Inhibition of (Na,K)-ATPase by Tetravalent Vanadium*

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Vanadyl, the tetravalent state of vanadium and a divalent cation, VO\(^{4+}\), was a relatively powerful inhibitor of highly purified membrane-bound sodium and potassium ion transport adenosine triphosphatase. The sensitivity of the ATPase activity to vanadyl characteristically correlated positively with the specific activity of the enzyme preparation. Inhibition ranged from nearly complete inhibition at less than 5 \(\mu\)M vanadyl for some of the purest fractions (specific activity ~45 \(\mu\)mol/min/mg of protein) to no observable inhibition at 300 \(\mu\)M vanadyl in one crude preparation of the enzyme with a specific activity of 10 \(\mu\)mol/min/mg of protein. The level of free vanadyl was reduced by incubation with these membranes, but this reduction was not sufficient to account for the low sensitivity to vanadyl observed in crude preparations. A reduction in specific activity by partial inactivation of a sensitive preparation by treatment with FeCl\(_3\) and ascorbate reduced its sensitivity to vanadyl.

Anionic ligands of the enzyme, vanadate or ATP, increased the rate of recovery from inhibition after chelation of free vanadyl. At pH 6.1, the inhibition was characteristically fully reversible (\(\sim 10\) min), whereas at pH 8.1 it was stable for hours. The degree and stability of enzyme inhibition by vanadyl increased for several hours during incubation of the vanadyl enzyme mixture, and at pH 6.1 the properties of the inhibitor itself also changed with time. Preincubation of the ion at that pH for 5 h before addition of the enzyme produced a more stable inhibition. The time- and pH-dependent changes in the degree and stability of enzyme inhibition probably relate to the complex chemistry of the vanadyl ion in solution.

The potential of vanadyl ion, the tetravalent state of vanadium, as a regulator of the activity of (Na,K)-ATPase has only recently been suggested. Vanadate ion, containing vanadium in the pentavalent oxidation state, is a potent inhibitor of the ATPase activity \((K_i \approx 0.5\ \mu M)\) with striking pharmacologic effects on cardiac contractility, blood pressure, and urine flow \((1)\). In addition, externally applied vanadate has insulin-like effects on glucose transport \((2)\), glucose oxidation \((3)\), and on glycogen synthetase and phosphorylation of the insulin receptor \((4)\). However, Cantley and co-workers \((5, 6)\) have shown that vanadate is reduced in red cells to vanadyl ion by intracellular glutathione following uptake through an anion transport system. Although free vanadyl ion is oxidized to vanadate in a few minutes in aqueous solution exposed to air at neutral pH, the tetravalent state appears to be stable when complexed with intracellular proteins, such as hemoglobin \((5, 6)\) or smaller molecules \((3, 7)\). The difficulties inherent in preventing oxidation of vanadyl at neutral pH have hampered study of the biochemical actions of vanadyl in \textit{vivo}. Based on transient kinetics of vanadyl interaction with (Na,K)-ATPase, Cantley and Aisen \((5)\) concluded that vanadyl is at most a less potent inhibitor of the enzyme than vanadate. Since vanadyl, rather than vanadate, is the major form of intracellular vanadium, we have taken a closer look at its interaction with (Na,K)-ATPase using an anaerobic assay system that avoids inhibition of the enzyme by contaminating vanadate.

EXPERIMENTAL PROCEDURES

Materials—Vanadyl sulfate (vanadium oxysulfate) was obtained from Alfa Inorganics, Inc. (Beverly, MA), and ammonium vanadate was from Fisher. VOSO\(_4\) and NH\(_4\)VO\(_3\) solutions were prepared at 10 mM concentration in 50 mM HCl or 1 mM Tris, respectively, and stored at room temperature. All buffers were from Sigma. Diodium ATP was from Boehringer Mannheim.

