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# THE EFFECTS OF VANADATE ON THE FLUXES OF SODIUM AND POTASSIUM IONS THROUGH THE SODIUM PUMP

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SUMMARY

1. The effects of sodium orthovanadate on the fluxes of sodium and potassium (or rubidium) ions through the sodium pump have been investigated in intact human red cells and in resealed ghosts prepared from them. Sodium–potassium exchange, potassium–potassium exchange, pump reversal, sodium–sodium exchange and uncoupled sodium efflux have each been studied.

2. When intact human red cells were incubated in high-sodium media containing vanadate in low concentrations, inhibition of potassium or rubidium influx was marked only if the potassium or rubidium concentration in the medium was sufficiently high to cause nearly maximal influx in the absence of vanadate. The absence of inhibition at lower potassium or rubidium concentrations cannot be explained by supposing that the onset of inhibition by vanadate is slower in these conditions.

3. Lowering the extracellular sodium concentration, or raising the vanadate concentration, decreased the minimum concentration of extracellular potassium or rubidium at which inhibition by vanadate was detected.

4. Experiments on potassium influx into intact red cells treated with the ionophore A23187 showed that magnesium ions act at intracellular sites to promote inhibition by vanadate.

5. Measurements of potassium efflux from intact red cells incubated in high-sodium media, with or without potassium, showed that potassium–potassium exchange was inhibited by vanadate at low concentrations whereas reversal of the pump was not.

6. Measurements of sodium efflux from intact red cells or resealed ghosts incubated in high-sodium media, with or without potassium, showed that vanadate had little or no effect on sodium–sodium exchange at concentrations at which sodium–potassium exchange was markedly reduced. Much higher concentrations of vanadate did cause partial inhibition of sodium–sodium exchange.

7. Experiments to determine whether vanadate in low concentrations inhibited uncoupled sodium efflux were inconclusive, but suggested that the flux was inhibited.

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Measurements of the ATP hydrolysis that is thought to be associated with the uncoupled sodium efflux showed that this hydrolysis was strongly inhibited.

8. The different effects of vanadate on the different fluxes are discussed, and related to the way in which vanadate is thought to act on the sodium pump.

## INTRODUCTION

In 1975, Charney, Silva & Epstein reported that Sigma 'Sigma-grade' ATP contained an impurity that partially inhibited Na,K-ATPase from various tissues of the rat; they were unable to identify the impurity, and they published their results mainly as a warning to other workers. The warning was not always effective, and in 1977 Beaugé & Glynn (1977*b*) found that an unusual response of Na,K-ATPase to potassium ions, which they had previously thought to be peculiar to Na,K-ATPase from kidney outer medulla (Beaugé & Glynn, 1977*a*), was in fact due to the use of 'Sigma-grade' ATP. The dramatic effect on the enzyme's response to potassium, and the fact that 'Sigma-grade' ATP was unusual in being extracted from muscle, led them to suggest that the active impurity might have a physiological role as a modifier of the sodium pump. This possibility also occurred to Cantley and his colleagues, who had concentrated the impurity, and who, shortly afterwards, identified it as orthovanadate (Cantley, Josephson, Warner, Yanagisawa, Lechene & Guidotti, 1977). Experiments on red cells, or on resealed ghosts prepared from them, showed that the vanadate acted intracellularly (Cantley, Resh & Guidotti, 1978) but that the effect of potassium ions on the sensitivity to vanadate occurred at extracellular sites (Beaugé & Glynn, 1978).

In the last two years there have been several further papers on the effects of vanadate on Na,K-ATPase activity (Cantley, Cantley & Josephson, 1978; Beaugé, 1979; Bond & Hudgins, 1979; Grantham & Glynn, 1979; Post, Hunt, Walderhaug, Perkins, Park & Beth, 1979), and Beaugé & DiPolo (1979) have recently described the effects of vanadate on sodium efflux from squid axons in different conditions. At the time the experiments to be reported here were done, however, there had been no attempt to investigate the effects of vanadate on the fluxes of sodium and potassium through the sodium pump mechanism acting in each of its various modes. These experiments were designed to fill that gap. They show that sodium–potassium exchange, potassium–potassium exchange and uncoupled sodium efflux (or, at least, the ATP hydrolysis that is associated with it) are inhibited by vanadate at very low concentrations, whereas sodium–sodium exchange and reversal of the pump are not. The significance of these findings will be discussed later.

## METHODS

### *Preparation of the cells*

Because procedures were different in the different experiments, details are given in the tables and figure legends. In experiments to investigate the effects of vanadate on fluxes in intact cells, the cells were gradually preincubated with vanadate for at least 30 min to allow time for the vanadate to equilibrate across the cell membrane ($t_{\frac{1}{2}}$ = 4 min; see Cantley, Resh & Guidotti, 1978). In the second experiment of Table 1, however, in order to minimize differences in composition between the vanadate and the control cells, vanadate was included only in the last wash solution and the final incubation medium. To allow time for equilibration, the period between

the start of the final incubation and the taking of the 'initial' samples was increased to 15 min. In experiments on resealed ghosts, vanadate was introduced at the moment of lysis, after lysis but before the incubation necessary for resealing, or only in the final incubation medium. In the last case, the period before the taking of the 'initial' samples was increased to 15 min, to allow time for the vanadate to equilibrate.

In the experiment of Fig. 5*B*, the sodium concentration in the incubation medium during the exposure of the cells to the ionophore A23187 (Reed & Lardy, 1972) was made similar to the intracellular sodium concentration, and the period in the 37 °C bath was limited to 14 min, in order to minimize the risk that, at the low magnesium concentration, the ionophore might catalyse appreciable movements of sodium (Flatman & Lew, 1977). (The ionophoretic activity of A23187 is greatly reduced at low temperatures (personal communication from V. L. Lew).) Since, with the ionophore concentration used, cell magnesium equilibrates with magnesium in the medium with a half time of about 1 min at 37 °C (unpublished experiments by J. D. Cavieres), the 14 min in the 37 °C bath should have been ample, even allowing for the time taken for the tube contents to reach bath temperature.

*Flux measurements*

Details of the different experimental designs are given in the Tables and Figure legends. With $^{42}K$ and $^{24}Na$, we measured Cerenkov radiation rather than gamma radiation because much lower backgrounds could be obtained. Haemoglobin was estimated by its absorbance at 541 nm in lysates in dilute ammonia (about 10 mM), and the optical density of packed red cells at this wavelength was taken to be 284.

