# Interaction of Trace Levels of Vanadium(IV) and Vanadium(V) in Biological Systems

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**Abstract:** Enzyme kinetics have been used to study interactions of trace-level concentrations of vanadate [V(V)] and vanadyl cation [V(IV)] in biological systems. A quantitative method based on the inhibition of alkaline or acid phosphatase by monomeric vanadate or vanadyl cation has been developed to determine the concentration of free monomeric vanadate or vanadyl cation at $10^{-5}$–$10^{-7}$ M vanadium concentrations. Interactions of vanadate and vanadyl cation with potential ligands including buffers, chelating agents, enzyme substrates, cofactors, amino acids, peptides, and proteins were examined. Seven out of 26 commonly used buffers were found to strongly complex vanadate, and an additional 11 buffers were found to complex vanadate to various degrees. The vanadyl cation generally interacts more strongly with these buffers than does vanadate. In contrast, the Tricine[1]–vanadate complex was 8 times more stable than the Tricine–vanadyl complex. The formation constant ($K^{eff}_{pH\ 8.0}$) for the EDTA–vanadate complex was determined to $1.4 \times 10^4$ L/mol in agreement with previous estimations in this pH range. The interaction of vanadate with enzyme substrates such as glycerol and glucose or with cofactors such as NAD was not observable at $10^{-6}$ M vanadate concentrations. However, enzyme substrates such as citrate, malate, and glycerate did show interaction with vanadate. Many amino acids and peptides such as lysine, glutamic acid, glycine, and glycylglycine showed evidence of weak vanadate interactions. Amides, amines, and carboxylic acids in amino acids or peptides show similar affinity for vanadate or vanadyl cation. Enzymes, including ribonuclease (in the presence of uridine), chymotrypsin, pepsin, aldolase, acetylcholinesterase, and myosin (in the presence of ADP), were found to bind vanadate considerably more strongly than did bovine serum albumin. The evidence presented suggests that spontaneous vanadate and vanadyl reactions may affect observed biological activities with vanadium present at $10^{-5}$–$10^{-7}$ M concentrations.

Biological studies involving vanadium in oxidation states IV and V have increased dramatically with the discovery of several potent biological effects of this element.[2] Vanadate mimics the effects of insulin and epidermal growth factor and elevates the cAMP levels in mammals.[2] Vanadate and vanadyl cation inhibit ribonuclease, many ATPases, and phosphatases including phosphotyrosine phosphatase.[2] Vanadate derivatives are reported as substrates for several enzymes in the glycolysis and glycogen metabolism.[3] Recently a vanadium-requiring nitrogenase has been discovered and characterized biochemically and genetically.[4] Vanadium is essential for vanadium-accumulating tunicates and mushrooms, and vanadium is a trace element in mammals and plants in the concentration range from $10^{-9}$ to $10^{-6}$ M.[2] Although the oxidation state of vanadium in biological environments is commonly presumed to be IV, accumulating evidence suggests that vanadium(V) is also present.[5]

Biological studies with vanadium are complicated by the aqueous chemistry of vanadium(IV) and vanadium(V).[6-11] Vanadium(IV) ($VO^{2+}$) is a well-characterized cation that has been examined with EPR spectroscopy and UV spectroscopy in biological systems. Vanadium(IV) will oligomerize or polymerize under physiological conditions at concentrations above $10^{-6}$ M, oxidize in the presence of oxygen, or generate complexes in the presence of various phosphates, sulfates, thiols, or carboxylic acids.[1,2] Vanadium(V), on the other hand, is more stable as free vanadate ($H_2VO_4^-$ or $HVO_4^{2-}$) in the presence of oxygen but is readily reduced by various organic molecules and oligomerizes at concentrations above $10^{-4}$ M.[2] For example, NADH or NADPH will chemically reduce vanadium(V) in the presence of buffers such as Tris and Hepes, presumably in conjunction with the oxidation of NADH or NADPH.[6] Such a reaction might account for the first report of a vanadium-dependent oxidase.[6] Functionalities such as thiols and reactive aldehydes or ketones in small or complex biomolecules will also reduce vanadium(V).[7,8] In addition to redox chemistry, vanadate can also undergo a series of spontaneous addition reactions with many organic molecules.[9-11]

There has been recent interest in chemical and biochemical interactions of biomolecules with vanadate.[9,10] The interactions of vanadate with many organic molecules have been studied at vanadate concentrations from 0.1 to 50 mM with $^{51}V$ NMR spectroscopy.[8-10] At these high vanadate and ligand concentra-

(1) Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris propane, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane, EPPS, *N*-(2-hydroxyethyl)piperazine-*N*'-3-propanesulfonic acid; TAPS, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); DEA, diethanolamine; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; MDEA, *N*-methyldiethanolamine; TEA, triethanolamine; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; DIPSO, 3-[*N*-[tris(hydroxymethyl)methyl]amino]-2-hydroxypropanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; MIDA, *N*-methyliminodiacetic acid.

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tions, vanadate generates various oligomeric anions. Since all the oligomeric anions are in equilibrium, complex formation of one species will affect the concentration of all species. Because of oligomerization, the monomeric vanadate concentration varies in a nonlinear relationship with the total vanadate concentration. The product ratio of complex (presumably formed from monomeric vanadate) to vanadate will accordingly vary with the total concentration of vanadate. Formation constants of complexes determined in solutions containing low vanadate concentrations give constant product ratios of complex to vanadate since the total vanadate present in solution is mainly monomeric vanadate. Observations of vanadate reactions at low concentrations therefore not only parallel physiological conditions in terms of vanadate concentrations but also simplify the interpretations of vanadate reactions because the oligomeric vanadate species under these conditions are negligible. This work concerns the chemical interactions of both vanadyl cation and vanadate with organic molecules at vanadium concentrations from $10^{-5}$ to $10^{-7}$ M.