Preparation and Assay of the Enzyme—Membrane-bound (Na,K)-ATPase was purified from the outer medulla of dog kidney by the zonal gradient centrifugation method of Jorgensen \((8)\). Ten to fifteen fractions were collected from the gradient and stored at 0°C in 30% glycerol (w/v), 20 mM imidazole HCl (pH 7.5), 0.7 mM EDTA, and 0.7 mM dithioerythritol; in some cases the enzyme was washed free of EDTA and dithioerythritol by centrifugation before use. The ATPase activity was stable for more than 6 months and was 99.9% inhibitable by ouabain. The specific activity of the collected fractions ranged from 10 to 44 \(\mu\)mol/min/mg of protein at 37°C in 30 mM imidazole/glycylglycine (pH 7.4), 100 mM NaCl, 25 mM KCl, 4 mM Na\(_2\)ATP, 3.9 mM MgCl\(_2\), 0.2 mM EDTA, and 0.2 mg/ml of fat-free bovine serum albumin. Activity in the presence of 0.33 mM ouabain was subtracted from that in its absence. All (Na,K)-ATPase activities reported are in units of micromoles of P\(_i\) released per min under these conditions. The protein concentration of the (Na,K)-ATPase was estimated by the method of Bradford \((9)\) with bovine serum albumin as a standard. The values were multiplied by 1.144 to correct for the ratio of the color factors of the two proteins as estimated in this laboratory by amino acid analysis.\(^2\) Inorganic phosphate split from ATP in the ATPase assay was estimated on a Technicon autoanalyzer by the method of Hegyvary \textit{et al.} \((10)\).

Membrane-bound (Na,K)-ATPase was prepared from the outer medulla of dog or guinea pig kidney by two modifications of the rapid method of Jorgensen \((8)\). The two procedures differed primarily in their method of dissection and SDS/protein ratio. The first used visual dissection of the outer medulla based on its red color and an SDS/protein ratio (w/w) of 0.32–0.37. It produced an enzyme with a specific activity of 10–20 units/mg of protein. In the second method, the dissection was done by scraping away the cortex from the inner medulla.

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\(^1\) The abbreviations used are: (Na,K)-ATPase, sodium and potassium ion transport adenosine triphosphatase (EC 3.6.1.3); SDS, sodium dodecyl sulfate; PIPES, piperoxane-N,N'-bis(2-ethanesulfonic acid); HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid.

\(^2\) Y. Hayashi and R. L. Post, unpublished data.
medulla, and the SDS/protein ratio was 0.43. This improved method yielded a (Na,K)-ATPase specific activity of 20–40 units/mg of protein. ATPase activity was measured as described above, and the enzyme was stored at 0°C in 30% glycerol containing either 24 mM imidazole, 16 mM Hepes, 0.5 mM EDTA, and 2 mM dithiothreitol (pH 7.0) or 15 mM imidazole/MOPS and 0.1 mM EDTA (pH 7.1). All membrane separations were stored at a protein concentration of 2.2–5.5 mg/ml.

Estimation of Vanadyl Inhibition of (Na,K)-ATPase—To minimize oxidation of vanadyl to vanadate, which occurs in air above pH 3, Warburg flasks containing a buffered suspension of the enzyme in the body of the flask and separate solutions of VOSO₄ and EDTA in two side arms were equilibrated under prepurified N₂ at 37°C for 1 h before mixing of the components. The solution of VOSO₄, in 1 mM HCl (0.4 ml) was then tipped into the enzyme suspension (2.0 ml) to start the reaction. Subsequently the vanadyl ion free in solution was chelated by tipping in the EDTA solution (0.4 ml). After 1 min or more the flask was opened and a solution of MgATP containing bovine serum albumin (0.4 ml) was added to start the ATPase reaction. In this way the recovery from vanadyl inhibition could be observed. Aliquots (0.5 ml) were removed at five successive time intervals after ATP addition for determination of inorganic phosphate released by means of the automated assay described above.