*Sources of materials*

A23187 was a gift from E. Lilly & Co., Indianapolis. Orthovanadate (trisodium salt) was obtained from B.D.H. Ltd. London, ATP (disodium salt) from Boehringer, Mannheim, and iodoacetamide, inosine, HEPES, creatine, phosphocreatine (disodium salt), 2-deoxyglucose, creatine phosphokinase (from rabbit muscle), hexokinase (type F 300 from yeast) and choline chloride from Sigma, London, Ltd. The choline chloride was recrystallized from hot ethanol before use. Sodium chloride and potassium chloride were the 'specpure' reagents from Johnson Matthey, Ltd., London. $^{24}Na$, $^{22}Na$, $^{42}K$, $^{86}Rb$ and [$\gamma^{32}P$]ATP were obtained from the Radiochemical Centre, Amersham.

## RESULTS

### *The effects of vanadate on potassium influx during sodium–potassium exchange (or rubidium influx during sodium–rubidium exchange)*

Fig. 1 shows the results of an experiment to investigate the effects of $2 \times 10^{-6}$ M-vanadate on rubidium influx when intact red cells were incubated in media containing rubidium at different concentrations. The cells were first incubated at 37 °C for 2 hr in high-Na, rubidium-free, glucose media, with or without vanadate and with or without ouabain; this preincubation allowed time for the vanadate to equilibrate across the cell membrane. Aliquots of the suspensions were then transferred to similar solutions containing between 0·2 and 30 mM [$^{86}Rb$]RbCl, and the uptake of radioactivity during the next 30 min was measured. The striking feature of the results is that inhibition was marked only at rubidium concentrations that were high enough to give nearly maximal rubidium influx in the absence of vanadate. This was a characteristic feature of experiments with low vanadate and high sodium concentrations, though there was some variation between experiments (cf. the experiment shown in Fig. 3, where, under conditions similar to those in the experiment of Fig. 1, vanadate caused 16% inhibition when the rubidium concentration was

10 mM). Lowering the sodium concentration in the suspending medium (Fig. 2), or increasing the concentration of vanadate (Fig. 3), reduced the level of rubidium at which inhibition appeared.

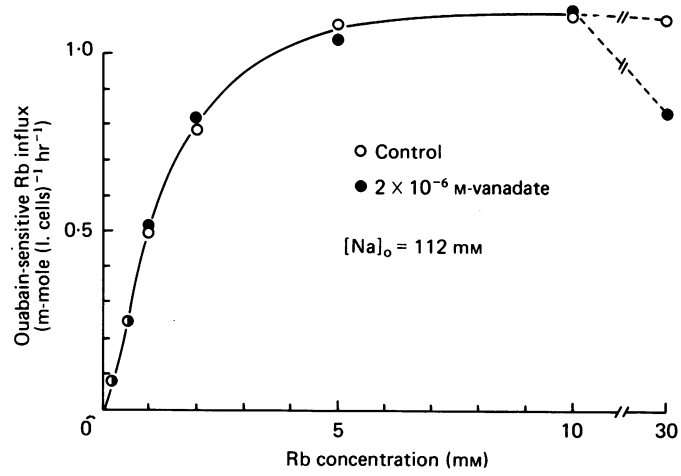


Fig. 1. The effect of $2{\cdot}2 \times 10^{-6}$ M-vanadate on the ouabain-sensitive influx of rubidium into human red cells incubated in high-sodium media containing different concentrations of rubidium. Red cells from freshly drawn heparinized blood were washed 5 times, at room temperature, with a solution containing 150 mM-sodium chloride, 1 mM-magnesium chloride, 10 mM-Tris/Tris Cl (pH 7·4 at 37 °C) and 0·1 mM-EGTA, and were resuspended at a haematocrit of 20 % in similar solutions containing 14 mM-glucose, with and without $2{\cdot}5 \times 10^{-6}$ M-sodium orthovanadate and with and without $10^{-4}$ M-ouabain. The suspensions were incubated at 37 °C for 2 hr, to allow the vanadate to equilibrate across the cell membranes, and were then cooled to 0 °C. Portions of the suspensions, 0·1 ml. in volume, were added to tubes containing 0·9 ml. volumes of ice-cold solutions whose composition was such that the final concentrations were: 112 mM-sodium chloride, 0·2–30 mM-[$^{86}$Rb]RbCl, 29·8–0 mM-choline chloride, 0·96 mM-magnesium chloride, 9·6 mM-Tris/Tris Cl (pH 7·6 at 37 °C), 0·1 mM-EGTA, 9 mM-glucose, 0 or $2{\cdot}2 \times 10^{-6}$ M-vanadate, and 0 or $2 \times 10^{-4}$ M-ouabain. After the contents of each tube had been mixed, the tubes were incubated at 37 °C for 30 min and returned to the ice-bath. The cells were centrifuged and washed 3 times with an ice-cold wash solution like that used to wash the cells originally, and were then lysed by the addition of 0·9 ml. water. The protein was removed by precipitation with trichloracetic acid (final concentration 5 %, w/v), and the $^{86}$Rb content of the supernatants was determined by scintillation counting. Each point in the Figure represents the mean of two duplicates.

Vanadate might seem to be less effective, or ineffective, as an inhibitor at low external rubidium concentrations, if the onset of inhibition were slow and were accelerated by increasing the rubidium concentration in the relevant range. To test that hypothesis, we arranged that unlabelled rubidium, at a concentration of 10 or 30 mM, should be present during the last 0, 20 or 40 min of the preincubation period. [$^{86}$Rb]RbCl were then added, and the uptake of radioactivity during the next 30 min was measured. The results are summarized in Fig. 4 and show that the presence of rubidium during the preincubation did not affect the degree of inhibition. The effect of rubidium cannot, therefore, be attributed to an acceleration of the rate of onset of inhibition.

The experiments described above were all done using rubidium, because $^{86}Rb$ is a more convenient isotope than $^{42}K$, but essentially similar results were also obtained with potassium (see, for example, Fig. 5).

