The Effects of Vanadate on the Plasma Membrane ATPase of 
Neurospora crassa*

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The plasma membrane ATPase of Neurospora crassa, which is believed to function as an electrogenic proton pump, is extremely sensitive to inhibition by vanadium in the +5 oxidation state (vanadate). At pH 6.7 (the pH optimum of the ATPase), inhibition is half-maximal between 0.45 and 1.0 μM vanadate, depending upon the ionic composition of the reaction medium. Inhibition also depends upon pH, with the result that 1.5 μM vanadate causes an apparent shift of pH optimum to 6.0.

EDTA prevents inhibition of the Neurospora ATPase by vanadate. It does not act by chelating Mg2+, since other chelators (trans-1,2-diaminocyclohexanetetraacetic acid (CDTA), ethyleneglycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA)) are ineffective and since, over a broad range of concentrations, MgCl2 has little effect on the degree of inhibition by vanadate. Rather, EDTA appears to complex vanadate directly, as has previously been reported in the chemical literature.

We have used vanadate to explore the reaction mechanism of the Neurospora ATPase in two ways. 1) When assayed with vanadate-free ATP, the ATPase shows a sigmoid dependence upon substrate concentration, consistent with the notion that the enzyme has two or more substrate binding sites acting cooperatively. Vanadate stimulates ATPase activity at low substrate concentrations and inhibits at high concentrations, thus reducing the apparent cooperativity between sites.

2) We have previously reported (Bowman, B. J., and Slayman, C. W. (1977) J. Biol. Chem. 252, 3357-3363) that Neurospora plasma membrand ATPase activity is stimulated 20 to 62% by K+ or NH4+. Under these conditions, vanadate still inhibits more than 95% of the activity, and Dixon plots of the data are linear. The results can best be accounted for in terms of a single plasma membrane ATPase which is subject to non-specific salt effects, and are difficult to reconcile with the idea that the K+-stimulated portion of ATPase activity represents a discrete enzyme involved in K+ transport. The dynein ATPase from cilia and flagella (3, 4). Two features of inhibition by vanadate are of particular interest: its site of action (a low affinity binding site at the inner surface of the membrane, in the case of the (Na+, K+)-ATPase; 5, 6) and its specificity. Other ATPases tested, the (Ca2+)-ATPase of sarcoplasmic reticulum (1), actomyosin ATPase (1), and mitochondrial ATPase (1, 7), are unaffected by vanadate.

Thus, we were interested to discover recently that the plasma membrane ATPase of Neurospora is extremely sensitive to vanadate (7). Physiologically, this ATPase (8, 9) and a similar one in yeast (10) are analogous to the ATPase complexes of mitochondria and bacteria. Their function is to transport protons outwards across the plasma membrane, generating a large electrochemical H+ gradient (11-14); the H+ gradient, in turn, drives the cotransport of sugars, amino acids, and inorganic ions (15-18). Biochemically, however, the fungal plasma membrane ATPases are more closely related to the (Na+, K+)-ATPase of animal cells. Both are integral membrane proteins, solubilized only with the aid of detergents. In addition, Dufour and Goffeau (19) have recently shown that the plasma membrane ATPase from Schizosaccharomyces pombe contains a single large subunit of M, = 100,000 reminiscent of the large 95,000-dalton polypeptide of the (Na+, K+)-ATPase (20); preliminary studies in our laboratory point to a similar subunit composition for the Neurospora enzyme (21).

In their response to inhibitors, the fungal plasma membrane ATPases show a distinct pattern. They are insensitive to oligomycin and to ouabain, but sensitive to DCCI and (as we have recently found for the Neurospora enzyme) to vanadate (7). The present report explores the mechanism by which vanadate acts on the plasma membrane ATPase of Neurospora. We find that the degree of inhibition depends upon the ionic composition of the medium and the ATP concentration at which the enzyme is assayed, and that inhibition is reversed by norepinephrine or by EDTA.