Less than 20% of the ATP was split. ATPase activity in a duplicate flask containing 0.33 mM ouabain was subtracted from the activity in its absence to estimate the (Na,K)-ATPase activity. The reaction medium was buffered with PIPES/Tris at pH 6.1 or 6.5 or with Tris/glycylglycine in pH buffers, 0.05 M KCl, 5 mM ATP, 4 mM MgCl₂, 0.1–0.5 mM EDTA, 0.1 mg/ml of bovine serum albumin, and 400 μM VOSO₄. These ionic conditions were chosen to minimize possible inhibition of the enzyme by vanadate formed by inadvertent oxidation of vanadyl ion (see under "Results"). The coefficient of variation of enzymatic activity in replicate flasks estimated under these conditions was 4.0%. Unless otherwise stated, the Na⁺ was present initially with the enzyme, and the K⁺ was added together with the Mg/ATP. However, in some cases Na₅ATP or one or more of the ions and ammonium vanadate (NH₄VO₃) were added with or shortly following the EDTA in order to assess their effect on the rate of recovery from vanadyl inhibition (cf. figure legends). Complete chelation of free vanadyl by EDTA was verified by demonstrating that addition of the EDTA to the enzyme suspension before addition of the vanadyl completely prevented inhibition. At low pH (pH 6.1) EDTA was a relatively effective chelator also of vanadate, completely preventing vanadate inhibition of the enzyme (measured in the presence of 80 mM NaCl and 80 mM KCl) at 1 h time points. In the absence of divalent cations, EDTA could be made insignificant when necessary (for instance, when the effect of vanadate on the vanadyl recovery rate was measured) by increasing the pH to 6.5 and lowering the EDTA/vanadate ratio to 3.

The data for the inhibited samples in most cases were normalized against control data in order to provide a format that was independent of the rate in the control samples and that corrected the test data for an occasional small curvature in the control activity curve. The concentrations of released Pi from the control flasks were fitted to an equation of the form \( [F] = a + b t^2 \), where \( a \) and \( b \) are fitted constants and \( t \) is time. For each concentration of Pi, released by the inhibited enzyme a time was calculated from the inverse of the equation. This time was that which would have been required for the control enzyme to release the same amount of Pi. The ratio of this time to the actual time was taken as the ratio of Pi released in the inhibited sample to that in the control. The control data are represented on a graph by a straight line, and the data from the inhibited samples are plotted as a fraction of these normalized control values. Data points without normalization are shown in Figs. 4a, 6, and 8.

To estimate the initial inhibition present at the time of ATP addition it was assumed that a fraction, F, of the enzyme was inhibited at zero time and that this inhibition subsequently disappeared as a single exponential function of time, \( t \), with a rate constant, \( k \), i.e., \( [F]/[F]₀ = 1 - e^{-kt} \), where \( [F]₀ \) is the activity of the uninhibited enzyme. Trial values were inserted for \( F \) and \( k \), and the error was computed as the sum of the square of each difference between a data point and its computed value divided by the number of data points and \( k \) was adjusted repeatedly by successive approximations until the minimum error was found. Since two constants were estimated from five data points and since the validity of the assumptions could not be tested independently, this method of data analysis is no more than a heuristic device.

Inactivation by Ascorbate and FeCl₃—The membrane-bound (Na,K)-ATPase was inactivated by a modification of the method used by Levine (11) for glutamine synthetase. Purified enzyme (0.5 mg of protein/ml; 40 units/mg of protein) was incubated at 37°C in a 30/12 mM imidazole/MOPS buffer (pH 7.2) containing freshly prepared aliquots of ascorbic acid (5 mM) and FeCl₃ (0.01 mM). Inactivation was stable for at least 2 weeks after dilution with the incubation buffer. ATPase activity was measured under standard conditions as described above, and the remaining degree of inhibition and the rate of ATPase activity were measured after addition of (NH₄)VO₃ were added with or shortly following the EDTA in order to block Va/VO₃⁺ complexes.

Measurement of the Rate of Vanadyl Oxidation—Oxidation of the blue vanadyl ion at pH 6.1 or 6.1 was monitored by observing the disappearance of the characteristic vanadate absorbance at 766 nm. The extinction coefficient at this wavelength was approximately 17.6 M⁻¹ cm⁻¹. It was not possible to measure the vanadate absorbance directly after stopping incubations using ion exchange chromatography following acidification of the samples to pH 2 with H₂SO₄. After a brief centrifugation to remove the VANADYL/phosphate mixture, the absorbance was measured at 280 nm. The relative proportions of each ion were then estimated based on the approximate extinction coefficients of vanadate (260 M⁻¹ cm⁻¹) and vanadyl (100 M⁻¹ cm⁻¹) at 280 nm.