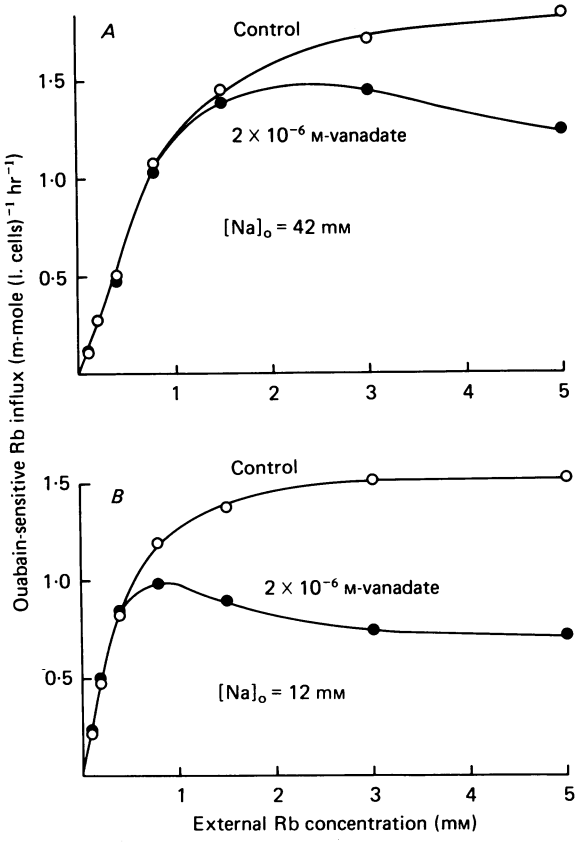


Fig. 2. The relation between ouabain-sensitive rubidium influx and the external rubidium concentration, in the presence and absence of vanadate ($2 \times 10^{-6}$ M), in media containing 42 mM-sodium (*A*) or 12 mM-sodium (*B*). The procedure was similar to that described in the legend to Fig. 1 except that (i) the sodium concentration in the final incubation medium was 42 or 12 mM, (ii) the highest rubidium concentration used was 5 mM, and (iii) at the end of the 2 hr preincubation, the cell suspensions were not cooled, but 0·1 ml. portions were transferred at timed intervals to 0·9 ml. portions of pre-warmed final incubation media in Eppendorf centrifuge tubes.

*The dependence of inhibition on intracellular magnesium*

The inhibitory effect of vanadate on Na,K-ATPase is known to be highly sensitive to the magnesium concentration (Hudgins & Bond, 1977). To discover whether the magnesium ions act at the inner or outer surface of the cell membrane, we first measured potassium influx into red cells, at several different external potassium concentrations, in the presence of $3{\cdot}3 \times 10^{-6}$ M-vanadate, with either 6 mM-magnesium or no magnesium in the suspending media. The results are summarized in Fig. 5*A*, and show that extracellular magnesium had no effect on the vandate inhibition. To

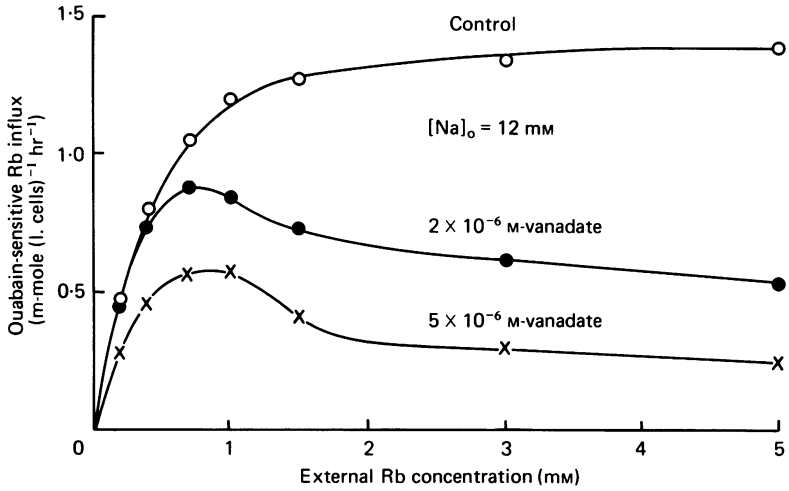


Fig. 3. The effects of vanadate, at two concentrations, on the ouabain-sensitive influx of rubidium into intact red cells incubated in media containing 12 mM-sodium and different concentrations of rubidium. The procedure was similar to that described in the legend to Fig. 2, except that (i) the preincubation media contained 0, $2{\cdot}5 \times 10^{-6}$ or $6{\cdot}2 \times 10^{-6}$ M-vanadate, (ii) the final incubation media contained 0, $2{\cdot}2 \times 10^{-6}$ or $5{\cdot}5 \times 10^{-6}$ M-vanadate, and (iii) the sodium concentration in the final incubation media was only 12 mM.

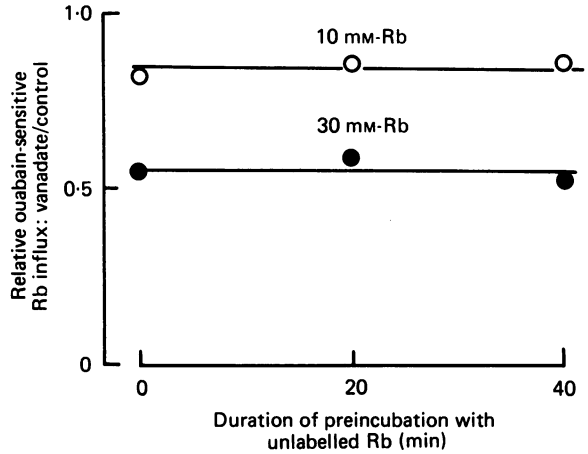


Fig. 4. An experiment showing that the greater effectiveness of vanadate at high rubidium concentrations is not the result of a faster onset of inhibition. Cells were prepared, and preincubated with $2{\cdot}5 \times 10^{-6}$ M-vanadate, as described in Fig. 1. At 120, 140 or 160 min after the start of the preincubation period, 0·1 ml. samples of the suspensions were transferred, in duplicate, to Eppendorf centrifuge tubes containing 0·9 ml. portions of warm media whose composition was such as to give final concentrations of 10 or 30 mM-rubidium chloride (unlabelled), 20 or 0 mM-choline chloride, 127 mM-sodium chloride, 1 mM-magnesium chloride, 10 mM-Tris/Tris Cl (pH 7·4 at 37 °C), 0·09 mM-EGTA, 9 mM-glucose, 0 or $2{\cdot}2 \times 10^{-6}$ M-sodium orthovanadate, and 0 or $1{\cdot}2 \times 10^{-4}$ M-ouabain. These tubes were kept at 37 °C. At 160 min after the start of the preincubation, 0·02 ml. 1 mM-[$^{86}$Rb]RbCl was added to each of the Eppendorf tubes, and the incubation was continued for a further 30 min. The subsequent procedure was as described in the legend to Fig. 1. Thus the cells in each of the vanadate tubes were preincubated with vanadate for 160 min, of which the last 0, 20 or 40 were in the presence of unlabelled rudidium at a concentration of 10 or 30 mM.

test the effect of intracellular magnesium, we did a similar experiment with red cells which had been treated with the ionophore A23187, and preincubated in high-magnesium or low-magnesium media so that they contained either about 500 $\mu$M-free magnesium or about 26 $\mu$M-free magnesium. From the results, which are summarized in Fig. 5*B*, it is clear that only the high-magnesium cells showed the inhibition at high potassium levels, characteristic of cells under the influence of

TABLE 1. The effects of vanadate (3·3 $\mu$M) on the ouabain-sensitive efflux of potassium from red cells incubated in high-sodium, potassium-free media (pump reversal), or high-sodium media containing 5 or 14 mM-potassium (potassium–potassium exchange)

| | Rate constants for ouabain-sensitive potassium efflux* | | |
|---|---|---|---|
| | Potassium-free medium ($hr^{-1}$) | 5 mM-potassium medium ($hr^{-1}$) | 14 mM-potassium medium ($hr^{-1}$) |
| Experiment 1 | | | |
| Control | 0·0078 ± 0·0009 | 0·0112 ± 0·0011 | 0·0110 ± 0·0011 |
| Vanadate | 0·0073 ± 0·0006 | 0·0096 ± 0·0006 | 0·0048 ± 0·0007 |
| Experiment 2 | | | |
| Control | 0·0071 ± 0·0008 | 0·0129 ± 0·0006 | 0·0118 ± 0·0007 |
| Vanadate | 0·0083 ± 0·0004 | 0·0108 ± 0·0008 | 0·0050 ± 0·0007 |

* In these experiments, vanadate had no significant effect on the potassium efflux in the presence of ouabain under any conditions.

