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STUDIES ON OUABAIN-COMPLEXED ($Na^+ + K^+$)-ATPase CARRIED OUT WITH VANADATE

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Vanadate is able to promote the binding of ouabain to ($Na^+ + K^+$)-ATPase and it is shown that vanadate is trapped in the enzyme-ouabain complex. Also ouabain-bound enzyme, the formation of which was facilitated by ($Mg^{2+} + Na^+ + ATP$) or ($Mg^{2+} + P_i$), is accessible to vanadate when washed free of competing ligands used for the promotion of ouabain binding. For vanadate binding to ($Na^+ + K^+$)-ATPase and to enzyme-ouabain complexes a divalent cation (Mg^{2+} or Mn^{2+}) is indispensable, indicating that the cation does not remain attached to the ouabain-bound enzyme. K^+ further increases vanadate binding in the absence of ouabain, but seems to have no additional role in case of vanadate binding to enzyme-ouabain complexes. Mn^{2+} is more efficient than Mg^{2+} in promoting binding of vanadate and ouabain to ($Na^+ + K^+$)-ATPase. That K^+ in combination with Mn^{2+}, in analogy with the effect in combination with Mg^{2+}, increases the equilibrium binding level of vanadate and decreases that of ouabain does not seem to favour the hypothesis of selection of a special E_2-subconformation by Mn^{2+}. The vanadate-trapped enzyme-ouabain complex was examined for simultaneous nucleotide binding which could demonstrate a two-substrate mechanism per functional unit of the enzyme. The acceleration by ($Na^+ + ATP$) of ouabain release from the ($Mg^{2+} + P_i$)-facilitated enzyme-ouabain complex does not, as anticipated, support such a mechanism. On the other hand, the deceleration of vanadate release as well as of ouabain release from a (Mg^{2+} + vanadate)-promoted complex could be consistent with a two-substrate mechanism working out-of-phase.

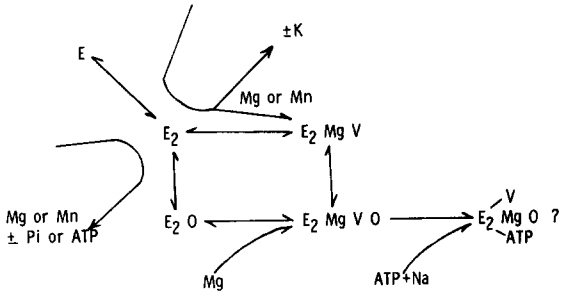
Introduction

Cardiac glycosides, for example, ouabain, are specific inhibitors of the sodium pump and the ($Na^+ + K^+$)-activated ATPase [1,2]. For high-affinity binding of ouabain to take place, certain conditions should be fulfilled, and studies on the factors which promote or impede ouabain binding to ($Na^+ + K^+$)-ATPase have given most useful information on the enzyme [3–8]. Many open questions as to the way ouabain binding takes place remain, however. Among them are the real nature of the K^+-ouabain antagonism [8,9] and whether phosphorylation is a prerequisite for ouabain interaction with ($Na^+ + K^+$)-ATPase, though the origin of phosphate would be obscure in cases where Mg^{2+} is the only ligand added for promotion of glycoside binding [4,10]. Furthermore, whether two distinct enzyme-ouabain complexes exist, a ($Mg^{2+} + Na^+$-ATP)-facilitated and a ($Mg^{2+} + P_i$)-facilitated one, or whether sliding conversion between them is possible and the real nature of their differences are likewise debated [11–14].

Recently, a supposed congener of phosphate, the pentavalent oxy-derivative of vanadium, vanadate, was discovered to be a high-affinity inhibitor of ($Na^+ + K^+$)-ATPase [15,16] and in combination with Mg^{2+} a very efficient ligand for promotion of ouabain binding [17]. The signal

initiated by the high-affinity binding of vanadate from the cytoplasmic side of the membrane gives rise to the emergence of a high-affinity ouabain receptor on the extracellular aspect of the cell membrane. In the present publication the simultaneous or sequential binding to $(Na^+ + K^+)$-ATPase of two high-affinity inhibitors has been exploited in an attempt to gain new insight into the multiplicity of enzyme-ouabain complexes. A number of ligands which are necessary for or will facilitate vanadate and ouabain interaction with the enzyme are also analyzed.

To guide the reader through the experimental section, a diagram summarizing the experimental situations and their interpretations is provided here. E represents $(Na^+ + K^+)$-ATPase; E_2, the state induced by a number of more or less permanently interacting ligands (Mg^{2+} or Mn^{2+}, $\pm P_i$, etc.) which may give rise to vanadate or ouabain binding; E_2O, enzyme-ouabain complex; E_2MgV and E_2MgVO, enzyme-vanadate and enzyme-vanadate-ouabain complexes (in which Mg is probably retained).