Estimation of Vanadyl Binding—Total binding of vanadyl to the membrane-bound enzyme preparation was estimated in an indirect manner by first allowing vanadyl to complex with the preparation and then separating free and bound vanadyl by filtration. Free vanadyl in the filtrate was oxidized quantitatively to vanadate, and the (Na,K)-ATPase specific activity was determined in MgATP containing bovine serum albumin and KCl to give 0.8 mM K⁺. After addition of MgATP containing bovine serum albumin and KCl to give 0.8 mM K⁺, the sample was incubated at 37°C for 5 min. The mixture was centrifuged at 8000 rpm for 5 min, and the supernatant was removed for further detection of vanadate. The (Na,K)-ATPase specific activity was then determined in MgATP containing bovine serum albumin and KCl to give 0.8 mM K⁺.
of 4 mM Na₂ATP (in 0.4 ml) was then compared to that produced by standard vanadate concentrations (1-4 µM). The degree of enzyme inhibition varied linearly with the logarithm of vanadate concentration in this range.

RESULTS

Vanadyl in micromolar concentrations was a powerful and reversible inhibitor of highly purified membrane-bound (Na,K)-ATPase (Fig. 1). It was possible to study inhibition of (Na,K)-ATPase activity by vanadyl in effective isolation from inhibition by contaminating vanadate by working under a nitrogen atmosphere and in the presence of low concentrations of K⁺ (0.8 mM) and free Mg²⁺ (4 mM with 5 mM ATP).

Under these ionic conditions (Na,K)-ATPase activity was about 20% of that in the standard system, and inhibition by 0.1 mM NH₄VO₃ was less than 5%. EDTA was added to the vanadyl-enzyme mixture in order to chelate free vanadyl just prior to addition of Mg/ATP. This allowed estimation of the rate of recovery from vanadyl inhibition as well as the initial degree of inhibition (see under “Experimental Procedures”). For the data shown in Fig. 1, the initial degree of inhibition after a 15-min incubation with 50 µM vanadyl was close to 100%, and the half-time for disappearance of the inhibition was about 6 min. The degree and stability of (Na,K)-ATPase inhibition increased with the time of incubation of the vanadyl-enzyme mixture (Fig. 2).

The sensitivity of (Na,K)-ATPase activity to vanadyl varied with the degree of enzyme purity. Among different fractions from a given zonal preparation of the enzyme, increasing sensitivity to vanadyl correlated roughly with increasing specific activity (Fig. 3) and, therefore, with position in the sucrose density gradient (denser fractions possessing both higher specific activity and higher sensitivity to vanadyl). Typically, the best fractions of most zonal preparations of the enzyme (specific activity, 30-44 units/mg of protein) were inhibited 50% after a 30-min incubation with about 10 µM vanadyl, with complete inhibition occurring at higher vanadyl concentrations. However, no inhibition was observed at 300 µM vanadyl in one crude enzyme preparation with a specific activity of 10 units/mg (Fig. 4A). Some preparations showed an atypical response to vanadyl that was characterized by relatively high sensitivity (Kᵢ < 5 µM) and high stability of inhibition (Fig. 4B). For enzyme fractions of high specific activity, a 30-min incubation at pH 7.1 with various concentrations of vanadyl showed Michaelis-Menten kinetics. A few early experiments indicated more complex kinetics with cruder poorly sensitive enzyme preparations (not shown).

We were interested in determining whether the variation of vanadyl sensitivity with enzyme purity reflected some intrinsic difference in the state of the enzyme in the different preparations or rather simply a reduction of the free vanadyl concentration by the cruder preparations. This reduction might be due to binding of vanadyl species. A nonspecific effect was suggested by early experiments showing reduction of vanadyl sensitivity with increase in the concentration of membrane (enzyme) protein in the reaction mixture (not shown). In order to measure the concentration of free vanadyl after incubation with the enzyme preparation, we separated free from bound vanadyl by filtration, quantitatively oxidized...
In order to examine the correlation between low enzyme specific activity and low sensitivity to vanadyl in an isolated enzyme preparation, we compared the vanadyl sensitivity of a high specific activity preparation and that of the same preparation after partial inactivation by the method applied by Levine (11) to glutamine synthetase. In this method incubation of the enzyme in the presence of ascorbate and FeCl₃ for either 70 or 240 min produced 65% or nearly complete (>99%) inactivation of (Na,K)-ATPase activity, respectively. The response of the 65% inactivated enzyme to 10 μM vanadyl was compared to that of the untreated enzyme and to a 65:35 mixture (based on protein) of the completely inactivated enzyme and the untreated enzyme. The initial degree of inhibition of the 65% inactivated enzyme was much lower than that of either the untreated enzyme or the 65:35 mixture of the completely inactivated and untreated enzymes (Table I). A possible interpretation of these results in terms of interaction between inactive and active enzyme molecules within the membrane bilayer is offered under "Discussion."