Spontaneous interactions of both vanadyl cation and vanadate with proteins may be key to understanding the action of vanadium both in vivo and in vitro. Vanadyl cation binds tightly to various proteins including serum albumin, carboxypeptidase, nucleases, and phosphatases.[2,12] Vanadate has previously been shown to associate randomly with proteins such as bovine serum albumin,[13] and recently the interactions between vanadate and dipeptides have been reported.[14] Vanadate can potentially interact with amine and amide groups in addition to hydroxyl and carboxylic acid functionalities on the surface of the protein. It is possible the vanadate will be maintained in an inactive form through such reactions and therefore not be available for biological reactions. Alternatively, such vanadate interactions may be key for establishing the initial contact between protein and vanadate, or between substrate and vanadate, and eventually lead to biological activity. Vanadate has previously be found to interact with biomolecules including enzyme substrates and cofactors such as uridine, AMP, lactate, and citric acid;[10,15] buffers such as Tricine, TEA, AMPSO, and citric acid;[11,15] metal chelators such as EDTA and EGTA;[11,15] and enzymes such as ribonuclease and myosin.[16-19]

This paper presents a method for quantitatively determining low levels of free monomeric vanadate or vanadyl cations using an acid or alkaline phosphatase catalyzed enzyme assay. We use this technique to determine the extent $10^{-7}$–$10^{-5}$ M vanadium interacts with selected organic molecules including enzyme substrates and cofactors, metal chelators, buffers, amino acids, di- and tripeptides, and selected proteins. Differences in the interactions of vanadyl cation and vanadate with biomolecules are identified and discussed with respect to the mechanism by which vanadium can act both in vitro and in vivo.

## Experimental Section

**General. (A) Reagents and Enzymes.** Chemicals were of reagent grade (Fisher, Aldrich) and used without further purification. Water was distilled and deionized. Wheat germ acid phosphatase (EC 3.1.3.2), alkaline phosphatase from bovine intestinal mucosa (EC 3.1.3.1), and magnesium *p*-nitrophenyl phosphate were purchased from Sigma and used without further purification. A vanadate stock solution was prepared by dissolving vanadium pentaoxide with 2 equiv of sodium hydroxide to generate a vanadate solution of 0.25 M; this solution was stored at 4 °C. The concentrations of the vanadate standard solutions were monitored by UV spectroscopy (at wavelengths in the 260–270-nm range), and no changes in concentrations were observed over the course of 6 months. At no time was acid added to a solution containing vanadate, since vanadate in the presence of acid generates the orange decamer.[9,11] The experiments using vanadyl cation were carried out as described previously.[12] All solutions were purged with nitrogen immediately before use. The vanadyl stock solution was maintained at pH 1 to avoid air oxidation, and concentrations were determined as described previously.[12]

**(B) Kinetic Measurements.** Spectrophotometric determinations of initial rates of hydrolysis were obtained at 25 °C and 405 nm on a Lambda 4B Perkin-Elmer double-beam spectrophotometer equipped with a constant-temperature cell. The rates were determined by hydrolysis of *p*-nitrophenyl phosphate catalyzed by acid phosphatase at pH 5 or catalyzed by alkaline phosphatase at pH 8.[18,19] Acid phosphatase rates were measured with an end-point assay where $T_0$ was measured before substrate was added and $T_t$ was measured after 5-min incubation at 25 °C. Alkaline phosphatase rates were measured with a 100-s kinetic run, absorbances being measured every 10 s. The addition of substrate initiated the enzyme reaction in this assay. The rates were normalized at the beginning of each set of kinetic measurements. They were determined in duplicate or triplicate and at several substrate concentrations from 0.010 to 0.50 mM. Inhibition resulting from the production of inorganic phosphate was kept to a minimum by monitoring the formation of *p*-nitrophenol to only about 1–10% conversion of substrate. KCl was added to the assay solution (1 M) in order to maintain a constant ionic strength. The rates were calculated with $\epsilon = 18.5$ L mmol$^{-1}$ cm$^{-1}$ for *p*-nitrophenol anion. Protein concentration of enzyme solutions approached approximately 0.5 mg/mL and was quantified by the method of Lowry.[20]

**Enzyme Assays. (A) Acid Phosphatase Assay (General).**[18] The rate of hydrolysis $V_0$ was determined in acetate buffer as the quantity of *p*-nitrophenyl phosphate hydrolyzed during a 5-min incubation at 25 °C and pH 5.0. The assay solution contained 50 mM acetate, 0.25 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.03–0.06 mg/mL acid phosphatase. $V_0$ determined in the presence of various biomolecules or buffers contained 5 mM acetate and the various concentrations of biomolecules of interest. The inhibited rates, $V_i$, for phosphate or arsenate were determined as the inhibited rate for vanadate except for substitution of the 0.0025 mM vanadate by 0.30 mM phosphate or 0.075 mM arsenate. The $V_0/V_i$ ratios were calculated from these two rates.

The changes in absorbance were determined against a blank solution, and controls were run with each set of samples. If the controls were above 0.01 OD unit, the samples were repeated with freshly prepared *p*-nitrophenyl phosphate.

**(B) Acid Phosphatase Assay in Citric Acid.** The rate of hydrolysis was determined in 50 mM citric acid as the quantity of *p*-nitrophenyl phosphate that was hydrolyzed during 5-min incubation at 25 °C and pH 5.0. The assay solution contained 50 mM citric acid, 0.25 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.03–0.06 mg/mL acid phosphatase. The inhibited rate of hydrolysis was determined in 50 mM citric acid as the quantity of *p*-nitrophenyl phosphate hydrolysis during 5-min incubation at 25 °C and pH 5.0 in the presence of 0.0025 mM vanadate. The $V_0/V_i$ ratio was calculated from these two rates.