Under the assumption that vanadate binding resembles phosphorylation, whether obtained from P_i-binding or ATP-phosphorylation, and takes place at a nucleotide substrate site, the possibility of verifying by the use of vanadate and ATP the hypothesis of coexistence of two nucleotide substrate sites [18] is next dealt with (the complex with the question mark on the right-hand side of the diagram). The hypothesis implies one high and one low affinity site per functional unit of the $(Na^+ + K^+)$-ATPase working out of phase [18] and thus, a doubling of the number of substrate sites compared to the number of ouabain-binding sites. These extra sites should not be confused with the subdivision of the basic number of substrate sites (equal to the number of ouabain binding sites) in case of, for example, anticooperativity.

Some of the observations have been reported briefly elsewhere [19,20].

Materials and Methods

$(Na^+ + K^+)$-ATPase was prepared from pig kidney outer medulla as described by Jørgensen [21]. Alternatively, enzyme was prepared according to the first steps in Jørgensen's procedure. Microsomal enzyme suspended in 30 mM imidazole/250 mM sucrose/2 mM EDTA (pH 7.4, 20°C) was diluted in H_2O plus SDS to a final protein concentration of 3 mg/ml containing 0.03% SDS. After 20 h at room temperature the enzyme was spun down, resuspended in the above-mentioned buffer diluted 1:3, and finally washed twice by centrifugation in the dilute buffer. Enzyme was resuspended and stored in the buffer mentioned. The specific activity of the former preparation was 17.6 and of the latter 6.8 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$.

[^{48}V]Vanadyl chloride in HCl was obtained from Amersham International. The solution was neutralized by NaOH, buffered with Tris at pH 8.5, and stirred in the presence of air for conversion of vanadyl to vanadate. A stock solution of 400 mM vanadate was obtained by dissolving $NaVO_3$ (Merck) in 1 M NaOH. [^{3}H]Ouabain was obtained from New England Nuclear Corp. The isotope was purified by chromatography on $(Na^+ + K^+)$-ATPase as described elsewhere [22]. The extractability of [^{48}V]vanadate by $(Na^+ + K^+)$-ATPase was examined by a method similar to that described for [^{3}H]ouabain. Only batches that were 100% extractable were used.

Unless otherwise stated, vanadate binding took place at 25°C in the presence of 5 mM Mg^{2+}, 10 mM K^+ and 40 mM Tris-HCl (pH 7.25). Equilibrium binding was achieved in less than 60 min. Ouabain binding took place at 37°C in the presence of 3–5 mM Mg^{2+}, 40 mM Tris and with either the phosphorylating ligand indicated or vanadate. Ouabain binding was determined after 2 h incubation, at which time equilibrium had been reached. Bound and non-bound [^{48}V]vanadate or [^{3}H]ouabain were separated by filtration as described [22] except that Seitz membrane filters (pore size 0.8 μm) were used.

Illustrations presented are representative of at least three experiments.

Results

Does the enzyme-ouabain complex trap vanadate?

It was previously shown that vanadate in the same concentration range as that of $(Na^+ + K^+)$-ATPase (defined as the ouabain-binding capacity) is capable of promoting the binding of ouabain provided Mg^{2+} is also present [17]. The rate of ouabain binding and probably also the equilibrium binding level are a function of the vanadate concentration. It was suggested that an enzyme-vanadate-ouabain complex with a 1 : 1 vanadate/ouabain ratio was created, though a repetitive role of one vanadate ion in rapid exchange with several receptors could be postulated.

From Fig. 1 it is seen that vanadate is retained in a relatively stable enzyme-vanadate-ouabain compex. In the presence of $(Mg^{2+} + K^+)$ and the absence of ouabain there takes place a fast binding of [^{48}V]vanadate, which reaches a steady-state level. In the absence of K^+, a slower binding of vanadate takes place and it levels off at a lower degree of saturation of sites. Addition of a surplus of unlabelled vanadate at 37°C causes a rapid K^+-sensitive release of the isotope.