MATERIALS AND METHODS

Growth of Cells and Cell Fractionation—Wild type strain HL21a of Neurospora crassa was used in these experiments and grown for 13 h in liquid minimal medium with vigorous aeration (7). Cells were treated with snail digestive juice (Sigma β-glucuronidase) to weaken their cell walls and disrupted by osmotic lysis (22). As previously described (7), unbroken cells and large fragments were removed by centrifugation at 1,000 × g, and mitochondria, by two cycles of centrifugation at 10,000 × g. Finally, a particulate fraction containing the plasma membrane ATPase activity was obtained by centrifugation at 48,000 × g (7). The 48,000 × g pellet was suspended at 25 mg of protein/ml in 10 mM Tris, 5 mM MgCl2, pH 7.5, and stored at

Vanadate has been found to be a potent inhibitor of the (Na+, K+)-ATPase of animal cells (1, 2) and, more recently,* this work was supported by Research Grant GM-15761 from the National Institute of General Medical Sciences and Grant PCM 7725199 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* The abbreviations used are: (Na+, K+)-ATPase, sodium and potassium ion-dependent adenosine triphosphatase; DCCI, NN'-dicyclohexylcarbodiimide; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; CDTA, trans-1,2-diaminocyclohexanetetraacetic acid; S, substrate; v, velocity in μmol/min/mg of protein.
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-70°C. This plasma membrane fraction was used for all ATPase measurements.

ATPase Assays—In most experiments, ATPase activity was assayed at 30°C in 0.5 ml of the following reaction mixture: 5 mM Na₂ATP (Boehringer), 5 mM MgCl₂, 5 mM phosphoenolpyruvate, 2.5 µl of Sigma pyruvate kinase (25 µg of protein, 11 mM (NH₄)₂SO₄, final concentration), 5 mM potassium azide (to inhibit residual mitochondrial ATPase; Ref. 7), and 10 mM Pipes, adjusted to pH 6.7 with Tris. The reaction was started by the addition of enzyme and stopped after 10 min by the addition of 0.1 ml of 50% (w/v) trichloroacetic acid. The tubes were then centrifuged at 2000 × g for 5 min, and inorganic phosphate was measured in a portion of the supernatant by the method of Lowry et al. (24). High concentrations of vanadate (above 20 µM) interfere with the phosphate assay, causing a decrease in absorbance at 710 nm. Corrections were made by assaying phosphate standards in the presence of appropriate concentrations of vanadate.

A second problem involving vanadate is that a slight delay in the onset of inhibition causes an error of about 8% in a 10-min assay. We have ignored this error in the experiments depicted in Figs. 1, 2, 4, 5, and 9. In the experiment of Fig. 10, where such an error could be significant, we included a 1-min preincubation in the presence of vanadate.

To assay ATPase activity at low substrate concentrations (see Figs. 6 to 8), 3.5 ml of reaction mixture was used, and the concentration of Tris/ATP was equal to the concentration of MgCl₂. In some experiments, the mixture contained oligomycin (1 µg/ml, to inhibit residual mitochondrial ATPase; Refs. 7 and 8) and 10 mM Pipes buffer, titrated to pH 6.7 with Tris. Hydrolysis of ATP was measured from ¼ to 6½ min. This procedure was adopted as a compromise which would partially allow for the delay in the onset of vanadate inhibition while keeping total ATP hydrolysis below 4%. Control experiments showed that under these conditions, a steady state rate of hydrolysis had been achieved by ½ min at low substrate concentrations. At higher substrate concentrations, longer times were required to achieve a true steady state rate in the presence of vanadate (Fig. 3), and the compromise procedure used in this experiment resulted in a slight overestimation of rates (at 5 mM MgATP and 1.5 µM vanadate, for example, by about 12%).

Tris/ATP was prepared from Na₂ATP (Boehringer) by ion exchange chromatography on Dowex-50 resin.

Estimation of Protein—Protein was assayed by the method of Lowry et al. (24) with bovine serum albumin as standard.