In *Experiment 1*, red cells from freshly drawn heparinized blood were washed three times, at room temperature, with a solution containing 146 mM-choline chloride, 1·5 mM-magnesium chloride, and 10 mM-imidazole (pH 7·4 at 20 °C), and were then loaded with $^{42}K$ by incubating them at a haematocrit of 50 %, at 37 °C, in a solution containing 62 mM-[$^{42}K$]KCl, 82 mM-choline chloride, 1 mM-magnesium chloride, 0·1 mM-EGTA, 2·5 mM-potassium hydrogen phosphate and 5 mM-HEPES, adjusted to pH 7·4 (at 20 °C), with Tris base. After 3·5 hr, sodium orthovanadate was added to one half of the suspension to give a final concentration of $4 \times 10^{-6}$ M, and the incubation was continued for another 30 min. The suspensions were cooled to 0 °C and the cells centrifuged and washed four times with an ice-cold solution containing 140 mM-sodium chloride, 1 mM-magnesium chloride, 0·1 mM-EGTA, 2·5 mM-sodium hydrogen phosphate, and 5 mM-HEPES (pH 7·4 at 20 °C), with or without vanadate ($4 \times 10^{-6}$ M) as appropriate. The washed cells were resuspended in more of the same wash solutions, at a haematocrit of 6 %, and 1 ml. aliquots were dispensed into Eppendorf centrifuge tubes which already contained 0·2 ml. portions of similar media in which sufficient sodium chloride had been replaced by potassium chloride to give final potassium concentrations of 0, 5 or 14 mM. Half of the tubes also contained ouabain (final concentration $10^{-4}$ M). The final vanadate concentration in the vanadate tubes was $3·3 \times 10^{-6}$ M. The tube contents were mixed by inversion of the racks, which were then transferred to the 37 °C bath. After 5 min and after 65 min tubes were removed, cooled and centrifuged, and the $^{42}K$ contents of the supernatants were determined by measuring the Čerenkov radiation. (The 5 min and 65 min losses of $^{42}K$ were determined in quadruplicate.) The 541 nm absorbance of each supernatant was also measured in order to allow corrections for haemolysis to be made. The $^{42}K$ contents of the original cell suspensions were estimated after treatment with trichloracetic acid (5 %, w/v) and removal of the precipitate. In *Experiment 2*, the procedure was similar except that, in order to minimize differences in composition between the vanadate and the control cells, vanadate was not added during the preincubation period but was present in the solution used for the fourth wash and in the final incubation medium. To allow time for the vanadate to enter the cells the 'initial' tubes were removed after 15 min instead of 5 min and the final tubes were removed after 75 min. The figures in the Table represent the differences between the means of rate constants for potassium efflux in the presence and absence of ouabain ± S.E. of the differences of means.

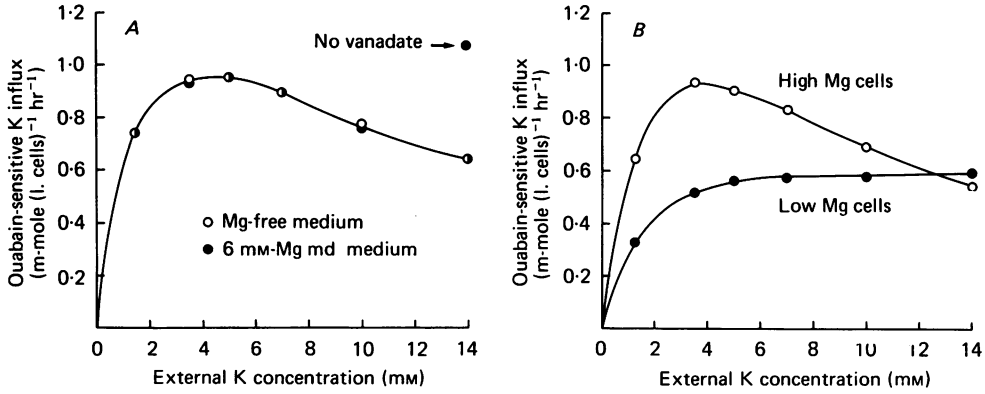


Fig. 5. The effects of extracellular and intracellular magnesium on the inhibition by vanadate of ouabain-sensitive potassium influx into red cells incubated in media of different potassium concentrations. *A, the effect of extracellular magnesium.* Red cells from freshly drawn heparinized blood were washed three times with an ice-cold magnesium-free wash solution containing 135 mM-sodium chloride, 9 mM-choline chloride, 10 mM-imidazole (pH 7·4 at 20 °C), and 10 mM-glucose, and were incubated at a haematocrit of 5 % at 37 °C for 1 hr in similar media with or without $4 \times 10^{-6}$ M-sodium orthovanadate. After this preincubation, the cells were washed with more of the same media, with or without vanadate as appropriate, and were resuspended, at haematocrits of about 3 %, as follows: the vanadate-free cells were suspended in more of the ice-cold magnesium-free wash solution; the vanadate-containing cells were suspended in similar media with or without 7·2 mM-magnesium chloride (magnesium chloride replacing choline chloride). Portions of each cell suspension, 2·5 ml. in volume, were squirted, in triplicate, into glass tubes to which had been added 0·5 ml. of solutions containing 135 mM-sodium chloride, 10 mM-imidazole (pH 7·4 at 37 °C) 10 mM-glucose, appropriate concentrations of [$^{42}$K]KCl (replacing sodium chloride), and between 6·0 and 8·3 mM-choline chloride. Some of the tubes also contained sufficient ouabain to give a final concentration of $10^{-4}$ M. In the tubes containing vanadate, its final concentration was $3{\cdot}3 \times 10^{-6}$ M; in the tubes containing magnesium its final concentration was 6 mM. All the tubes were incubated at 37 °C for 1 hr and then replaced in the ice-bath. The cells were centrifuged and washed three times with an ice-cold solution containing 106 mM-magnesium chloride and 10 mM-Tris/Tris Cl (pH 7·4 at 20 °C). The cell pellet was extracted with 7 ml. 5 % (w/v) trichloracetic acid, the precipitate removed by centrifuging, and the $^{42}$K in the supernatant estimated by its Cerenkov radiation.