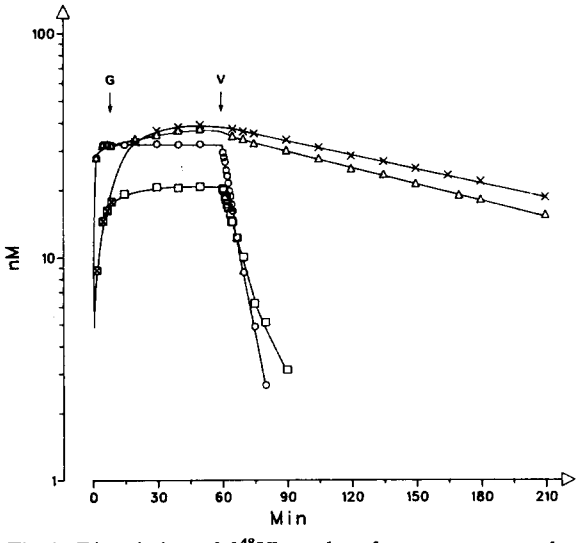


Fig. 1. Dissociation of [^{48}V]vanadate from enzyme-vanadate and enzyme-vanadate-ouabain complexes after addition of an excess of unlabelled vanadate. 62.5 μg enzyme protein/ml, corresponding to an ouabain capacity of 45 nM, was incubated with 5 mM Mg^{2+}, 40 mM Tris-HCl (pH 7.25) and $5 \cdot 10^{-8}$ M [^{48}V]vanadate (×, □) or plus 10 mM (K^+ (○, △). After 10 min, $1 \cdot 10^{-4}$ M ouabain was added in the case of two of the experiments (×, △). After 60 min of incubation, $1 \cdot 10^{-4}$ M unlabelled vanadate was added in all four experimental situations. [^{48}V]Vanadate binding was determined by a filtration technique as described [22].

Stepwise addition of ouabain at [^{48}V]vanadate equilibrium binding succeeded by an excess of unlabelled vanadate is reflected in an increased [^{48}V]vanadate binding, especially in the absence of K^+, and then a much retarded release of the isotope compared to the situation in the absence of ouabain. The rate of release of vanadate is very similar to that of ouabain (vide infra) which means that vanadate is trapped in a relatively stable enzyme-vanadate-ouabain complex.

Vanadate binding to preformed enzyme-ouabain complexes

In contrast to vanadate, binding of inorganic phosphate to the $(Na^+ + K^+)$-ATPase protein is usually claimed to be insignificant. In the presence of Mg^{2+} and ouabain, however, a steady-state level of $^{32}P_i$ incorporation into an enzyme-ouabain complex is measurable in trichloroacetic acid precipitates [5,23], but even under these conditions the affinity of the enzyme for P_i is relatively low. In the $(Mg^{2+} + Na^+ + ATP)$-facilitated enzyme-ouabain complex, phosphorylation from the nucleotide is known to take place in parallel with ouabain binding. The nucleotide-donated phosphate is released much faster than ouabain, and rephosphorylation from P_i but not from ATP becomes possible [24,25,14].

The high-affinity congener of phosphate, vanadate, can probably be used to make some generalizations concerning the Mg^{2+}- and $(Mg^{2+} + P_i)$-facilitated enzyme-ouabain complexes. Incubation of the Mg^{2+}-promoted complex with vanadate showed that it would accept vanadate, although at a lower rate than the non-ouabain-bound enzyme [20]. The $(Mg^{2+} + P_i)$- and $(Mg^{2+} + Na^+ + ATP)$-promoted complexes apparently did not accept vanadate but this was due to simple competition with P_i (added or released during ATP hydrolysis) as indicated by the experiment shown in Fig. 2.

At low temperature the enzyme-ouabain com-

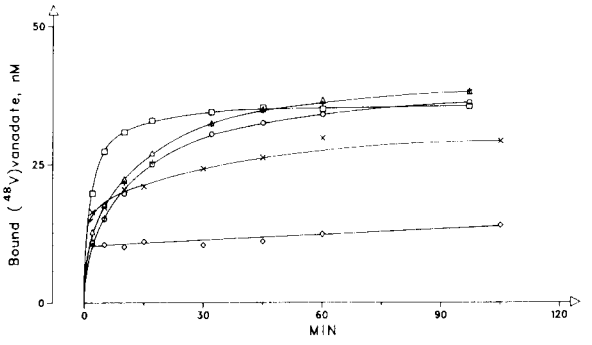


Fig. 2. [^{48}V]Vanadate binding to different enzyme-ouabain complexes after their separation from non-bound ligand that promoted complex formation. 62.5 μg enzyme protein/ml was incubated for 30 min at 37°C with 5 mM Mg^{2+}, 40 mM Tris-HCl (□) plus $1 \cdot 10^{-4}$ M ouabain (○), plus 1 mM P_i (△) or 50 nM unlabelled vanadate (◇). Alternatively, incubation took place with 3 mM Mg^{2+}, 120 mM Na^+, 3 mM ATP, 40 mM Tris-HCl and $1 \cdot 10^{-4}$ M ouabain (+). After cooling to 0°C enzyme was precipitated by centrifugation, washed twice in dilute Tris buffer at 0°C and reprecipitated. Resuspension took place in 5 mM Mg^{2+}, 10 mM K^+, 40 mM Tris and $5 \cdot 10^{-8}$ M [^{48}V]vanadate. Binding of vanadate at 25°C was determined as described [22] at the times indicated. In control experiment for vanadate lability during the washing procedure in the absence of ouabain, this compound was omitted during the preincubation (×).