Reagents—β-gluconoridase (type H-2), pyruvate kinase (type II), phosphoenolpyruvate, oligomycin, norepinephrine, ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(β-aminoethyl ether)N,N',N''-tetraacetic acid (EGTA), trans-1,2-diaminocyclohexanetetraacetic acid (CDTA), piperazine-N,N'-bis(2-ethanesulfonic acid) (Pipes), tri(hydroxymethyl)aminomethane (Tris), pyrophosphate, triisohosphosphate, and trimetaphosphate were purchased from Sigma Chemical Corp. In all experiments, Na₂ATP from Boehringer Mannheim was used. Sodium vanadate (ortho) was obtained from Fisher Scientific Co.

RESULTS

Dependence of ATPase Activity on the Source of ATP—As has been observed for the (Na⁺, K⁺)-ATPase of animal cells (1, 2, 25–27), the activity of the Neurospora plasma membrane ATPase depends upon the source of ATP used in the assay. In a preliminary experiment, we found ATPase activity to be 62% higher with Boehringer ATP than with Sigma ATP at pH 6.0 and 153% higher at pH 6.7. At both pH values, addition of EDTA (0.5 mM) to the reaction mixture increased the activity seen with Sigma ATP nearly to the level seen with Boehringer ATP. These results point to the presence of an inhibitor in Sigma ATP; the reversal of inhibition by EDTA will be discussed further in a later section.

Inhibition by Vanadate; Dependence on pH—Consistent with the results of Cantley et al. (2) on the (Na⁺, K⁺)-ATPase of animal cells, the inhibitor can be identified as vanadate, now known to be present in Sigma ATP purified from equine muscle. Five millimolar Sigma ATP, the concentration used in our standard assay, contains 0.3 to 2.4 µM vanadate (Sigma Technical Bulletin). Fig. 1 illustrates that 1.5 µM vanadate, when added to 5 mM Boehringer ATP, causes a significant inhibition of ATPase activity (60% at pH 6.7). In addition, because the degree of inhibition varies with pH, vanadate at this concentration brings about an apparent shift of pH optimum from 6.7 (the true optimum observed with vanadate-free ATP) to 6.0 (the value observed previously with Sigma ATP; Ref. 8). The reason for the pH dependence of vanadate inhibition is not clear. According to published data, at concentrations below 0.1 mM and at pH values between 4 and 8, vanadate exists in solution predominantly as H₂VO₄⁻ (28). The Neurospora ATPase is approximately 7-fold less sensitive to vanadate at pH 6.0 than at pH 6.7, however, with half-maximal inhibition produced by 4.5 and 0.75 µM vanadate, respectively, (pH 6.7, data shown in the next section; pH 6.0, data not shown).

Effect of Norepinephrine and EDTA—Norepinephrine and other catecholamines have been shown to prevent vanadate inhibition of the (Na⁺, K⁺)-ATPase (1, 2), and Fig. 2 shows that the same effect is seen with the Neurospora ATPase. Because high concentrations of norepinephrine are needed, and because norepinephrine is known to form a complex with vanadate (29), it is unlikely that this is a physiological effect of the hormone.

EDTA also prevents inhibition of the Neurospora ATPase by vanadate, an effect first observed in the preliminary exper-

![Fig. 1](http://www.jbc.org/fig1.jpg)  
Fig. 1. The effect of pH on ATPase activity in the presence and absence of 1.5 µM vanadate. The pH of the standard reaction medium (see "Materials and Methods") was varied from 5.2 to 8.4 by titration with Tris.

![Fig. 2](http://www.jbc.org/fig2.jpg)  
Fig. 2. Inhibition of ATPase activity by vanadate; prevention by norepinephrine (2.5 mM) and EDTA (1 mM). Activity was assayed as described under "Materials and Methods."
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The rate of the reaction during the first 5 min was 2.65 mmol/min/mg of protein. At the first arrow, 1.5 mM Na₃VO₄ was added; at the second arrow, 1.0 mM EDTA was added.

