover under no condition are the terms containing ATP and P_i separable into a product as is demanded by our experimental results.

The affinity for K regains its independence from ATP if it is assumed that

(i) for the system to cycle at measurable rate a second non-phosphorylating site has to be occupied by ATP;

(ii) in the range of ATP concentrations covered by our experimental conditions the phosphorylating site is fully saturated by ATP, changes in rate being the result of changes in the occupation of the non-phosphorylating site, whose affinity for ATP is much lower;

(iii) the second site exists and its affinity is constant in all the states of the pump that predominate during Na:K exchange. Its occupation therefore does not affect the apparent affinity for K.

When these additional assumptions are incorporated into our kinetic scheme, eqn. (3) becomes

$$\text{Flux} = \frac{k\,E_t}{(1+\beta_2/(\text{ATP}))\,(ABC(1+\alpha_1/(K^+)) + C(1+\alpha_2/(K^+))(1+B) + (1+\alpha_1/(K^+)))}, \quad (4)$$

where β_2 is the apparent dissociation constant of ATP from a second low-affinity site in the pump.

Though eqn. (4) predicts independence between the effects of ATP and P_i it still implies interactions between K and P_i. Further assumptions are therefore needed to adjust our kinetic scheme to the experimental results.

When non-limiting concentrations of K are present the steady-state level of phosphorylation of the pump is low (Gibbs, Roddy & Titus, 1965; Fahn, Koval & Albers, 1968) which makes it likely that in the presence of low concentrations of P_i $C < 1$. In addition the available experimental evidence strongly suggests that the equilibrium between E_1-P and E_2-P is displaced in favour of E_2-P (see Post *et al.*, 1969), so that $B < 1$. Moreover since (Fahn *et al.*, 1968) when the E_1-P to E_2-P transition is inhibited the level of phosphorylation is not much affected it seems reasonable to think that also $A < 1$. If these unequalities are taken into account it is clear that the affinity for K in eqn. (4) will be mainly governed by α_1, that is by that corresponding to the low affinity state of the pump. However in contrast with this prediction experimental evidence clearly shows (see for instance Garrahan & Glynn, 1967*d*) that the apparent affinity for K influx is near to that of the high affinity state (Robinson, 1973) of the pump.

A way out of this paradox is to suggest that not only the phosphorylated forms but also some of the dephosphorylated forms of the pump are endowed with high affinity for K. The most likely candidate for a dephospho form with high affinity for K is E_2 since all the experimental procedures which favour the view that phosphorylation increases the affinity for K result in the formation not only of E–P but also of E_2. If the affinity for E_2 is made equal to that of E–P and furthermore if the term containing α_1 in eqn. (4) is neglected on the basis that $ABC \ll 1$, eqn. (4) becomes

$$\text{Flux} = \frac{k\,E_t}{\left(1+\frac{\beta_2}{(\text{ATP})}\right)\left(1+\frac{\alpha_2}{(K^+)}\right)(1+(P_i)\,(H+HB))}, \tag{5}$$

in which the terms containing ATP, K and P_i are separated into a product as it is demanded by our experimental results. It may be worth while stressing at this point that though eqn. (5) was derived from a definite kinetic scheme, an equation of identical form will describe any transport mechanism in which the forms that predominate during Na:K exchange are those which are endowed with both high affinity for K and reactivity towards inorganic phosphate.

There are several lines of evidence which, independently of the demands of our kinetic studies, suggest that a low affinity site for ATP exists in

the Na pump and that its occupation is necessary for Na +K dependent ATP hydrolysis, i.e.

(i) in the presence of K the number of moles of ATP that are bound to the (Na +K)-ATPase exceeds in about twofold the amount of phosphate that can be incorporated from ATP (Hegyvary & Post, 1971);

(ii) the curve relating ATP hydrolysis to ATP concentration shows a biphasic response with an apparent K_m of 0·001 mM as well as a K_m near 0·5 mM. The V associated with the high affinity site is quite small (Neufeld & Levy, 1969);

(iii) the low affinity site for ATP has been implicated (Post *et al.* 1972) in the increase of the rate of reconversion of E_2 into E_1 and may be responsible for the nucleotide requirement of the K:K exchange catalysed by the Na pump (Simons, 1972).

If under physiological conditions the K_m of the phosphorylating site were similar to that detectable by ATP binding or phosphorylation studies (0·1–30 μM, see for instance Hegyvary & Post, 1971) its value would be considerably lower than the concentration attained in our experiments (see Table 1). This would give support to our assumption that in our conditions the phosphorylating site is fully saturated.

Moreover, if the distribution of the different states of the pump during Na:K exchange favoured a phosphate-reactive form of the pump as we have assumed to develop eqn. (5) our equation would be valid even if the low affinity site existed only in this conformation.

The Na pump as a 'V system'. In their analysis of allosteric transitions in proteins Monod, Wyman & Changeux (1965) pointed out that two classes of effects may be expected in allosteric systems:

(i) *K systems.* In which the presence of a ligand modifies the apparent affinity to other ligands, and conversely,

(ii) *V systems.* In which the interaction between sites leaves unchanged their affinity and is manifest only in variations of their catalytic properties.

The kinetic behaviour of the Na pump in what regards its interactions with inner and outer cations, ATP and P_i seems to indicate that the active transport system behaves as a V system. A similar conclusion was reached by Lieb & Stein (1970) for the case of the glucose transport system of red cells. Independence between the affinities of Ca, ATP and P_i has been observed in the Ca pump of sarcoplasmic reticulum (Kanazawa, Yamada, Yamamoto & Tonomura, 1971; Yamada & Tonomura, 1973; Meissner, 1973).

It may perhaps be in contrast with what happens with soluble regulatory proteins, that in transport systems ligand induced changes in reactivity are more important than ligand-induced changes in affinity.

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