**(C) Alkaline Phosphatase Assay (General).**[19] The $V_0$ in Hepes was determined as the rate of *p*-nitrophenyl phosphate hydrolysis at 25 °C and pH 8.0. The reaction solution contained 20 mM Hepes, 0.0256 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.003–0.007 mg/mL alkaline phosphatase. The $V_0$ was determined in the presence of various biomolecules or buffers in assay solutions containing 5 mM Hepes and various concentrations of biomolecules of interest. The $V_i$ in Hepes for vanadate was determined as the rate of *p*-nitrophenyl phosphate hydrolysis at 25 °C and pH 8.0 in the presence of vanadate. The assay solution contained 20 mM Hepes, 0.025 mM *p*-nitrophenyl phosphate, 1 M KCl, 0.010 mM vanadate, and 0.003–0.007 mg/mL alkaline phosphatase (the concentration range of vanadate convenient in this assay is 0.010–0.001 mM). $V_i$ was determined in the presence of various biomolecules or buffers in solutions that contained only 5 mM Hepes and the various concentrations of the biomolecule of interest. The $V_i$ for phosphate or arsenate was determined as for the $V_i$ for vanadate except the 0.010 mM vanadate was substituted with 0.30 mM phosphate or 0.075 mM arsenate.

**(D) Alkaline Phosphatase Assay in Tricine.** The $V_0$ in Tricine was determined as the rate of *p*-nitrophenyl phosphate hydrolysis at 25 °C and pH 8.0. The assay solution contained 20 mM Tricine, 0.0256 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.003–0.007 mg/mL alkaline

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phosphatase. The $V_i$ was determined as the rate of *p*-nitrophenyl phosphate hydrolysis in the presence of 0.010 mM vanadate at 25 °C and pH 8.0. The assay solution contained 20 mM Tricine, 0.0256 mM *p*-nitrophenyl phosphate, 1 M KCl, 0.010 mM vanadate, and 0.003–0.007 mg/mL alkaline phosphatase. The $V_0/V_i$ ratio was calculated from these two rates.

**(E) Assays Using Vanadyl Cation.**[12] Assays carried out with vanadyl cation were done as described above for vanadate with minor modifications. All solutions were purged with nitrogen before use; the assay was carried out under nitrogen in a cuvette with a rubber stopper such that contamination with oxygen was minimized. If the solutions were not purged sufficiently, the inhibition by vanadyl cation decreased. Vanadate is a weaker inhibitor than vanadyl cation, and oxidation of vanadyl cation will therefore increase reaction rates. The rates should therefore be checked at 30-min intervals, and only when the rate does not decrease further by purging are the solutions sufficiently purged. The concentration range of vanadyl cation that was used in the assay was $10^{-6}$–$10^{-8}$ M.

**$^{51}$V NMR Spectroscopy.**[21] Vanadium-51 is a NMR-active nucleus of 99.75% natural abundance. Although its spin is $^7/_2$, its line widths are relatively narrow and easily resolved in the vanadium window. $^{51}$V NMR is therefore a convenient and informative tool for studies of vanadium(V) species.[8,10,11] The $^{51}$V NMR spectra were recorded on a 200-MHz Bruker WPSY (4.7 T) spectrometer and a $^1$H 360-MHz Nicolet (8.45 T) spectrometer. We typically used spectrum widths of 16 000 Hz, a 90° pulse angle, an accumulation time of 0.2 s, and no relaxation delay. The chemical shifts are reported relative to the external reference standard $VOCl_3$ (0 ppm), although we in practice use an external reference solution (pH 7.5) containing the complex of vanadate and diethanolamine (−488 ppm).[11] The DEA complex is a convenient reference because the $^{51}$V NMR resonance only varies slightly with pH and ionic strength and the signal appears in the chemical shift range of interest. Conversely, the chemical shift of monomeric vanadate is very sensitive to pH, ionic strength, concentration, and temperature.

**(A) NMR Sample Preparation.** The vanadate solutions for $^{51}$V NMR studies with various buffers were prepared by mixing buffer, potassium chloride, and deuterium oxide. Sufficient vanadate was added from the standard vanadate solution to yield the final concentration of vanadate. The pH and volume were adjusted to give the final pH and volume. Unless otherwise specified, all NMR samples were prepared containing 1.0 M KCl. The vanadate solutions for determination of monomer, dimer, and tetramer concentrations were prepared without deuterium oxide in order to avoid any ambiguity in pH due to isotope effects. Thus, the $^{51}$V NMR spectra of these sample were recorded with an external lock. The solutions prepared for $^{51}$V NMR spectroscopy to determine the concentrations of vanadate oligomers in the enzyme assays did not contain *p*-nitrophenyl phosphate or enzyme. In separate experiments no differences in the distribution of vanadate oligomers were observed by addition of substrate or enzymes, so we did not include substrate or enzyme in the NMR samples for experimental simplification.

**Data Manipulation.** The uncertainties on kinetic rate determinations are approximately 3%. We estimate that the uncertainties in the $V_0/V_i$ ratios will not exceed 10%, and in general they were less than 5%. However, we have observed deviations in $V_0/V_i$ ratios of up to 15% with different chemical lots of, for example, Hepes (from Sigma or Aldrich). These variations were reproducible and suggest that even reagent-grade chemicals contain small amounts of impurities that affect the activity of alkaline phosphatase.[22]

## Results and Discussion

**Determination of Inhibitor Concentration Using $V_0/V_i$ Ratios.** The reaction rate of an enzyme reaction is dependent on the concentration of inhibitor present in the reaction solution. If the rate changes and the inhibition are constant, the rate can be used to determine the inhibitor concentration. Monomeric vanadate has been reported to inhibit many enzymes.[2] Many equilibria are established in vanadate solutions, and the resulting equilibrium mixture often contains mono- and oligovanadates.[9] If a complexing ligand is added to such a vanadate solution, the original equilibrium mixture will change to a new equilibrium mixture where the concentration of mono- and oligovanadates has decreased because some vanadate–ligand complex has formed. Since the concentration of monomeric vanadate is less in the solution containing the complexing ligand, the enzyme rate will increase. Thus, a simple measure for the vanadate inhibition is to determine the enzyme rate ratios $V_0/V_i$, where $V_0$ is the uninhibited rate and $V_i$ is the rate of the reaction inhibited by vanadate. The $V_0/V_i$ ratio of a reference solution compared to the $V_0/V_i$ ratio of a solution containing a ligand will reflect the extent vanadate interacts with this ligand.