Since pH has complex effects on the chemistry of vanadyl ion in solution (12), we compared vanadyl inhibition of (Na,K)-ATPase activity at pH values of 6.1 and 8.1. Comparisons between pH values were made for single preparations of the enzyme in order to avoid the variability of different enzyme preparations with regard to vanadyl inhibition. Characteristically inhibition was fully reversible after several minutes at pH 6.1, whereas at pH 8.1 it was stable for hours (Fig. 5). In addition, the initial inhibition was slightly greater at pH 6.1 than at pH 8.1 (Fig. 5). Control experiments confirmed that EDTA was an effective chelator of vanadyl at both pH values, even after a 60-min preincubation of the VOSO₄ at those pH values without enzyme (data not shown). Thus we assume that a true rate of recovery from vanadyl inhibition

![Fig. 3](https://example.com/fig3.png)

**FIG. 3.** Vanadyl inhibition of different membrane fractions of varying specific activity from a single zonal preparation of the enzyme. Reactions were performed at pH 6.5 as described under "Experimental Procedures" and Fig. 1. VOSO₄ (30 μM) was allowed to equilibrate with the enzyme samples (9.4 μg of protein/ml) for 45 min before addition of 0.3 mM EDTA. Mg/ATP was added to start the test of activity 4 min after addition of the EDTA. The data points shown are the averages of duplicate determinations and are plotted normalized against their respective controls (incubated in the absence of VOSO₄, depicted by the solid reference line marked un inhibited enzyme. The specific activities of the samples under standard (Na,K)-ATPase assay conditions (see under "Experimental Procedures") were 10.4 units/mg of protein (●), 12.0 units/mg (▲), 15.8 units/mg (○), 23.1 units/mg (×), and 33.5 units/mg (◆).

![Fig. 4](https://example.com/fig4.png)

**FIG. 4.** Extremes from the range of responses of (Na,K)-ATPase activity to vanadyl observed with different enzyme preparations. A, a crude preparation of the enzyme from guinea pig kidney made by the rapid method (21.5 μg of protein/ml; 10.1 units/mg of protein) buffered at pH 8.1 with Tris/glycylglycine was incubated under a N₂ atmosphere as described under "Experimental Procedures" with either 0 (●), 200 (▲), or 300 (×) μM VOSO₄ for 2 h before addition of 1 mM EDTA. Mg/ATP was added to start the test of activity 4 min after addition of the EDTA. B, two separate preparations of the enzyme from hog kidney (●, 2.9 μg of protein/ml; 28.3 units/mg of protein) or dog kidney (×, 3.1 μg of protein/ml; 38.7 units/mg of protein) were incubated at pH 6.5 under a N₂ atmosphere with either 5 (●) or 10 μM (×) VOSO₄ for 15 (●) or 30 (×) min before addition of EDTA. Mg/ATP was added to start the test of activity 10 (●) or 6 (×) min after addition of the EDTA. The data points for the inhibited samples are plotted normalized against their respective controls, depicted by the solid straight reference line.
TABLE 1

Reduction in sensitivity to vanadyl produced by partial inactivation with ascorbate/FeCl₃

| System                        | Specific activity* | Membrane initial inhibition | Half-time of recovery† |
|-------------------------------|--------------------|----------------------------|------------------------|
| Protein concentration        | units/mg protein   | µg/ml                      | %                      | min                     |
| 1. Untreated enzyme           | 40                 | 4.06                       | 89 ± 1.3               | >75                     |
| 2. 65% inactivated enzyme     | 26                 | 4.06                       | 45 ± 2.2               | 15 ± 1                  |
| 3. 65:35 mixture of untreated and dead enzyme | 26 | 4.06 | 91 ± 1.2 | >75 |