*B, the effect of intracellular magnesium.* Red cells from freshly drawn heparinized blood were washed three times with an ice-cold solution containing 135 mM-sodium chloride, 5 mM-potassium chloride, 20 mM-imidazole (pH 7·4 at 20 °C) and 10 mM-glucose, and 8 ml. of the washed cells were then incubated, at a haematocrit of 5 %, for 1 hr at 37 °C in a similar medium supplemented with $4 \times 10^{-6}$ M-vanadate. The vanadate-loaded cells were washed 3 times in two 100 ml. tubes with an ice-cold 'choline, vanadate wash solution' containing 140 mM-choline chloride, 20 mM-imidazole (pH 7·4 at 20 °C), 10 mM-glucose, 20 μM-EGTA (Tris salt) and $4 \times 10^{-6}$ M-vanadate. The cells in one tube were resuspended in 140 ml. of a similar solution which had been supplemented with 3·5 mM-ATP (disodium salt), 0·826 mM-magnesium chloride and 0·2 mg of the ionophore A23187. The mixture of ATP and magnesium chloride was calculated to give a free-magnesium concentration of 26 μM. The cells of the other tube were suspended in a similar solution supplemented with 3·5 mM-ATP, 3·5 mM-magnesium chloride and 0·2 mg A23187. This mixture was calculated to give a free-magnesium concentration of 507 μM. Each suspension was divided between two 100 ml. glass centrifuge tubes which were transferred to the 37 °C bath for 14 min, to allow magnesium ions to equilibrate

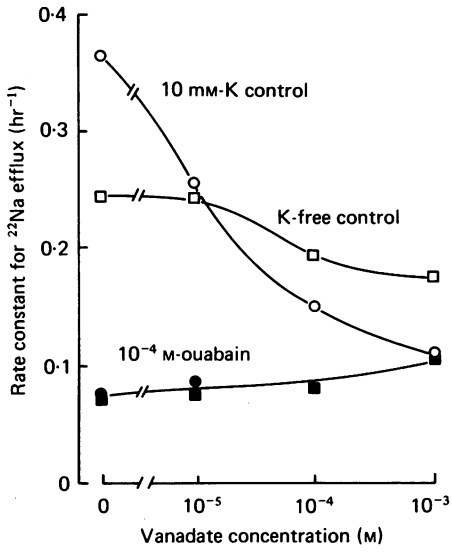


Fig. 6. The effects of vanadate, at different concentrations, on the Na efflux associated with sodium–potassium exchange and sodium–sodium exchange in intact red cells. Red cells from freshly drawn heparinized blood were washed five times, at room temperature, with a 'high sodium, potassium-free wash solution' containing 150 mM-sodium chloride, 1 mM-magnesium chloride, 10 mM-Tris/Tris Cl (pH 7·4 at 20 °C), 0·1 mM-EGTA and 11 mM-glucose. The washed cells were resuspended at a haematocrit of about 12 % in more of the same wash solution supplemented with 0, $10^{-5}$, $10^{-4}$ or $10^{-3}$ M-sodium orthovanadate, and with tracer amounts of $^{22}$Na. The cells were incubated in these solutions at 37 °C for 2 hr, in order to load them with $^{22}$Na and to allow the vanadate to equilibrate, and were then washed seven times at 0 °C in similar solutions lacking tracer. The loaded, washed cells were finally suspended, at a haematocrit of 20 %, in more of the 'high sodium, potassium-free wash solution' supplemented as appropriate with vanadate. Aliquots (0·05 ml.) of the suspensions were added, in triplicate, to Eppendorf centrifuge tubes containing 0·95 ml. portions of the 'high sodium, potassium-free wash solution' or of a similar solution with 11 mM-potassium chloride replacing an equivalent amount of sodium chloride, supplemented as appropriate with vanadate, and with ouabain. The final haematocrit was 1 %, the final potassium concentration was 0 or 10·6 mM, and the final ouabain concentration was 0 or $10^{-4}$ M. The tubes were incubated at 37 °C for 1 hr, cooled in an ice-bath, and centrifuged. The $^{22}$Na contents of the supernatants were then determined by scintillation counting.

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across the membrane, and were then returned to the ice-bath. The magnesium-loaded cells were washed three times with ice-cold 'choline, vanadate wash solution' supplemented *either* with 0·5 mM-ATP and 0·14 mM-magnesium chloride (giving 26 $\mu$M-free magnesium) *or* 0·5 mM-ATP and 0·93 mM-magnesium chloride (giving 503 $\mu$M-free magnesium), and were finally suspended, at a haematocrit of 4 % in similar ice-cold media containing 140 mM-sodium chloride instead of 140 mM-choline chloride. Portions of each cell suspension, 2·5 ml. in volume, were dispensed, in triplicate, into a series of glass tubes containing 0·5 ml. of similar ice-cold Na medium in which enough of the sodium chloride had been replaced by [$^{42}$K]KCl to give the desired final potassium concentrations. The final vanadate concentration was $3{\cdot}3 \times 10^{-6}$ M. Half of the tubes also contained sufficient ouabain to give a final concentration of $10^{-4}$ M. The tubes were incubated at 37 °C for 1 hr and potassium influx was estimated in the usual way (see *A*).

vanadate. Although the suspending media, in this experiment, also differed in their magnesium contents – having free magnesium ion concentrations similar to those in the cells – the effect observed must have been due to the intracellular magnesium since extracellular magnesium was shown to be ineffective by the experiment of Fig. 4*A*. The low level of potassium influx, at saturating potassium concentrations, in the low-magnesium cells was almost certainly due to the low intracellular magnesium concentration (Flatman & Lew, 1979), and not to inhibition by vanadate.