Assuming both vanadate and vanadyl cation are competitive inhibitors, Michaelis–Menten kinetics can be used to describe the $V_0/V_i$ ratio (eq 1) as a function of $K_m$, $V_{max}$, $K_i$, [S], and [I].[23] $K_m$ and $V_{max}$ are the Michaelis–Menten parameters for the enzyme reaction in the presence of the ligand, $K_i$ is the inhibition constant for vanadate under the same conditions, [S] is the enzyme substrate concentration, and [I] is the concentration of inhibitor (e.g., monomeric vanadate). Under conditions where substrate and inhibitor concentrations are constant, and $K_m$ and $K_i$ are constant, eq 1 simplifies to eq 2. A $V_0/V_i$ ratio identical with the reference $V_0/V_i$ value will be observed when there are no changes in inhibitor concentration. Alternatively, if an organic molecule interacts with the inhibitor, the concentration of available inhibitor will decrease, and therefore, the overall $V_0/V_i$ ratio will decrease. Although Michaelis–Menten constants are steady-state constants and not thermodynamic constants, this does not affect the determination of vanadate or vanadyl concentration, if the vanadium species in the solutions under investigation are at equilibrium. It was verified experimentally that the concentration of inhibitor did not change with time under the reaction conditions used in this work.

$$\frac{V_0}{V_i} = \frac{V_{max}[S]/(K_m + [S])}{V_{max}[S]/[K_m(1 + [I]/K_i) + [S]]} \quad (1)$$

$$\frac{V_0}{V_i} = 1 + \frac{K_m([I]/K_i)}{K_m + [S]} \quad (2)$$

In many cases the environments of the protein, substrate, and inhibitor are fairly constant, and the $K_m$, $K_i$, and $K_i/K_m$ are constant. Using the assumption that $K_m$, $K_i$, and the $K_i/K_m$ are constant (eq 3)[24] can be derived from eq 2, and the $V_0/V_i$ ratio can be used to calculate the concentration of the effective inhibitor $[I]_{eff}$. Since all buffers have the potential to interact with vanadate, the choice of reference buffers was based on the buffers that showed the least interaction. We chose Hepes and acetate as the reference buffers because high $V_0/V_i$ ratios, for vanadate, were obtained in these buffers and only monomeric vanadate was observed with $^{51}$V NMR spectroscopy.

$$[I]_{ref} - [I]_{eff} = \left(\frac{V_{0,ref}}{V_{i,ref}} - \frac{V_0}{V_i}\right)\left(K_i + \frac{K_i[S]}{K_m}\right) \quad (3)$$

The concentration of vanadate monomer obtained from the $V_0/V_i$ ratio can be used to calculate the concentration of complexed vanadate by use of $[I_{tot}] = [I_{free}] + [I_{complexed}]$. Such calculations are most easily carried out if the major free vanadate species is the monomer ($[I_{free}] = [V_1]$). Although equilibrium considerations predict oligovanadates will form in solution, their concentrations should be so low that $[V_{tot}] \approx [V_1]$. Such conditions are obtained at vanadate concentrations below $5 \times 10^{-5}$ M.[2,9]

$V_0/V_i$ ratios could be used to calculate a wide range of vanadate or vanadyl cation concentrations if the appropriate enzyme reactions were employed. Suitable enzymes inhibited by vanadate or vanadyl cation in the concentration rate $10^{-7}$–$10^{-5}$ M vanadium include ribonuclease and acid and alkaline phosphatase. Acid and alkaline phosphatases have simple and convenient enzyme assays in which the hydrolysis of *p*-nitrophenyl phosphate is monitored. Both acid and alkaline phosphatases are therefore excellent candidates for measuring vanadium concentration at $10^{-5}$–$10^{-7}$ M vanadium.

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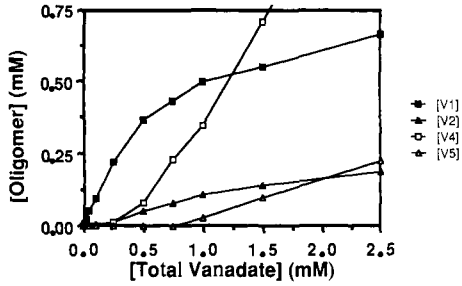


**Figure 1.** Concentrations of vanadate monomer, dimer, tetramer, and pentamer are shown as a function of total vanadate concentration. The concentrations of the vanadium species were determined with $^{51}V$ NMR spectroscopy of vanadate solutions prepared as dictated by the enzyme assay conditions for alkaline phosphatase. The solutions contained 20 mM Hepes, 1 M KCl, and various concentrations of vanadate (from 0.01 to 2.5 mM). The solutions did not contain deuterium oxide since the samples were run with external lock. The vanadate derivatives are presented as monomer (■), dimer (▲), tetramer (□), and pentamer (△).

**Mono- and Oligovanadate Equilibria.** At vanadate concentrations above 0.2 mM the monomer forms significant concentrations of dimer, tetramer, and pentamer, and these species can be observed with $^{51}V$ NMR spectroscopy.[9,21] We therefore recorded the $^{51}V$ NMR spectra at various vanadate concentrations in 20 mM Hepes, 1 M KCl, pH 8.0 (alkaline phosphatase assay conditions). The concentrations were obtained by calculating the mole fraction from the integrated $^{51}V$ NMR spectra in samples where the total vanadate concentrations are known.[10,11,15,21] The concentrations of various vanadate species are plotted as a function of total vanadate concentration in Figure 1. When concentrations of vanadate monomer, dimer, and tetramer are expressed as shown in eq 4–6, the following equilibrium constants are calculated for $K_{14}$, $K_{12}$, and $K_{24}$: 7.3 (±0.5) × $10^9$ $M^{-3}$, 4.4 (±0.4) × $10^2$ $M^{-1}$, and 3.8 (±0.4) × $10^4$ $M^{-1}$, respectively. These values correspond to those reported in the literature.[15,21]

$$2V_1 \overset{K_{12}}{\rightleftharpoons} V_2 \quad (4)$$

$$4V_1 \overset{K_{14}}{\rightleftharpoons} V_4 \quad (5)$$

$$2V_2 \overset{K_{24}}{\rightleftharpoons} V_4 \quad (6)$$

**Vanadate Monomer as Inhibiting Species for Alkaline Phosphatase.** Although monomeric vanadate is presumed to be the inhibiting species in an equilibrium mixture of mono- and oligovanadates, this presumption has not actually been demonstrated experimentally.[2] We have therefore used enzyme kinetics and $^{51}V$ NMR to verify the correlation between enzyme rates and concentration of the presumed inhibiting vanadate species (monomeric vanadate).