* Specific activity under standard (Na,K)-ATPase assay conditions.
† The degree of initial inhibition in the presence of vanadyl is expressed as a percentage of the activity of the same system when measured without vanadyl, extrapolated back to the start of ATP hydrolysis (see under "Experimental Procedures").
‡ Half-times for recovery from vanadyl inhibition that are greater than 75 min represent >99% stable inhibition within the time frame of these measurements.
$ The mixture contained 1.4% µg of protein/ml of the untreated enzyme and 2.6% µg of protein/ml of the dead enzyme.

under these conditions was observed. Earlier experiments with different preparations of the enzyme at an intermediate pH (pH 7.1) indicated two components of inhibition corresponding to the patterns observed at pH values of 6.1 and 8.1. About 65% of the inhibition was reversible in less than 30 min, but a residual component of stable inhibition was present several hours after addition of 1 mM EDTA (data not shown).

At pH 6.1 the properties of the vanadyl ion itself changed with time. Preincubation of the ion at that pH for 5 h before addition of enzyme produced a more stable inhibition (Fig. 6), mimicking that seen at pH 8.1 without the preincubation (cf. Fig. 5).

The tetravalent vanadyl species is stable as the divalent cation, VO²⁺, below pH 3, but becomes increasingly susceptible to oxidation and hydroxylase as pH increases (12). We detected no oxidation of vanadyl (0.4 mM) to vanadate after 2 h of incubation at 37 °C under a N₂ atmosphere at pH 6.1. In this measurement the relative proportions of the vanadyl and vanadate species were estimated by stabilizing both oxidation states by acidification, then separating them by Dowex chromatography (see under "Experimental Procedures"). Vanadyl and vanadate were monitored in the column effluent by their ultraviolet absorption. We also monitored the rate of oxidation of vanadyl solutions in air at 37 °C by observing the disappearance of the characteristic blue vanadyl absorbance at 766 nm. In a 4.2 mM vanadyl solution, half of the vanadyl was oxidized in air at pH 6.1 in 60 min, whereas at pH 8.1 half was oxidized in 12 min (Fig. 7). The rate of oxidation was dependent on the vanadyl concentration, being over twice as rapid at pH 6.1 in a 0.4 mM vanadyl solution as compared to a 4.2 mM solution (Fig. 7). In another experiment, enzymatic assay of the conversion of 5 µM vanadyl to vanadate at pH 7.1 and 20 °C showed a half-time of about 5 min.

The effect of other ligands of the (Na,K)-ATPase on vanadyl inhibition was tested. We found that after vanadyl was allowed to complex with the enzyme preparation, a 10-min incubation with 4 mM ATP in the presence of 0.5 mM EDTA (before addition of Mg²⁺ to start ATP hydrolysis) significantly lowered the degree of inhibition compared to controls preincubated with EDTA alone (Fig. 8). Addition of 0.1 mM ammonium vanadate to the vanadyl-enzyme complex 2 min after chelation of the free vanadyl with EDTA and 13 min prior to addition of ATP to start the test of activity also resulted in a lower degree of initial inhibition and a higher rate of recovery from vanadyl compared to controls (Fig. 9). Initial inhibition refers to that extrapolated back to the start of the activity test. Acceleration of recovery from vanadyl inhibition by vanadate was favored by K⁺ and Mg²⁺ more than by Na⁺. It was readily observable in the presence of K⁺ (0.8 mM) and Mg²⁺ (4 mM) without Na⁺, but was small or nonexistent in the presence of Na⁺ (160 mM) alone (Fig. 9). Na⁺ or K⁺ alone with or without Mg²⁺ did not appear to modify vanadyl inhibition in the absence of vanadate (Fig. 9).