TABLE 2. The effects of vanadate ($3{\cdot}3 \times 10^{-6}$ M) on the efflux of sodium from resealed ghosts incubated in high-sodium, potassium-free media (sodium–sodium exchange) or high-sodium 14 mM-potassium media (sodium–potassium exchange)

| | | Rate constants for sodium efflux | |
|---|---|---|---|
| | | Ouabain-resistant ($hr^{-1}$) | Ouabain-sensitive ($hr^{-1}$) |
| Potassium-free media | Control | $0{\cdot}226 \pm 0{\cdot}003$ | $0{\cdot}120 \pm 0{\cdot}005$ |
| | Vanadate | $0{\cdot}188 \pm 0{\cdot}002$ | $0{\cdot}123 \pm 0{\cdot}004$ |
| 14 mM-potassium media | Control | $0{\cdot}241 \pm 0{\cdot}003$ | $0{\cdot}268 \pm 0{\cdot}008$ |
| | Vanadate | $0{\cdot}193 \pm 0{\cdot}002$ | $0{\cdot}179 \pm 0{\cdot}004$ |

Red cells from freshly drawn heparinized blood were washed 3 times at room temperature with a solution containing 146 mM-choline chloride, 1·5 mM-magnesium chloride, and 10 mM-imidazole (pH 7·4 at 20 °C), and packed. One ml. samples of the packed cells were lysed in 10 ml. volumes of ice-cold lysing solution containing 3 mM-ATP (disodium salt), 3·5 mM-magnesium chloride, 20 mM-imidazole, 0·2 mM-EGTA (Tris salt), and 4 mM-[$^{22}$Na]NaCl, with or without $4 \times 10^{-6}$ M-sodium orthovanadate. The pH of the lysing solution had been adjusted with Tris base to pH 7·4 at 20 °C. After 5 min, tonicity was restored to 310 ideal m-osmole/l. by the addition of 3 M-choline chloride, and the ghosts were incubated at 37 °C for 40 min to allow resealing to occur. The two lots of resealed ghosts were washed five times with an ice-cold wash solution containing 140 mM-sodium chloride, 1·5 mM-magnesium chloride, 1 mM-calcium chloride, 10 mM-imidazole (pH 7·4 at 20 °C) 10 mM-glucose and, where appropriate, $4 \times 10^{-6}$ M-vanadate. The washed ghosts of each lot were finally resuspended in 40 ml. of the appropriate wash solution, and 1·25 ml. aliquots were dispensed into tubes which already contained 0·25 ml. portions of wash solution without vanadate, and, where appropriate, with sufficient sodium chloride replaced by potassium chloride to give a final potassium concentration of 14 mM. Half of the tubes also contained ouabain (final concentration $10^{-4}$ M). The final vanadate concentration was $3{\cdot}3 \times 10^{-6}$ M. The tube contents were mixed by inversion of the racks, which were then transferred to the 37 °C bath. After 5 min and after 45 min, tubes were removed, cooled and centrifuged, and the $^{22}$Na contents of the supernatants were measured by scintillation counting. The $^{22}$Na contents of the original cell suspensions were estimated after treatment with trichloracetic acid (5 %, w/v) and removal of the precipitate.

*The effects of vanadate on potassium–potassium exchange and pump reversal*

To investigate the effects of vanadate on potassium–potassium exchange and on pump reversal we loaded intact red cells with $^{42}$K and measured the ouabain-sensitive potassium efflux into high-sodium media containing 5 or 14 mM-potassium (potassium–potassium exchange) or no potassium (pump reversal). The results of two experiments are summarized in Table 1. They show that, at a concentration of $3{\cdot}3 \times 10^{-6}$ M, vanadate inhibited potassium–potassium exchange by rather more than 50 %, but had no significant effect on pump reversal. It is also worth noting that the inhibition of ouabain-sensitive potassium efflux by vanadate was much more marked

with 14 mM-potassium in the medium than with 5 mM-potassium, even though 5 mM-potassium is sufficient to allow potassium–potassium exchange to occur at nearly its maximal rate (see fig. 5 of Glynn, Lew & Lüthi (1970)).

*The effects of vanadate on sodium efflux associated with* (i) *sodium–potassium exchange, and* (*ii*) *sodium–sodium exchange*

Fig. 6 shows the effects of vanadate on the efflux of sodium from intact red cells incubated in high-sodium media containing 10 mM-potassium or no potassium. It is clear that the efflux of sodium into the 10 mM-potassium medium, i.e. sodium efflux associated with sodium–potassium exchange, was much more sensitive to inhibition by vanadate than sodium efflux into the potassium-free medium, i.e. sodium efflux associated with sodium–sodium exchange. A similar result was obtained in an experiment on resealed red cell ghosts (see Table 2). In this experiment, the vana-

TABLE 3. The effects of vanadate (3·3 $\mu$M) on the ouabain-sensitive efflux of sodium from resealed ghosts incubated in Na-free media containing no potassium (uncoupled sodium efflux) or 5 mM-potassium (sodium–potassium exchange)

| | Rate constants for ouabain-sensitive sodium efflux* | |
|---|---|---|
| | Potassium-free medium ($hr^{-1}$) | 5 mM-potassium medium ($hr^{-1}$) |
| Control | 0·048 ± 0·011 | 0·242 ± 0·011 |
| Vanadate | 0·013 ± 0·009 | 0·010 ± 0·006 |

* In this experiment, vanadate had no significant effect on the efflux of sodium in the presence of ouabain, either with no potassium or with 5 mM-potassium in the medium.

Red cells from freshly drawn heparinized blood were washed 3 times, at room temperature with a medium containing 150 mM-choline chloride, 1 mM-magnesium chloride, 2 mM-HEPES (Tris salt; pH 7·4 at 20 °C) and 0·1 mM-EGTA (Tris salt), and were then lysed in 50 volumes of ice-cold lysing solution containing 1 mM-ATP (disodium salt), 6 mM-[$^{24}$Na]NaCl, 1·5 mM-magnesium chloride, 5 mM-HEPES (Tris salt; pH 7·3 at 20 °C) and 0·2 mM-EGTA (Tris salt). After 5 min, sufficient 3 M-choline chloride was added to restore the tonicity to 107 ideal m-osmole/l., and the suspension was incubated at 37 °C for 20 min to allow resealing to occur. The ghosts were then washed 5 times with a hypotonic wash solution containing 50 mM-choline chloride, 1 mM-magnesium chloride, 2 mM-HEPES (Tris salt; pH 7·4) and 0·2 mM-EGTA (Tris salt). The wash solution was ice-cold for the first, second and fourth washes, and at 37 °C for the third and fifth wash; and the suspension was incubated at 37 °C for 5 min after each of the warm washes and then diluted with cold wash solution before the centrifuging. (The aim of the warm washes, and of the incubation, was to reduce the initial loss of $^{24}$Na when the ghosts were warmed in the final incubation medium.) The washed ghosts were finally suspended, at a haematocrit of about 0·4%, in more of the ice-cold hypotonic wash solution, with or without sodium orthovanadate ($4 \times 10^{-6}$ M), and 2·5 ml. portions were added to glass tubes which already contained 0·5 ml. of the same hypotonic wash solution supplemented where appropriate with sufficient potassium chloride to give a final concentration of 5 mM, and sufficient ouabain to give a final concentration of $10^{-4}$ M. The final concentration of vanadate in the vanadate tubes was $3{\cdot}3 \times 10^{-6}$ M. The tubes were incubated at 37 °C for 15 min (initials) or 60 min (finals), and were then cooled to 0 °C and centrifuged. $^{24}$Na was estimated, by measurement of the Čerenkov radiation, in the supernatants, and also in the whole cell suspensions after treatment with trichloracetic acid (final concentration 5%, w/v) and removal of the precipitate. The rate constants for Na efflux were calculated from the fractional losses of radioactivity from the ghosts between 15 and 60 min. The flux in each set of conditions was measured in quintuplicate.

date concentration was only $3 \cdot 3 \times 10^{-6}$ M, and there was no significant inhibition of ouabain-sensitive efflux of sodium into the potassium-free medium, although efflux into a 14 mM-potassium medium was reduced to two thirds of the control value.