The hydrolysis of 0.025 mM *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase (from 0.0067 to 0.0072 mg/mL) was measured in 20 mM Hepes, 1 M KCl at pH 8.0 and 25 °C at various concentrations of vanadate ranging from 0 to 0.5 mM. The rate was monitored by the absorption of the hydrolysis product, *p*-nitrophenol, at 405 nm. The concentrations of various vanadate species in the assay solutions were also determined with $^{51}V$ NMR spectroscopy as described above.

Figure 2 was obtained by plotting the reciprocal rate ($1/v$) as a function of total vanadate, monomer, dimer, and tetramer concentrations. Figure 2 shows that only the concentration of monomer is linearly proportional to the rate of substrate hydrolysis. The observed inhibition of alkaline phosphatase can therefore be correlated to the vanadate monomer. Although this observation does not eliminate the possibility that other vanadium species can be active as inhibitors, it is unlikely that other vanadium complexes will be more potent inhibitors than monomeric vanadate.

**Dependency of $V_0/V_i$ Ratios on Inhibitor Concentration, Substrate Concentration, Enzyme Concentration, and pH.** Details in testing and describing the method for determining vanadate and vanadyl cation concentration will be reported elsewhere.[24]

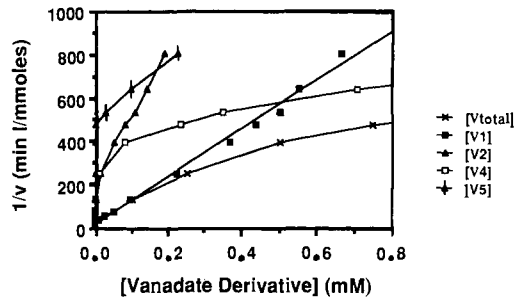


**Figure 2.** Reciprocal hydrolysis rate of the *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase is plotted as a function of total vanadate concentration (×), vanadate monomer concentration (■), vanadate dimer concentration (▲), vanadate tetramer concentration (□), and vanadate pentamer concentration (▲). The concentrations of various vanadate derivatives were determined with $^{51}V$ NMR spectroscopy (±5% error limit), and the rates of hydrolysis were determined at 25 °C and pH 8.0 at 0.250 mM *p*-nitrophenyl phosphate ($10K_m$), in 20 mM Hepes, 1 M KCl and with 0.0077 mg/mL alkaline phosphatase. The correlation coefficient of the linear relationship between $1/v$ as a function of monomeric vanadate was 1.00.

**Table I.** Quantitative Determination of Monomeric Vandate Concentrations Using the Alkaline Phosphatase Assay[a]

| $[V_{tot}]$ (mM) | $V_0/V_i$ | $[V_1]_{assay}$ (mM)[b] | $[V_1]_{^{51}V\ NMR}$ (mM)[c] | deviation (%) |
|---|---|---|---|---|
| 0.010 | 1.7 | 0.010 | 0.010 | |
| 0.025 | 2.1 | 0.024 | 0.025 | 4 |
| 0.050 | 3.0 | 0.048 | 0.050 | 4 |
| 0.100 | 4.7 | 0.097 | 0.095 | 2 |
| 0.250 | 9.2 | 0.23 | 0.22 | 3 |
| 0.500 | 14.5 | 0.38 | 0.38 | 0 |

[a] The assay was conducted at pH 8.0 at 25 °C in 20 mM Hepes, 0.256 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.0077 mg/mL alkaline phosphatase. The experimental uncertainty on the $V_0/V_i$ ratio was 5%. [b] $(K_i + K_i[S]/K_m) = 0.029$ was determined at 0.256 mM *p*-nitrophenyl phosphate under the assay conditions described in footnote *a*. [c] The spectra from which these concentrations were calculated had in the worst case a signal to noise ratio of 10 to 1. Since the total vanadate concentration was known, the mole fraction obtained by integration was used to calculate the monomeric vanadate concentration. Repeated recordings of spectra would give vanadate concentrations within 5% of the first determination. We therefore estima.e the experimental uncertainty on the concentrations determined by $^{51}V$ NMR in these experiments as 5%.

Several points are discussed briefly below in order to demonstrate the validity of the described method.

The enzyme rate measured without inhibitor ($V_0$) is constant, and therefore, $V_0/V_i$ should be proportional with $1/V_i$. Since Figure 2 shows $1/v$ ($=1/V_i$) is proportional to $[V_1]$, $V_0/V_i$ should therefore be proportional to $[V_1]$. In addition to demonstrating that only monomeric vanadate is an active inhibitor, Figure 2 also shows eq 2 is valid for the alkaline phosphatase assay system up to 0.6 mM monomeric vanadate.

A direct comparison was carried out between $[V_1]$ determined by $^{51}V$ NMR and $[V_1]$ determined with the enzyme assay. Table I shows the $V_0/V_i$ ratios and $[V_1]$ as calculated from the known $[V_{tot}]$ and the $^{51}V$ NMR spectra or from the *p*-nitrophenyl phosphate hydrolysis rates obtained with the alkaline phosphatase assay. The $[V_{tot}]$ varies from 0.010 to 0.50 mM vanadate, whereas the monomeric vanadate concentration as determined by $^{51}V$ NMR varies from 0.010 to 0.38 mM. The monomeric vanadate concentration varied from 0.010 to 0.38 mM when obtained from the $V_0/V_i$ ratios. As seen from Table I, the two methods are in agreement since the deviations are within the error limits.