In the absence of EDTA, the ATPase was inhibited over the range of 0.05 to 5.0 μM vanadate, with half-maximal inhibition at 0.75 μM. In the presence of 1 mM EDTA, much higher concentrations of vanadate (greater than 5 μM) were required to produce a significant effect, with half-maximal inhibition at 25 μM.

In addition to preventing vanadate inhibition, EDTA can also reverse inhibition, as shown in Fig. 3. In this experiment, plasma membrane ATPase was added to the standard reaction mixture and a linear rate of Pi production was measured for the first 5 min. At that time, vanadate (1.5 μM) was added; after a lag of about 1 min, enzyme activity was reduced to 25% of the initial rate. EDTA was then added to the inhibited enzyme and a slow increase in activity was observed, such that 8 min after EDTA addition, the reaction was proceeding at 80% of the initial rate.

One possible interpretation of these results is that the sensitivity of the ATPase to vanadate might depend critically upon the concentration of free Mg²⁺ (in addition to that present as Mg-ATP); EDTA could then act by chelating the excess Mg²⁺. This explanation is made unlikely by the fact that two structurally different chelators (EGTA and CDTA) are unable to prevent inhibition of the ATPase by vanadate (Fig. 4). Thus, in the presence of 1.5 μM vanadate (which gave 83% inhibition of enzyme activity), addition of up to 3 mM EGTA or CDTA had very little effect. By contrast, EDTA restored activity to nearly the control level; restoration was half-maximal at 0.5 mM EDTA.

Effect of Mg²⁺—Further evidence that EDTA does not act by lowering the free Mg²⁺ concentration comes from an experiment in which [MgCl₂] was varied in the presence and absence of vanadate (1.5 μM) and EDTA (1 mM). This experiment also provides information about the complexity of the interactions of vanadate, MgATP, and free Mg²⁺ with the ATPase.

In the absence of vanadate and EDTA (C, Fig. 5), as [MgCl₂] was raised from 0 to 15 mM (with ATP fixed at 5 mM), ATPase activity increased to a maximum at approximately 5 mM MgCl₂ (Mg:ATP = 1:1), and then declined gradually to approximately 66% of the maximal rate at 15 mM MgCl₂ (10 mM free Mg²⁺). Thus, the Neurospora ATPase is inhibited by excess free Mg²⁺, but not drastically so.

When 1.5 μM vanadate was added (C, Fig. 5), its effect depended upon the MgCl₂ concentration in an unexpected way. At low [MgCl₂], there was no inhibition of enzyme activity by vanadate (and, in fact, an indication of a slight stimulation, which will be discussed further in the next section). As [MgCl₂] was raised, vanadate became progressively more inhibitory, until at 5 mM MgCl₂, it gave the 73% inhibition seen in earlier experiments (Figs. 1 to 4). Above 5 mM MgCl₂, the vanadate curve declined in parallel with the control curve, and inhibition remained roughly constant at 73 to 78%. Thus, the action of vanadate does not appear to depend critically on the presence of MgCl₂ in excess of the ATP concentration.

In the remaining two curves in Fig. 5, MgCl₂ was also raised from 0 to 15 mM, this time in the presence of 1 mM EDTA, with and without 1.5 μM vanadate. The addition of EDTA alone (V, Fig. 5) shifted the control curve to the right by an amount corresponding to the removal of 1 mM MgCl₂, but did not otherwise change the shape of the curve. When EDTA was added together with vanadate (Δ, Fig. 5), its ability to protect against vanadate inhibition depended upon the MgCl₂ concentration. Below 5 mM MgCl₂, vanadate produced no significant inhibition (compared with the curve obtained in the presence of EDTA alone), and thus protection was essentially complete. This result is consistent with the notion that, at low MgCl₂ concentrations, essentially all of the free vana-
date as well as nearly 1 mM of the Mg$^{2+}$ were complexed by the EDTA. Above 5 mM MgCl$_2$, however, the protective effect of EDTA progressively diminished. Presumably in this range, excess free Mg$^{2+}$ began to compete with vanadate for the EDTA, the concentration of free vanadate in the reaction mixture increased, and inhibition was seen again.