The small effects of vanadate on the ouabain-*in*sensitive effluxes of sodium in the experiments of Fig. 6 and of Table 2 are puzzling, since they are in opposite directions. They could presumably reflect changes in leakiness, changes in the ouabain-insensitive facilitated transfer mechanism for univalent cations, or changes in the composition of the cells due to the presence of vanadate during the preincubation.

TABLE 4. The effects of vanadate ($3 \cdot 3 \times 10^{-6}$ M) on the Na-ATPase activity and on the Na,K-ATPase activity, of resealed ghosts incubated in sodium-free media

| | Rate of ouabain-sensitive hydrolysis of ATP | |
|---|---|---|
| | Potassium-free medium (m-mole (l.cells)$^{-1}$ hr$^{-1}$) | 5 mM-potassium medium (m-mole (l.cells)$^{-1}$ hr$^{-1}$) |
| Control | $0 \cdot 201 \pm 0 \cdot 013$ | $0 \cdot 631 \pm 0 \cdot 018$ |
| Vanadate | $0 \cdot 020 \pm 0 \cdot 010$ | $0 \cdot 012 \pm 0 \cdot 007$ |

Red cells from freshly drawn heparinized blood were washed once, at room temperature, with a solution containing 130 mM-sodium chloride, 10 mM-potassium chloride, 1 mM-magnesium chloride, 10 mM-Tris/Tris Cl (pH 7·4 at 20 °C), 0·1 mM-EGTA (Tris salt) and chloramphenicol (25 $\mu$g/ml.), and were then incubated in more of the same solution, at a haematocrit of 2·5 %, at 37 °C for 40 hr. About 4 ml. of the cells were then washed in more of the same medium, resuspended in 50 ml. of similar medium which had been supplemented with iodoacetamide (6 mM) and inosine (6 mM), and incubated at 37 °C for a further 3 hr in order to get rid of the remaining ATP, ADP, and glycolytic intermediates. The cells were then washed three times, at room temperature, with a solution containing 150 mM-choline chloride, 1 mM-magnesium chloride, 1 mM-HEPES (Tris salt; pH 7·4 at 20 °C), and 0·1 mM-EGTA (Tris salt), packed, and lysed by squirting 1 ml. packed cells into 30 ml. ice-cold lysing solution. The lysing solution contained 0·1 mM-[$\gamma^{32}$P]ATP (disodium salt), 5 mM-[$^{32}$P]creatine phosphate (sodium salt), 0·05 mM-creatine, 2 mM-inosine, 2·5 mM-magnesium chloride, 5 mM-HEPES (Tris salt; pH 7·3 at 20 °C), 0·1 mM-EGTA (Tris salt), creatine kinase (5 u./ml.), and hexokinase (5 u./ml.). (The specific activity of the creatine phosphate and of the $\gamma$ phosphate groups in the ATP were identical, because the creatine phosphate, ATP, creatine and enzymes had previously been incubated together, for 1 hr at 37 °C, at 5 times their concentrations in the lysing solution – see Glynn & Karlish, 1976). Five min after lysis, sufficient 3 M-choline chloride was added to restore the tonicity to 306 ideal m-osmole/l. The suspension was divided into two lots, and sodium orthovanadate added to one lot to give a final concentration of $4 \times 10^{-6}$ M. Both lots were incubated at 37 °C for 20 min, to allow resealing to occur, and the resealed ghosts were then washed five times with a choline wash solution identical with that used to wash the cells before lysis, supplemented where appropriate with vanadate ($4 \times 10^{-6}$ M). These washes were done with ice-cold wash solution, except for the third, which was done with wash solution at 37 °C in order to facilitate the exit of inosine from the ghosts. The washed ghosts were finally resuspended in more of the ice-cold choline wash solution, with or without vanadate ($4 \times 10^{-6}$ M), at a haematocrit of 1 % (on the basis of the original cell volume). Portions of the suspension, 2·67 ml. in volume, were dispensed into glass tubes which already contained 0·33 ml. of ice-cold vanadate-free choline wash medium, with or without sufficient potassium chloride to give a final concentration of 5 mM, and with or without sufficient ouabain to give a final concentration of $6 \cdot 7 \times 10^{-5}$ M. The final vanadate concentration in the vanadate tubes was $3 \cdot 3 \times 10^{-6}$ M. The tubes were incubated at 37 °C for 30 min, and were then returned to the ice bath. Each received 100 $\mu$l 300 mM-2-deoxyglucose and was reincubated at 37 °C for 15 min in order to allow $^{32}$P from the relatively unstable creatine phosphate to be transferred, via ATP, to the more stable 2-deoxyglucose-6-phosphate. Extraction of [$^{32}$P]orthophosphate, and the estimation of its radioactivity and of the total radioactivity were done as described by Glynn & Karlish, 1976. The figures represent the ouabain-sensitive release of $^{32}$P orthophosphate per litre of original cells per hour $\pm$ S.E. of mean ($n = 5$).

*The effects of vanadate on uncoupled sodium efflux and on the associated hydrolysis of ATP*

Uncoupled sodium efflux refers to the ouabain-sensitive efflux of sodium that is observed when cells are incubated in media lacking both sodium and potassium. When intact red cells are incubated in such media, some of the ouabain-sensitive sodium efflux that is observed probably represents an exchange of sodium for potassium that has leaked from the cells; for this reason, uncoupled sodium efflux is best studied using resealed red cell ghosts that contain little potassium. We have made several attempts to discover whether vanadate inhibits the uncoupled efflux of sodium from such ghosts, but the flux is very small and in all but one experiment the scatter in the results made them inconclusive. The one successful experiment is summarized in Table 3, and suggests that vanadate, at a concentration of $3{\cdot}3 \times 10^{-6}$ M does inhibit the uncoupled sodium efflux, though the extent of the inhibition cannot be assessed at all accurately.

In view of the difficulties in measuring uncoupled sodium efflux, we decided to investigate the effect of vanadate on the hydrolysis of ATP that is associated with that flux (Glynn & Karlish, 1976). Using the technique developed by Glynn & Karlish, we prepared resealed red cell ghosts containing a system capable of regenerating [$\gamma^{32}$P]ATP of constant specific activity, and we measured the rate of ouabain-sensitive ATP hydrolysis, in the presence and absence of vanadate, when the ghosts were incubated in a choline medium lacking both sodium and potassium. The results are summarized in Table 4, and show that vanadate, at a concentration of $3{\cdot}3 \times 10^{-6}$ M strongly inhibited the ATP hydrolysis.