plexes are rather stable and may be washed free of non-bound ligands without significant loss of bound ouabain. After resuspension in a medium containing [^{48}V]vanadate and ligands necessary to promote its binding, vanadate is taken up by the enzyme-ouabain complex in amounts almost corresponding to the enzyme's total vanadate binding capacity, irrespective of whether ouabain binding had been promoted by Mg^{2+}, ($Mg^{2+} + P_i$) or ($Mg^{2+} + Na^+ + ATP$). Small differences in binding in some experiments are attributed to slightly less than 100% enzyme recovery after washing procedure. In contrast, the vanadate-facilitated enzyme-ouabain complex will not accept [^{48}V]vanadate, unless ouabain dissociation and simultaneous or subsequent release of unlabelled vanadate has taken place. At low temperature and in the absence of ouabain, vanadate is also rather firmly bound to ($Na^+ + K^+$)-ATPase and relatively resistant to the washing procedure. Enzyme exposed to unlabelled vanadate in the absence of ouabain during preincubation is available for [^{48}V]vanadate binding after the washing procedure, however, as seen from the control experiment. The fraction of unlabelled vanadate retained thus seems much lower than in the presence of ouabain.

Enzyme-ouabain complexes, whether formed with the help of ATP or of P_i, lose their phosphorylation and can react with P_i or vanadate: the latter then remains bound to the enzyme-drug complex. The complex formed in the presence of Mg^{2+} + *p*-nitrophenyl phosphate is also accessible to vanadate (not shown). From the above data it can be concluded also that Mg^{2+}-facilitated ouabain binding in the absence of other added ligands may have had the cooperation of traces of P_i, but cannot have involved traces of vanadate in the binding process.

Ligand requirements for vanadate binding to enzyme-ouabain complexes

For separate binding of vanadate or ouabain, some ligands are indispensable whereas others will promote or impede binding. If there are ligand requirements for vanadate binding to enzyme-ouabain complexes (after washing the complexes free of the ligands that facilitated their formation), this is an indication that the ligand in question is not a compulsory part of the receptor complex. The ligand may have been lost from the complex. The absence of such requirements, on the other hand, may mean that the ligand is an integrated part of the complex or that ouabain has stabilized a conformation suitable for vanadate binding accompanied by release of the ligand.

For vanadate binding to ($Na^+ + K^+$)-ATPase, a divalent cation such as Mg^{2+} or Mn^{2+} is indispensable and the rate and extent of binding is further increased by K^+ (Fig. 3). Due to the high affinity with which Mn^{2+} promotes vanadate binding, any additional effect of K^+ is difficult to demonstrate [26], however. K^+ is usually considered antagonistic to ouabain binding.

From Fig. 3 it is seen that very little vanadate is bound to an enzyme-ouabain complex formed in the presence of ($Mg^{2+} + P_i$) and washed free of these ligands. Upon addition of Mg^{2+}, vanadate binding is much improved, though the rate of binding is relatively slow and similar to the rate seen with ouabain-free enzyme in the absence of K^+. The binding level eventually reached is similar to that obtained with ouabain-free enzyme in