The $V_0/V_i$ ratio should be inversely dependent on the substrate concentration ([S]) according to eq 2. The rate of *p*-nitrophenyl phosphate hydrolysis was therefore measured at pH 8.0 in 20 mM Hepes, 1 M KCl, and from 0.0025 to 0.50 mM *p*-nitrophenyl phosphate with approximately 0.007 mg/mL alkaline phosphatase. The $V_0/V_i$ ratios could therefore be calculated at various substrate concentrations and plotted as a function of $1/(K_m + [S])$ (Figure

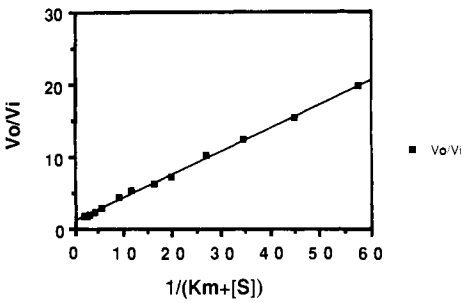


**Figure 3.** $V_0/V_i$ ratios in Hepes are plotted as a function of $1/(K_m + [S])$. The intercept on the $Y$ axis is $1/K_m$. The $K_m$ is found to be 0.012 mM. The rates of hydrolysis of the *p*-nitrophenyl phosphate (±3%) were determined at 25 °C at pH 8.0 in 20 mM Hepes, 1 M KCl, and 0.0065 mg/mL alkaline phosphatase with substrate concentrations varying from 0.0025 to 0.50 mM. The inhibited rates of hydrolysis, $V_i$, were determined under the above conditions by adding 0.010 mM vanadate. The correlation coefficient for the line was 1.00.

3). As predicted from eq 2 the relationship shown in Figure 3 was linear.

We also examined how the $V_0/V_i$ ratio varied with changes in both the enzyme concentrations and pH by measuring the rates at various enzyme concentrations and various pH.[24] Although the hydrolysis rates changed with alkaline phosphatase concentration, the $V_0/V_i$ ratio remained constant. The hydrolysis rates were also very sensitive to changes in pH, and the $V_0/V_i$ ratio varied considerably with pH.[24] Alkaline phosphatase has previously shown significant variation in substrate specificity ($K_m$), and large differences in reaction rates are observed by changes in pH.[25] Our observations are in agreement with these experiments and suggest that pH should be maintained constant within ±0.05 pH unit.

In summary, the $V_0/V_i$ ratio varies as predicted by eq 2, and therefore, [S] and [I] should be maintained constant for each series of measurements. Variations in alkaline phosphatase concentrations will not affect the $V_0/V_i$ ratio; however, it is crucial that the pH be held constant within ±0.05 pH unit.

**Probing the Applicability of the $V_0/V_i$ Ratio for Determination of Inhibitor Concentration in Variable Environments.** If the described approach is to be successful, the $V_0/V_i$ ratio must be constant under various reaction conditions. The method would then be applicable for quantitative determinations and offer advantages over other available methods for determination of inhibitor concentration (for example, $^{51}V$ NMR spectroscopy for determination of monomeric vanadate).

The constancy of the $V_0/V_i$ ratio was demonstrated by determining the $V_0/V_i$ ratio in various buffers with an inhibitor (phosphate) that is known to maintain constant activity under various conditions. The rates of hydrolysis of *p*-nitrophenyl phosphate catalyzed by acid and alkaline phosphatase in the presence of various buffers were determined without inhibitors and with 0.30 mM phosphate. The alkaline phosphatase assay was conducted at pH 8.0 in 20 mM Hepes, 1 M KCl, and 0.025 mM *p*-nitrophenyl phosphate catalyzed by 0.007 mg/mL alkaline phosphatase. The acid phosphatase assay was conducted at pH 5.0 in 50 mM acetate, 1.0 M KCl, and 0.25 mM *p*-nitrophenyl phosphate catalyzed by 0.005 mg/mL acid phosphatase. The $V_0/V_i$ ratios calculated from these rates are shown in Table II. The $V_0/V_i$ ratios were constant within the experimental uncertainty for both the acid and the alkaline phosphatase catalyzed reactions. We therefore conclude the $V_0/V_i$ ratios are constant under variable conditions when the inhibitor concentration is constant.

The corresponding $V_0/V_i$ ratios calculated from rate measurements containing 0.075 mM arsenate or 0.010 mM vanadate in the alkaline phosphatase assay and 0.060 mM arsenate or 0.0025 mM vanadate in the acid phosphatase assay are also included in Table II. The $V_0/V_i$ ratios for arsenate are fairly constant, whereas the $V_0/V_i$ ratios for vanadate vary considerably. To confirm the observations with vanadate, $^{51}V$ NMR spectra of

(25) (a) Lopez, V.; Stevens, T.; Lindquist, R. N. *Arch. Biochem. Biophys.* **1976**, *175*, 31–8. (b) Morton, R. K. *Biochem. J.* **1955**, *61*, 232–40. (c) Fernley, H. N.; Walker, P. G. *Biochem. J.* **1967**, *104*, 1011–8. (d) McComb, R. B.; Bowers, G. N., Jr. *Clin. Chem.* **1972**, *18*, 97–104.