DISCUSSION

Vanadyl was a relatively potent inhibitor of membrane-bound kidney (Na,K)-ATPase producing nearly complete inhibition at a concentration of less than 5 µM in some highly purified preparations of the enzyme. It was not possible to define an equilibrium constant for the inhibition since the degree of inhibition under a given set of conditions varied...
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FIG. 6. Change in kinetics of recovery from vanadyl inhibition due to preincubation of the vanadyl. Reactions were performed under N₂ at pH 6.1 as described under "Experimental Procedures" with the following modifications. O—O, the enzyme (8.6 μg of protein/ml; 25 units/mg of protein) was incubated in the buffer for 5 min in the absence of VOSO₄, then EDTA (1 mM) was added, followed 1 min later by Mg/ATP. ■—■, the enzyme was incubated with 50 μM VOSO₄ for 5 min before addition of EDTA and Mg/ATP as described above. ▲—▲, the vanadyl ion was preincubated for 5 h in the solution buffered at pH 6.1, but without enzyme. Enzyme was then added and allowed to equilibrate with the vanadyl for 5 min before addition of EDTA and Mg/ATP. X—X, the vanadyl ion was preincubated for 5 h in the solution buffered at pH 6.1, but without enzyme. Enzyme was then added and allowed to equilibrate with the vanadyl for 5 min before addition of EDTA and Mg/ATP as described above.

FIG. 7. Oxidation of vanadyl in air at pH 6.1 and 8.1. Oxidation of the blue vanadyl ion buffered at pH 6.1 (●) or pH 8.1 (▲) under air was monitored by following the disappearance of the characteristic vanadyl absorbance at 766 nm as described under "Experimental Procedures." The starting concentrations of the VOSO₄ solutions were 4.2 (●) and 0.4 (▲) mM.

greatly between different enzyme preparations, correlating positively with enzyme purity (cf. Figs. 3 and 4). In addition to this variation, the inhibitory properties of the vanadyl ion changed within the time frame of these experiments (cf. Fig. 6). Also note in Fig. 2 that inhibition did not saturate at 100% initial inhibition (which would show complete occupancy of one site), but that further inhibition developed at longer times of incubation as indicated by a slower rate of recovery (showing a second mechanism of inhibition). The dependence of vanadyl inhibition on enzyme purity was not simply due to a greater reduction of the free vanadyl concentration by cruder enzyme preparations. The variation between the response of different enzyme preparations to vanadyl may reflect some difference in the intrinsic state of the enzyme in these preparations.

The time- and pH-dependent changes in the degree and stability of (Na,K)-ATPase inhibition can be attributed to the complex chemistry of the vanadyl ion in solution. The important oxidation states of vanadium under physiologic conditions are the tetravalent (vanadyl) and pentavalent (vanadate) species, each of which forms hydroxide compounds with increasing pH. The major form of the vanadate species
between pH 6 and 8 and below a concentration of about 0.3 mM is the monovalent anion, \(\text{H}_2\text{VO}_4^-\) (13). The state of hydroxylation of the vanadyl ion near neutral pH is much less certain, although vanadyl exists as the divalent cation \(\text{VO}^{2+}\) in strong acid conditions and as a monovalent anion, \(\text{VO}^{\text{OH}}_2^-\), in highly alkaline conditions (12). Intermediate hydroxide forms of vanadyl presumably exist, but neither these nor their rates of equilibrium have been clearly defined.

The electron paramagnetic resonance of vanadyl demonstrated metal binding site conformational states in carboxypeptidase A (18) and transferrin (19). The ion inhibits alkaline phosphatase (20). Complexes of both vanadate and vanadyl with uridine inhibit ribonuclease (14).

Vanadyl ion forms stable complexes with several enzymes. The electron paramagnetic resonance of vanadyl demonstrated metal binding site conformational states in carboxypeptidase A (18) and transferrin (19). The ion inhibits alkaline phosphatase (20). Complexes of both vanadate and vanadyl with uridine inhibit ribonuclease (14).

The potential of vanadyl as a physiologic inhibitor of the native cellular (Na,K)-ATPase requires further scrutiny since the inhibitory potency of vanadyl was much diminished in crude enzyme preparations. However, we are encouraged that inhibition by vanadyl may be useful for study of the mechanism of the enzyme since binding of other ligands (vanadate or ATP) or partial inactivation by chemical treatment affected vanadyl inhibition. The data shown are averages from two separate experiments, each run in duplicate, and are plotted normalized against their respective uninhibited enzyme controls. The control rates of inorganic phosphate release were: 0.217 \(\mu\text{mol/min (}\Delta--\Delta); 0.169 \mu\text{mol/min (}\text{O}--\text{O}); 0.166 \mu\text{mol/min (}\Delta--\text{O}); 0.166 \mu\text{mol/min (}\text{O}--\text{O})\).
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