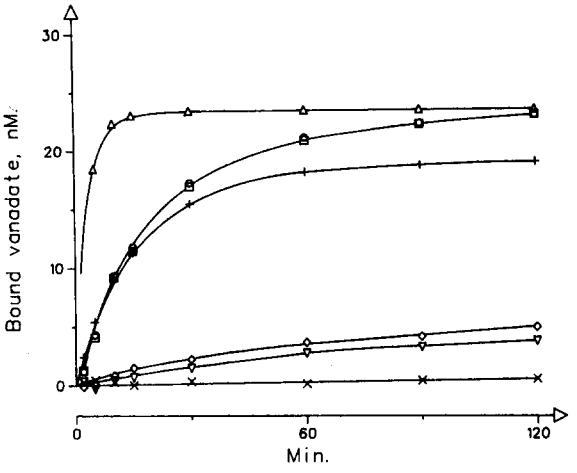


Fig. 3. Effect of Mg^{2+} and K^+ on vanadate binding to ouabain complexed ($Na^+ + K^+$)-ATPase and to control enzyme. 62.5 μg enzyme/ml, corresponding to an ouabain binding capacity of 45 nM, was preincubated for 30 min at 37°C with 5 mM Mg^{2+}, 1 mM P_i, 40 mM Tris-HCl and $1 \cdot 10^{-4}$ M ouabain (□, ○, ◇, ▽) or with Tris buffer only (×). After cooling to 0°C, enzyme was precipitated by centrifugation, resuspended in ice-cold, dilute Tris buffer and reprecipitated twice. Final resuspension and incubation at 25°C took place with 40 mM Tris-HCl, $2.5 \cdot 10^{-8}$ M [^{48}V]vanadate (◇, ×) plus 5 mM Mg^{2+} (□) and 10 mM K^+ (○) or plus 10 mM K^+ only (▽). [^{48}V]Vanadate binding to control enzyme without preincubation took place at 25°C in the presence of 5 mM Mg^{2+}, 40 mM Tris-HCl, $2.5 \cdot 10^{-8}$ M [^{48}V]vanadate (+) plus 10 mM K^+ (△).

the presence of both Mg^{2+} and K^+ (actually exceeding that level except here due to an inevitable loss of protein during the washing procedure). The further addition of K^+ affects neither the rate nor the extent of binding.

Results in the preceding section suggest that phosphate is released from the enzyme-ouabain complex before or during the washing of the complex. From the experiments presented in Fig. 3, it must be concluded that Mg^{2+} is not a part of the enzyme-ouabain complex, either. For binding of vanadate (or rephosphorylation by P_i) Mg^{2+} still has an essential role unrelated to the reactive state of the enzyme. While a conformation favourable to ouabain binding is induced by ($Mg^{2+} + P_i$), the reactive state is maintained by ouabain bound to the enzyme. This conformation is adequate for binding of vanadate in the presence of Mg^{2+}, but K^+ has now become superfluous.

Two-substrate mechanisms examined with vanadate

The kinetics of substrate protection of the inhibition of ($Na^+ + K^+$)-ATPase by substances such as 7-chloro-4-nitrobenzoxa-1,3-diazole chloride [27] and the biphasic nature of reversible plots of hydrolytic activity versus substrate concentration [28] have been interpreted as indications of one high-affinity and one low-affinity site per functional unit of the enzyme. In some of the proposed models for the ($Na^+ + K^+$)-ATPase the concept of two substrate sites has been combined with a half-of-the-sites or alternating site mechanism, by some authors also a flip-flop mechanism (for references, see reviews, Refs. 29–32).

Investigators have looked for suitable binding experiments that might support a half-of-the-sites reactivity mechanism. The trapped vanadate on ($Na^+ + K^+$)-ATPase in combination with ouabain could represent a situation that would approach a doubling of vanadate binding. On the other hand, substituting Mg^{2+} with Ca^{2+} has been reported to halve the P_i phosphorylation capacity induced by ouabain [33]. Since vanadate is supposed to behave as a high-affinity congener of phosphate,

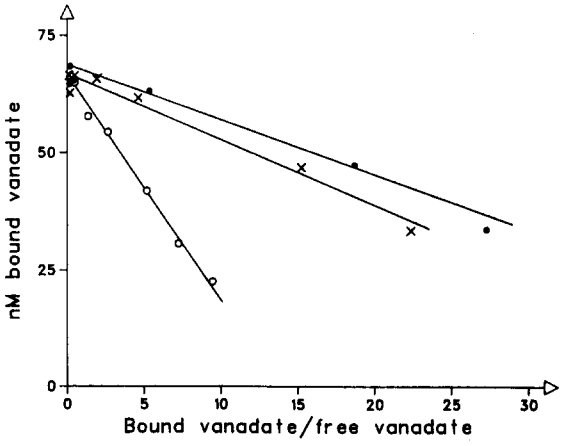


Fig. 4. Scatchard plot of equilibrium binding data of [^{48}V]vanadate obtained in the presence of Mg^{2+} or Ca^{2+} in combination with ouabain. 32.2 μg enzyme protein/ml with a specific activity of 17.6 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ was incubated at 25°C with 5 mM Mg^{2+}, 10 mM K^+, 40 mM Tris-HCl and $2.5 \cdot 10^{-8}$–$6 \cdot 10^{-7}$ M [^{48}V]vanadate (×) plus 10^{-4} M ouabain (●) or with 1 mM Ca^{2+}, 40 mM Tris-HCl, $1 \cdot 10^{-4}$ M ouabain and $2.5 \cdot 10^{-8}$–$6 \cdot 10^{-7}$ M [^{48}V]vanadate (○) for 60, 120 and 120 min, respectively. Binding was determined as described [22].

vanadate binding under similar conditions might be expected to confirm the above observation. Equilibrium values of vanadate binding obtained under standard conditions ($Mg^{2+} + K^+$), both in the presence and in the absence of ouabain, and under conditions in which Mg^{2+} and K^+ were substituted by Ca^{2+} are shown in Fig. 4 in the form of a Scatchard plot. It is seen that there is no indication of any effect on the total number of vanadate-binding sites. Since the values of the slopes indicate the apparent dissociation constants of the enzyme-vanadate complex, it can be seen that the highest vanadate affinity is obtained in the presence of ouabain. An apparent dissociation constant of 1 nM is obtained in that situation.