**Table II.** $V_0/V_i$ Ratios for Hydrolysis of *p*-Nitrophenyl Phosphate Catalyzed by Acid (Wheat Germ) and Alkaline Phosphatase (Bovine Intestinal Mucosa) in Various Buffers

| | $V_0/V_i$[a,b] | | | |
|---|---|---|---|---|
| buffer | $P_i$ | As | $V_i$ | $VO_2^{+c}$ |
| **Acid Phosphatase** | | | | |
| acetate | 1.9 | 1.9 | 2.5 | |
| TEA | 1.9 | 1.8 | 2.0 | |
| Tricine | 1.7 | 1.7 | 1.04 | |
| MIDA | 1.9 | 1.7 | 1.2 | |
| citrate | 1.5 | 1.6 | 1.12 | |
| malate | 1.9 | 1.8 | 1.06 | |
| Bis-Tris | 1.8 | 1.6 | 1.6 | |
| Tris | 1.8 | 1.7 | 2.3 | |
| glycine | 1.9 | 2.0 | 2.1 | |
| **Alkaline Phosphatase** | | | | |
| Hepes | 2.9 | 5.4 | 11.4 | 2.5 |
| TES | 2.3 | 5.1 | 5.4 | |
| Tris | 2.9 | 5.4 | 7.1 | 2.5 |
| TEA | 2.5 | 5.7 | 2.9 | |
| Tricine | 2.5 | 5.8 | 1.1 | 1.9 |
| barbitol | 2.5 | 5.7 | 6.4 | 4.5 |
| *N*-ethylmorpholine | 2.6 | 5.0 | 8.6 | 3.5 |
| EPPS | 2.5 | 4.9 | 6.5 | 3.0 |
| TAPS | 2.5 | 5.5 | 6.3 | 1.6 |
| DIPSO | 2.4 | 5.1 | 1.7 | 1.1 |
| glycylglycine | 2.3 | 5.1 | 4.4 | 1.1 |

[a] The concentrations of substrate and inhibitors in the acid phosphatase assay were as follows: *p*-nitrophenyl phosphate, 0.256 mM; phosphate, 0.30 mM; arsenate, 0.060 mM; vanadate, 0.0025 mM. The assay was conducted at pH 5.0, 25 °C, in 50 mM buffer, 1.0 M KCl and with 0.03–0.06 mg/mL acid phosphatase. We measure $K_m$ under these conditions to $1.4 \times 10^{-4}$ M, which corresponds to that previously reported of $1.3 \times 10^{-4}$ M.[16] We estimate the experimental uncertainty was within 10% on the $V_0/V_i$ ratio. [b] The concentrations of substrate and inhibitors in the alkaline phosphatase assay were as follows: *p*-nitrophenyl phosphate, 0.025 mM; phosphate, 0.30 mM; arsenate, 0.075 mM; vanadate, 0.010 mM. The assay was conducted at pH 8.0, 25 °C, in 20 mM buffer, 1.0 M KCl and with approximately 0.0067 mg/mL alkaline phosphatase. The experimental uncertainty on the $V_0/V_i$ was estimated to be 5%. [c] The assay with vanadium(IV) was conducted at pH 8.0 at 25 °C in 20 mM buffer, 0.25 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.077 mg/mL alkaline phosphatase. All solutions were freshly purged with nitrogen, and the vanadyl cation concentration was, when present, 0.001 mM. The experimental uncertainty on the $V_0/V_i$ for vanadyl cation was estimated to be 10%.

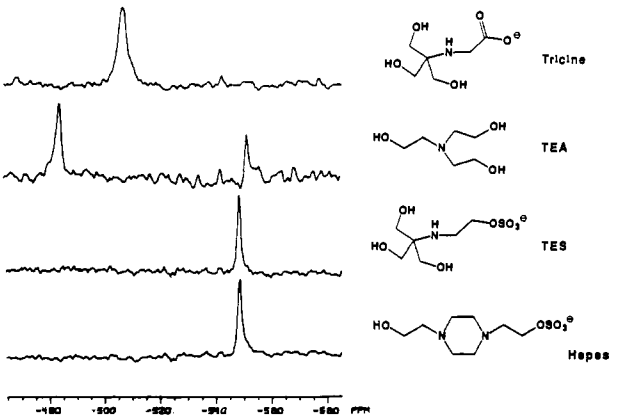


**Figure 4.** Spontaneous interactions between vanadium and buffers at pH 8 are observed by $^{51}V$ NMR spectroscopy. Hepes, TES, TEA, and Tricine were used as buffers. The resonance at −555 ppm is assigned to monomeric vanadate; signals downfield of this resonance are assigned to buffer–vanadate species. The vanadate concentration was 0.010 mM, and the respective buffer concentration was 20 mM and 1 M KCl. These conditions were also used for the inhibition studies with alkaline phosphatase. Integration of these spectra yields concentrations of various vanadate derivatives (Table III).

**Table III.** Concentrations of Monomeric Vanadate and Vanadate Complex in the Alkaline Phosphatase Assay and the Acid Phosphatase Assay

| buffer | buffer concn (mM) | $[V_iL_x]$ by $^{51}V$ NMR (mM) | $[V_1]$ by $^{51}V$ NMR (mM) | $V_0/V_i$ (mM) | $[V_iL_x]$ by assay | $[V_1]$ by assay | overall deviation (% of $[V_{tot}]$) |
|---|---|---|---|---|---|---|---|
| | | | Alkaline Phosphatase[a] | | | | |
| Hepes | 20 | 0.0000 | 0.010 | 11.4 | 0.0000 | 0.010 | |
| TES | 20 | 0.0000 | 0.010 | 5.4 | 0.0058 | 0.004 2 | 42 |
| TEA | 20 | 0.0079 | 0.002 1 | 2.9 | 0.0082 | 0.001 8 | 3 |
| Tricine | 20 | 0.0095 | 0.000 50 | 1.1 | 0.0099 | 0.000 10 | 4 |
| | | | Acid Phosphatase[b] | | | | |
| acetate | 50 | 0.0000 | 0.002 5 | 2.5 | 0.0000 | 0.002 5 | |
| TEA | 50 | 0.00030 | 0.002 2 | 2.1 | 0.00067 | 0.001 9 | 12 |
| MIDA | 50 | 0.0021 | 0.000 38 | 1.2 | 0.0022 | 0.000 33 | 4 |
| malate | 50 | 0.0021 | 0.000 38 | 1.3 | 0.0020 | 0.000 50 | 4 |
| citrate | 50 | 0.0023 | 0.000 25 | 1.08 | 0.0024 | 0.000 13 | 4 |
| Tricine | 50 | 0.0024 | 0.000 13 | 1.04 | 0.0024 | 0.000 067 | 0.2 |