The complexity of the reaction mixture means that the concentrations of all of the relevant species are impossible for us to calculate quantitatively. EDTA-vanadate complexes have been described in the chemical literature, however, with association constants in the range of $10^6$ to $10^{15}$ (30). Furthermore, we have observed that the addition of 1 mM EDTA to reaction buffer containing 150 mM vanadate (pH 6.7) leads to a pronounced change in the UV absorption spectrum of vanadate, with an increase in absorbance at 280 nm; addition of excess of MgCl$_2$ (5 mM) causes a return to the control spectrum. The spectral results support the notion that under the conditions of these experiments EDTA can bind vanadate reversibly, and that Mg$^{2+}$ competes with vanadate for the EDTA.

**Effect of Substrate Concentration**—One puzzling aspect of the results in Fig. 5 was the slight stimulation of ATPase activity by vanadate at low MgCl$_2$ concentrations. To explore this phenomenon further, the enzyme was assayed as a function of Mg-ATP concentration at 0, 1.5, and 15 mM vanadate (Fig. 6).

The control curve illustrates that, at its pH optimum (6.7) and in the absence of vanadate, the ATPase displays a sigmoid dependence upon Mg-ATP concentration (Fig. 6a). A double reciprocal plot of the same data is concave upward (Fig. 6b) but can be linearized by plotting $1/v$ versus $1/S$ (not shown), consistent with the notion that the ATPase possesses two or more binding sites with positive cooperativity between the sites (31). A Hill plot of the data (Fig. 7) was linear over the range 0.3 to 2 mM MgATP, and gave values of $n = 1.9$ and $K_{H} = 1.8$ mM.

The addition of vanadate to the reaction mixture caused a

**TABLE I**

<table>
<thead>
<tr>
<th>Concentration tested</th>
<th>% Inhibition</th>
</tr>
</thead>
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<tr>
<td>PO$_4$</td>
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<tr>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>10.0</td>
<td>7</td>
</tr>
<tr>
<td>AsO$_4$</td>
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</tr>
<tr>
<td>0.01</td>
<td>(4)</td>
</tr>
<tr>
<td>0.1</td>
<td>7</td>
</tr>
<tr>
<td>1.0</td>
<td>7</td>
</tr>
<tr>
<td>10.0</td>
<td>19</td>
</tr>
<tr>
<td>Pyrophosphate</td>
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</tr>
<tr>
<td>0.01</td>
<td>9</td>
</tr>
<tr>
<td>0.1</td>
<td>4</td>
</tr>
<tr>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>10.0</td>
<td>9</td>
</tr>
<tr>
<td>Tripolyphosphate (linear)</td>
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</tr>
<tr>
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<td>14</td>
</tr>
<tr>
<td>0.1</td>
<td>16</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>10.0</td>
<td>89</td>
</tr>
<tr>
<td>Trimetaphosphate (cyclic)</td>
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</tr>
<tr>
<td>0.01</td>
<td>16</td>
</tr>
<tr>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
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<tr>
<td>K$_2$CrO$_4$</td>
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<td>(2)</td>
</tr>
<tr>
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<td>(3)</td>
</tr>
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<td>0.1</td>
<td>6</td>
</tr>
<tr>
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<td>2</td>
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<tr>
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<td>1</td>
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<tr>
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**Fig. 6.** Effect of Mg-ATP concentration ($S$) on ATPase activity. All assays were done by the procedure described under "Materials and Methods" for low substrate concentrations.