Another approach to the question of the active site stoichiometry per functional unit would be to look for situations in which simultaneous nucleotide and P_i binding is supposed to take place. One model predicts that when two substrate sites per functional unit work simultaneously and out-of-phase, binding of ATP and P_i should take place at the same time on the enzyme [18]. Glynn and Karlish [29] had suggested that the acceleration by ATP of ouabain release from the ($Mg^{2+} + P_i$)-induced enzyme-ouabain complex was an example with simultaneous binding of ATP and P_i. If the enzyme-ouabain complex is washed free of non-bound ligands, the addition of ATP, especially if Na^+ and EDTA are also present, will accelerate ouabain release [34,35]. Under such conditions, rephosphorylation is negligible and the experiment would indicate simultaneous P_i- and ATP-binding provided P_i is still part of the enzyme-ouabain complex. From the preceding section, it is seen that the latter requirement is unlikely to be fulfilled, however.

A more clearcut experiment can be carried out if the phosphate analogue, vanadate, rather than phosphate is used to promote ouabain binding, since vanadate is known to be retained in the enzyme-ouabain complex. Acceleration of ouabain release in that situation could indicate two interacting substrate sites. An enzyme-vanadate-ouabain complex was prepared by using [^{3}H]ouabain, [^{48}V]vanadate and Mg^{2+}. The complex was washed free of non-bound ligands and was resuspended in dilute Tris buffer or in buffer + ATP + Na^+. It is seen from Fig. 5 that neither

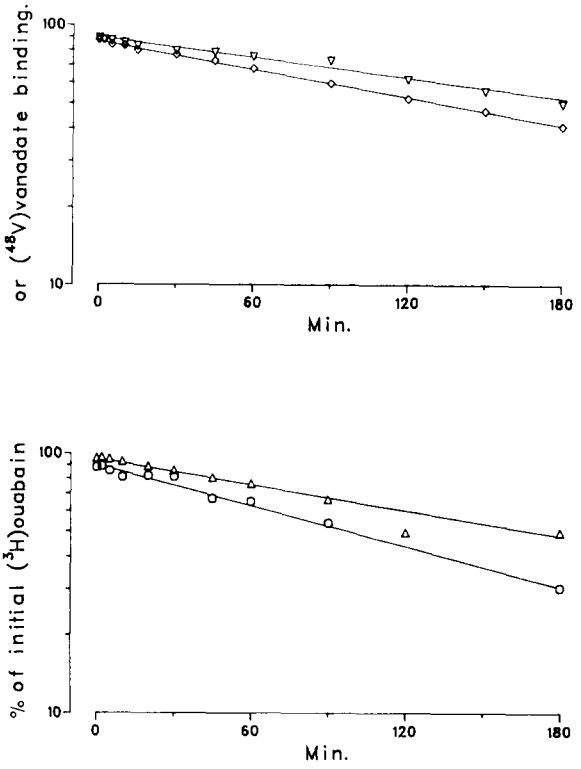


Fig. 5. Effect of (Na^+ + ATP) on [^{48}V]vanadate and [^{3}H]ouabain dissociation from the enzyme-vanadate-ouabain complex. Enzyme corresponding to an ouabain-binding capacity of 260 nM was incubated for 60 min at 37°C with 5 mM Mg^{2+}, 40 mM Tris-HCl plus 10 μM vanadate and $3 \cdot 10^{-7}$ M [^{3}H]ouabain (○, △) or plus 10 μM ouabain and $3 \cdot 10^{-7}$ M [^{48}V]vanadate (◇, ▽). After cooling to 0°C, enzyme was precipitated by centrifugation, washed twice in dilute Tris buffer and reprecipitated. Resuspension took place in 5-times the original volume containing 4 mM Tris-HCl (○, ◇) or plus 3 mM ATP and 120 mM Na^+ (▽, △). [^{48}V]Vanadate and [^{3}H]ouabain dissociation was determined at 37°C as described [22].

vanadate nor ouabain dissociation is accelerated by ATP + Na^+. On the contrary, the release of both is retarded to a minor degree. In control experiments this could be shown to be due to Na^+ in case of ouabain, but not in case of vanadate, the release of which was accelerated somewhat by Na^+. The release of vanadate from enzyme-vanadate (without ouabain) would behave in this way [36]. In another control experiment the dissociation of ouabain from the ($Mg^{2+} + P_i$)-promoted enzyme-ouabain complex was accelerated considerably by Na^+ + ATP. The above-

mentioned behaviour of the vanadate-ouabain complex of $(Na^+ + K^+)$-ATPase from pig kidney can be reproduced also with ox brain $(Na^+ + K^+)$-ATPase.