Table 4 also shows the powerful inhibitory effect of vanadate on the rate of ouabain-sensitive ATP hydrolysis when the same resealed ghosts were incubated in a choline medium containing 5 mM-potassium. In this medium, the Na,K-ATPase in the ghost membranes would have been catalysing sodium–potassium exchange, and the potency of the vanadate at a relatively low external potassium concentration is presumably explained by the absence of sodium from the medium.

## DISCUSSION

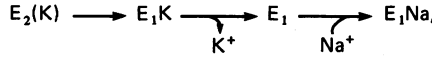
The results described above show that the sensitivity of the sodium pump mechanism to inhibition by vanadate depends on which mode of behaviour the mechanism is displaying. Sodium–potassium exchange, potassium–potassium exchange and uncoupled sodium efflux can all be inhibited by vanadate at very low concentrations; sodium–sodium exchange and reversal of the pump cannot.

This pattern is not what one would have expected from previous work on the inhibition of Na,K-ATPase by vanadate, for that work suggested that vanadate could inhibit only if there were sufficient potassium in the solution bathing the extracellular surface of the membrane. The unexpected feature of the present results is the powerful inhibition of the uncoupled sodium efflux and the ATPase activity associated with it; unexpected, first, because the cells were incubated in potassium-free media, secondly, because the Na-ATPase activity of partially purified Na,K-ATPase preparations has been found to be rather insensitive to vanadate (Cantley,

Cantley & Josephson, 1978; Beaugé, 1979), and thirdly because the efflux of sodium from squid axons perfused with ATP without ADP, and bathed in high-sodium, potassium-free, media, was insensitive to vanadate (Beaugé & DiPolo, 1979).

An obvious way to reconcile the present and past results is to suppose that extracellular potassium is necessary for vanadate to inhibit, only if sodium is present in the extracellular medium. If that is correct, the implication would seem to be that potassium promotes inhibition by displacing sodium ions from extracellular sites at which they prevent vanadate from acting. These sites cannot be the sites, or cannot only be the sites, from which potassium is transported into the cell, for at low vanadate and high sodium concentrations, the range of concentrations in which external potassium promotes inhibition by vanadate is rather higher than the range in which increasing the concentration of potassium greatly accelerates the rate of potassium influx (see Fig. 1). A similar disparity in the effective concentration ranges was noted in experiments in which the rate of hydrolysis of ATP was measured (see Fig. 1 of Beaugé & Glynn, 1977*b*). It may be that the sodium ions act as sites of which we have been unaware, but a more economical hypothesis is to suppose that the relevant sites are those 'allosteric sites' at which extracellular sodium, in low concentrations, inhibits uncoupled sodium efflux (Garrahan & Glynn, 1967*a*, *b*; Lew, Hardy & Ellory, 1973; Karlish & Glynn, 1974; Glynn & Karlish, 1976), or reduces and makes equal the affinities for potassium of the two external binding sites from which potassium ions are transported into the cell (Garrahan & Glynn, 1967*b*; Sachs, 1967; Lew, Hardy & Ellory, 1973; Cavieres & Ellory, 1975). Because of interaction between the 'allosteric sites' and the sites from which potassium is transported into the cell, the sensitivity to vanadate should depend on the loading of all the external sites, even if the nature of the cations occupying the potassium-transporting sites does not directly affect the action of vanadate. The relation between vanadate inhibition and the external sodium and potassium concentrations will therefore be complicated. It is worth noting, however, that the similarity between the relative effectiveness of different potassium congeners in catalysing ATP hydrolysis and in promoting vanadate inhibition (Bond & Hudgins, 1979; Grantham & Glynn, 1979) cannot be used as an argument that the binding of potassium ions at the extracellular sites from which they are transported, directly affects vanadate inhibition. Because of the allosteric interaction, such binding would help to displace sodium from the 'allosteric sites'.

We must next consider how vanadate inhibits. Recent experiments by Karlish, Beaugé & Glynn (1979), using Na,K-ATPase labelled with fluorescein, have shown that vanadate slows the conformational change that occurs when sodium in high concentration is added to a suspension of the enzyme in a sodium-free, low-potassium medium in the absence of ATP. The sequence of reactions in the absence of vanadate is thought to be.




where $E_2(K)$ denotes a form of the enzyme containing occluded potassium ions (see Beaugé & Glynn, 1979), and the first step – i.e. the step inhibited by vanadate – is rate-limiting.

Since the conversion of $E_2(K)$ to $E_1K$ is thought to be an essential step in potassium entry, both during the normal working of the pump and during potassium–potassium exchange (Karlish, Yates & Glynn, 1978; Karlish & Yates, 1978; Glynn, Karlish & Yates, 1979; Beaugé & Glynn, 1979, 1980), the inhibitory effect of vanadate on sodium–potassium exchange and on potassium–potassium exchange is readily explained. The explanation of the inhibition of uncoupled sodium efflux is less clear. One possibility is that, although $E_2(K)$ is not, in this case, an intermediate in the reaction cycle, some could be formed from intracellular potassium; if vanadate prevented its breakdown, most of the enzyme could eventually be trapped as $E_2(K)$ and therefore made unavailable for phosphorylation by ATP. An alternative possibility is that vanadate acts by stabilizing the enzyme in the $E_2$ form without potassium. In their experiments with fluorescein-labelled enzyme, Karlish *et al.* (1979) found that vanadate could convert the enzyme into the $E_2$ form in the absence of potassium, and although it promptly reverted to the $E_1$ form when sodium was added (to a final concentration of 80 mM), it is not clear whether sodium would have had this effect at lower concentrations or if it had had access only to the intracellular surface of the Na,K-ATPase.

The failure of vanadate, in low concentrations, to inhibit pump reversal is understandable, since the conversion of $E_2(K)$ to $E_1K$ is thought not to occur in the reversed cycle. It implies, however, that, in stabilizing $E_2(K)$, vanadate does not prevent it from reacting with orthophosphate to form $E_2P.K$, and hence proceeding backwards round the cycle. The failure of vanadate, in low concentrations, to inhibit sodium–sodium exchange is also understandable, since at any time nearly all of the enzyme should be in the phosphorylated form. It is not clear, however, why sodium–sodium exchange is inhibited at higher vanadate concentrations. It may be that, at high enough concentrations, vanadate can trap a substantial fraction of the enzyme in the $E_2(K)$ form even though any single enzyme molecule is vulnerable for only a very small fraction of the time. Another possibility, is that the inhibition of sodium–sodium exchange at high vanadate concentrations is a consequence of the binding of vanadate to a different site with a lower affinity (cf. Cantley, Cantley & Josephson, 1978).

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