**Fig. 7.** Hill plot of substrate-velocity data, obtained as described in the legend to Fig. 6. $S$ is substrate concentration (mM), $v$ is ATPase activity (umol/min/mg), and $V_{max}$ is the maximal ATPase activity obtained from the intercept of a Lineweaver-Burk plot.
striking shift in the kinetic behavior of the ATPase. The $v$ versus $S$ plot became more hyperbolic (Fig. 6a); the double reciprocal plot, more nearly linear (Fig. 6b). The plots in the presence of 1.5 $\mu$M vanadate are equivalent to our previously published data on the concentration dependence of the Neurospora ATPase, obtained with Sigma ATP (8); they are also consistent with the results of Fig. 5, in the sense that 1.5 $\mu$M vanadate inhibited ATPase activity by 60% at 5 mM MgATP but stimulated it at MgATP concentrations below 1 mM. A comparison of the double reciprocal plots with 1.5 and 15 $\mu$M vanadate (Fig. 6b) reveals that the linear portions share a common intercept on the 1/$S$ axis, and thus that vanadate affects the maximal velocity of the enzyme but is not competitive with the substrate.

Effect of Other Oxyanions—A number of compounds which share some properties with vanadate were tested for their ability to inhibit the ATPase (Table I). Although it has been suggested (2, 5, 6) that vanadate may act as an analog of phosphate, neither phosphate nor arsenate (at concentrations up to 10 mM) significantly affected the Neurospora enzyme, nor did they alter inhibition by vanadate (data not shown).

Several polyphosphates were also tested because the ability of vanadate to form stable polymers (28) could conceivably allow it to mimic a portion of the ATP molecule. Of the three compounds tried, only linear tripolyphosphate, at 10 mM, significantly inhibited the enzyme. Pyrophosphate and cyclic trimetaphosphate were ineffective.

Three compounds which are neighbors of vanadate in the periodic table, chromate, molybdate, and tungstate, were also tested. None of these compounds significantly inhibited the ATPase. All of these results, taken together, indicate that the action of vanadate upon the ATPase is highly specific.

Effect of Monovalent Cations—Although there is no evidence that the Neurospora plasma membrane ATPase is directly linked to potassium or sodium transport, we had previously observed a moderate stimulation of ATPase activity by cations (8). In view of reports that cations can affect the sensitivity of the (Na+, K+)-ATPase to vanadate, it was important to test the effect of cations on the Neurospora ATPase assayed with vanadate-free ATP. Fig. 8 shows that ATPase activity was stimulated by NH$_4^+$ (90%) and K$^+$ (12%), and was inhibited by Na$^+$ (43%). None of these cations significantly changed the $K_{1/2}$ for MgATP, and combinations of the ions had no synergistic effect (data not shown).

To test the possibility that cations may alter the sensitivity of the enzyme to vanadate, a series of assays was done with increasing concentrations of NH$_4^+$, K$^+$, or Na$^+$, each in the presence or absence of vanadate (Fig. 9). When 1.5 $\mu$M vanadate was present, NH$_4^+$ and K$^+$ no longer stimulated but...
instead were slightly inhibitory. For example, the addition of 25 mM NH₄⁺ to a sample with vanadate resulted in a 23% loss of activity (Fig. 9). Na⁺, instead of inhibiting, had no effect on ATPase activity when assayed with vanadate (1.5 μM). Thus, although monovalent cations have a much smaller effect on the Neurospora ATPase than on the (Na⁺, K⁺)-ATPase of animal cells (1, 2, 5, 6, 25-27), the interaction between vanadate and cations is qualitatively similar in the two cases: ATPase activity assayed in the presence of K⁺ or NH₄⁺ is more sensitive to vanadate than is ATPase activity assayed in the presence of Na⁺.

To explore this interaction further, the activity of the Neurospora ATPase was measured as a function of vanadate concentration in the presence of K⁺ (25 mM), NH₄⁺ (25 mM), and Na⁺ (25 mM). The data with K⁺ and NH₄⁺ are illustrated in Fig. 10; the Na⁺ data (not shown) were superimposable with the control curve obtained in the absence of added alkali metal cations. All four inhibition curves were linear when plotted by the method of Dixon and Webb (32) with half-maximal inhibition produced by 1.0 μM vanadate in the absence of added cation, 1.1 μM with Na⁺, 0.64 μM with K⁺, and 0.45 μM with NH₄⁺.