The K^+-ouabain antagonism

Although ouabain and vanadate are bound to the E_2-conformation of the enzyme [24,37,38], K^+ decreases the rate and affinity of ouabain binding and increases these same two parameters with respect to vanadate binding, at least when Mg^{2+} is the divalent cation employed. Robinson and Mercer [26] noticed that K^+ did not appreciably stimulate vanadate binding in the presence of Mn^{2+}. If Mn^{2+} should select an E_2-subspecies more favourable for vanadate binding, i.e., nearer the conformation induced by $Mg^{2+} + K^+$, then it could be expected that Mn^{2+} should be less favourable for promotion of ouabain binding. Since P_i is precipitated by Mn^{2+} we took advantage of the fact that much lower concentrations of vanadate are known to be able to facilitate ouabain binding in combination with Mg^{2+}. From Table I are seen the well-known effects of K^+ on Mg^{2+}-promoted vanadate and ouabain equilibrium-binding level. In combination with Mn^{2+} in the same concentration range there is apparently no effect of K^+ on the two parameters. At much lower concentrations of Mn^{2+}, however, vanadate binding is increased by K^+ and ouabain binding is decreased by K^+. Qualitatively, Mn^{2+} seems to behave like Mg^{2+}, but much lower concentrations of Mn^{2+} are necessary for promotion of vanadate and ouabain binding. The synergistic action of K^+ in the former case and the antagonistic action in the latter thus seems to be hidden when Mn^{2+} is present in mM concentrations.

That K^+, in combination with Mn^{2+}, reacts with the $(Na^+ + K^+)$-ATPase is also indicated by the effect of K^+ on rate of vanadate binding. Likewise, having prepared an enzyme-vanadate complex in the presence of Mn^{2+} and the absence of K^+, the addition of an excess of unlabelled vanadate or vanadate + K^+ will accelerate the rate of vanadate release in latter case compared to the former case. At 37°C the decay constant was 0.063 min^{-1} in the presence of 10 mM K^+ and 0.026 min^{-1} in the absence of K^+.

TABLE I

K^+-EFFECT ON Mg^{2+}- OR Mn^{2+}-PROMOTED VANADATE BINDING AND ON (Mg^{2+} OR Mn^{2+} + VANADATE)-FACILITATED OUABAIN BINDING TO $(Na^+ + K^+)$-ATPase

Ouabain binding: Enzyme containing 20 μM EDTA was incubated with 40 mM Tris-HCl (pH 7.25), 3 mM Mg^{2+} or 3 mM Mn^{2+} or 12 μM Mn^{2+} plus 10 μM vanadate ± 1 mM K^+ and $2.5 \cdot 10^{-8}$ M [^{3}H]ouabain for 120 min at 37°C. Vanadate binding: Enzyme containing 20 μM EDTA was incubated with 40 mM Tris-HCl (pH 7.25), 5 mM Mg^{2+} or 5 mM Mn^{2+} or 12 μM Mn^{2+} ± 10 mM K^+ and $2.5 \cdot 10^{-8}$ M [^{48}V]vanadate for 60 min at 25°C. The results are given as mean values + S.E. and the number of observations in each group was five.

Binding conditions	Vanadate binding (nM)	Ouabain binding (nM)
Mg^{2+}	19.6 ± 0.1 [a]	21.6 ± 0.1 [a]
$Mg^{2+} + K^+$	23.3 ± 0.1 [a]	6.4 ± 0.5 [a]
Mn^{2+}	24.6 ± 0.0 [b]	21.7 ± 0.1 [b]
$Mn^{2+} + K^+$	24.6 ± 0.0 [b]	21.6 ± 0.1 [b]
Mn^{2+} (12 μM)	3.2 ± 0.5 [a]	5.7 ± 0.4 [a]
Mn^{2+} (12 μM) + K^+	9.3 ± 0.6 [a]	0.5 ± 0.1 [a]

[a] Values with and without K significantly different ($P < 0.001$).
[b] Values with and without K not significantly different ($P > 0.025$).

Discussion

The data that have been presented on vanadate binding to $(Na^+ + K^+)$-ATPase in combination with ouabain have two main aspects: a characterization of the ouabain-complexed enzyme and an examination of the number of substrate sites per functional unit of the enzyme, specifically whether simultaneous nucleotide and vanadate binding may take place.

As to the first issue and referring to the diagram given in the Introduction, it is shown that during the process of (Mg^{2+} + vanadate)-facilitation of ouabain binding, vanadate becomes trapped in the enzyme-ouabain complex. This can be considered to be in contrast with the ($Mg^{2+} + Na^+ + ATP$)-facilitated complex from which phosphorylation is rapidly lost [14,24,25], thus allowing the binding of vanadate. The ($Mg^{2+} + P_i$)-facilitated enzyme-ouabain complex in the absence of free P_i can also bind vanadate, indicating that this glycoside-bound form does not trap phosphate

either. The rate of vanadate binding to all sorts of enzyme-ouabain complexes is slower than to non-ouabain bound enzyme. Vanadate binding to enzyme-ouabain complex requires a divalent cation, showing that the divalent cation used in the formation of the enzyme-ouabain complex was not retained as part of this complex. K^+ seems to have no additive role in promotion of vanadate binding to the complex. The E_2-conformation of the enzyme to which ouabain binding takes place [24,37] is probably preserved. The proper conformation of the enzyme, however, is clearly not the only prerequisite for vanadate binding, as is also indicated by the lack of K^+-facilitation of vanadate binding to non-ouabain-bound enzyme in the absence of Mg^{2+}. In the presence of Mg^{2+} alone, ouabain produces an increase in affinity of the enzyme for vanadate. The affinity remains, however, finite. The concentration dependent binding reflected in the straight line obtained in the Scatchard plot must mean that vanadate is able to leave the ouabain-bound enzyme, since ouabain saturation must have been achieved at the concentration employed.

Mn^{2+} is more efficient than Mg^{2+} in promotion of vanadate binding and, probably because of this, it is also more efficient in promoting vanadate-supported ouabain binding. Mn^{2+} thus does not necessarily create an E_2-subconformation more favourable for vanadate binding than Mg^{2+} [26]. A K^+-antagonism to Mn^{2+} can clearly be shown with ouabain binding and a K^+-synergism to Mn^{2+} with respect to vanadate binding is seen at low concentrations of Mn^{2+}.

As to the second question: no final answer can be given from the present experiments with vanadate as to whether a two-substrate mechanism is operative with $(Na^+ + K^+)$-ATPase. Vanadate binding even in the presence of Ca^{2+} + ouabain notoriously gives exactly the same value for the vanadate-binding capacity of the enzyme as is obtained with other divalent cations in the presence as well as in the absence of ouabain. This is in contrast to observations with P_i under similar conditions [33].

The acceleration of ouabain release from an enzyme-ouabain complex formed in the presence of $Mg^{2+} + P_i$ by $(Na^+ + ATP)$ has been used as an argument for a two substrate-site mechanism [29]. The experiments certainly show that nucleotide is able to react with ouabain-bound enzyme though with a much reduced affinity compared to enzyme not inactivated by ouabain [39]. From the present results, however, it seems questionable whether P_i is retained in the $(Mg^{2+} + P_i)$-promoted enzyme-ouabain complex, since it is available for vanadate binding in the absence of competing free P_i (Fig. 2). Vanadate is much more firmly bound than P_i and vanadate complexed enzyme-ouabain seems a better choice for the decision on the possible existence of a mechanism with two substrate sites. As shown in Fig. 5 neither ouabain nor vanadate release from an enzyme-vanadate-ouabain complex is accelerated by $(Na^+ + ATP)$. Vanadate release is, on the other hand, stabilized somewhat by $(Na^+ + ATP)$ and by ATP alone. This effect of nucleotide, though less dramatic than the acceleration of ouabain release from the $(Mg^{2+} + P_i)$-facilitated enzyme-ouabain complex, clearly indicates that ATP somehow influences the membrane system. The creation of a vanadate-triphosphate complex at one and the same nucleotide site could be postulated. Interesting, quite similar reactions were observed with the putative nucleotide analogues suramin and eosin [40,41,22], i.e., acceleration of ouabain release from the $(Mg^{2+} + P_i)$-promoted complex and deceleration of ouabain dissociation from the $(Mg^{2+} + V)$-promoted enzyme-ouabain complex (unpublished observations). The decelerations seen may, of course, also indicate a two-substrate-site mechanism. While this manuscript was in preparation, Askari and Huang [42] published similar observations on P_i-release from ouabain-bound enzyme by nucleotides.

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