It seems likely that experiments with vanadate will provide useful new information about the reaction mechanisms of both enzymes. The recent ⁴⁰K binding studies of Cantley et al. (6), for example, have shown that vanadate acts at a low affinity ATP-binding site of the (Na⁺, K⁺)-ATPase, and have lent support to an alternating site model for that enzyme. In the case of Neurospora ATPase, there are indications of a sigmoid dependence of enzyme activity upon ATP concentration, which can be fitted nearly quantitatively by assuming that two ATP binding sites must be filled in order for the reaction to proceed (or alternatively, that the enzyme possesses an even larger number of ATP binding sites with a lesser degree of cooperativity). Vanadate causes a shift in concentration dependence from a sigmoid curve to a hyperbolic curve and, thus, clearly diminishes the cooperativity between the sites. The precise way in which this effect is brought about will have to await further studies with purified enzyme; in particular, measurements of ⁴⁰K binding (analogous to those of Cantley et al. (6)) should prove valuable.

**Effects of Monovalent Cations**—A second important piece of information regarding the reaction mechanism of the Neurospora ATPase concerns the role of monovalent cations. We reported earlier (8) that, although there is considerable ATPase activity in the absence of alkali metal cations, activity is stimulated moderately by K⁺ or NH₄⁺. Because of the physiological evidence that the Neurospora plasma membrane ATPase functions in proton translocation (11-14), we proposed that these minor ionic effects are unrelated to transport but, instead, represent the kinds of salt effects seen with many enzymes (for example, bacterial, chloroplast, and mitochondri al ATPases; 33-35). An alternative possibility, and one that has been advanced to explain similar monovalent cation effects on the plasma membrane ATPase of higher plants (36-38), is that the overall ATPase activity results from at least two enzymes: a basal level of (Mg⁺⁺)-ATPase, and a K⁺-activated ATPase which functions in K⁺ transport.

Two lines of evidence from the present study argue strongly against this view for the Neurospora ATPase. In the first place, neither the sigmoid shape of the v versus S curve nor the Kₐₐ is altered by K⁺ or NH₄⁺, even though the amount of activity does vary (Fig. 8). In the second place, inhibition of ATPase activity by vanadate follows a simple monotonic curve, interpretable in terms of a single Kₛ, whether or not K⁺ or NH₄⁺ is present (Fig. 10). Both of these results are most easily accounted for in terms of a single ATPase whose activity is modulated slightly by monovalent cations.

**Reversal of Inhibition by EDTA**—One finding of special interest is that the inhibition of the Neurospora plasma membrane ATPase by vanadate can be prevented (and reversed) by low concentrations of EDTA. Because neither EGTA nor CDTA is effective, and because small changes in the concentration of free Mg⁺⁺ have no significant influence on enzyme activity, we conclude that EDTA does not act by chelating Mg⁺⁺. Rather, it seems likely that EDTA complexes vanadate directly. In turn, excess free Mg⁺⁺ can compete with vanadate for EDTA. Such competition could well explain the failure of Cantley et al. (2) to observe an EDTA effect on vanadate inhibition of the (Na⁺, K⁺)-ATPase, since their standard reaction medium contained 28 mM MgCl₂.

Overall, the EDTA effects seen in the present experiments have important implications for the procedures used to prepare and assay vanadate-sensitive ATPases. Since many cells and tissues contain vanadate (39), the presence or absence of EDTA during the preparation of an ATPase may determine how much vanadate remains bound to the enzyme. Furthermore, if vanadate is bound to an ATPase, or if it is accidentally added during the assay (for example, via Sigma ATP), the presence or absence of EDTA in the assay medium may determine the activity that is measured. In the light of these possibilities, previously reported effects of EDTA on ATPases (for example, Ref. 40-43), as well as reported shifts in pH optimum with ATP of varying purity (44), may need to be reinvestigated.

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