[a] The interactions between vanadate and buffers at pH 8.0 are determined by $^{51}V$ NMR spectroscopy. The solutions contained 0.010 mM vanadate and 1 M KCl. The vanadate concentrations were calculated from the mole fractions obtained from the integrated spectra. Repeated concentration determinations were usually within 5% and not outside 10%. We therefore expect the concentrations are accurate within ±0.0005 mM and not outside 0.001 mM for the alkaline phosphatase assay solutions. [b] The interactions between vanadate and buffers at pH 5.0 are determined by $^{51}V$ NMR spectroscopy. The vanadate concentration was 0.025 mM (10-fold the concentration in the actual assay) and 1 M KCl was included. The values for the concentration of the monomeric vandate in this assay are in a concentration range where $[V_{tot}] = [V_1]$, and therefore, these spectra can be used to determine the concentration of monomeric vanadate and complex in solutions containing a total vanadate concentration of 0.0025 mM. Repeated recordings for TEA and Tricine gave spectra from which mole fractions within 10% accuracy were obtained. We therefore estimate the experimental uncertainty was within 10% of the total vanadate concentration except for malate and citrate where the uncertainty was within 25% of the total vanadate concentration (the higher experimental uncertainty is due to poorer signal to noise and overlapping signals). The reported 0.0005 mM concentration of monomeric vanadate in Tricine was a result of a spectrum that showed no observable signal for monomeric vanadate; however, the integrated spectrum showed the presence of some monomeric vanadate. Since the integrated mole fraction was supported by the fact that the lower limit for observation of a peak in a NMR spectrum with this signal to noise ratio is on the order of 5%, it is reasonable to suggest 0.0005 mM monomeric vanadate is present in the Tricine solution.

alkaline phosphatase assay solutions containing selected buffers and $10^{-5}$ M vanadate were recorded. To obtain sufficient signal to noise, these spectra required from 3- to 8-h accumulation time (Figure 4). The integration of the $^{51}V$ NMR spectra led to the concentrations of monomeric vanadate shown in Table III. The corresponding $V_0/V_i$ determinations were carried out in less than 20 min on a UV spectrometer, and the results are also shown in Table III. No interactions of vanadate were observed with Hepes, as judged from observation of only one signal at −555 ppm (monomeric vanadate) in the $^{51}V$ NMR spectrum and a high $V_0/V_i$ ratio. Interaction was observed between vanadate and Tricine or TEA. The $^{51}V$ NMR spectra of these buffer solutions show resonances for a species in addition to monomeric vanadate, suggesting the vanadate is complexed. Correspondingly, these buffers have low $V_0/V_i$ ratios. Figure 4 shows no interaction was observed between vanadate and TES by $^{51}V$ NMR, in contrast to the low $V_0/V_i$ ratio obtained from the alkaline phosphatase assay which suggested a significant concentration of vanadate was complexed to TES. These observations suggest TES is interacting rapidly with vanadate on the $^{51}V$ NMR time scale whereas the interaction is slow on the enzyme reaction time scale. Detailed $^{51}V$ NMR studies with TES and vanadate solutions were consistent with this interpretation.[26] We conclude that the $[V_1]$ determined by alkaline phosphatase assay are in agreement with the $[V_1]$ obtained by $^{51}V$ NMR.

Table III also shows the $V_0/V_i$ ratios, $[V_1]$, and $[V_iL_x]$ for selected buffers at pH 5.0 as determined by $^{51}V$ NMR spectroscopy for comparison with the acid phosphatase assay. The solutions used in the acid phosphatase assay contained $2.5 \times 10^{-6}$ M vanadate, and even after 8 h of accumulation, the $^{51}V$ NMR spectra were very poor. Under the conditions the $^{51}V$ NMR spectra were recorded, $[V_1] \cong [V_{tot}]$, and therefore, the ratio between the concentrations of vanadate monomer and the vanadate complex should be constant. $^{51}V$ NMR spectra recorded of solutions containing $2.5 \times 10^{-5}$ M vanadate (Figure 5) could therefore be used to estimate the vanadate concentrations in the acid phosphatase assay. The integration of the spectra shown in Figure 5 led to the concentrations listed in Table III. The reference

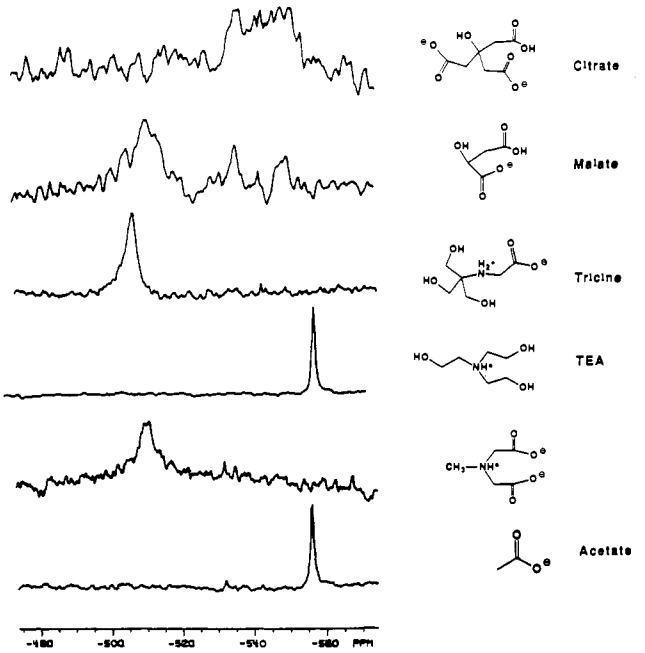


**Figure 5.** Interactions between vanadate and buffers at pH 5 are observed by $^{51}V$ NMR spectroscopy. Acetate, *N*-methyliminodiacetic acid, TEA, Tricine, L-malic acid, and citric acid are used as buffers. The resonance at −555 ppm is assigned to monomeric vanadate, signals downfield of this resonance are assigned to buffer–vanadate species. The vanadate concentration was 0.025 mM, and the respective buffer concentration was 50 mM (pH 5) and 1 M KCl. These conditions mimic the conditions used in inhibition studies with acid phosphatase with the exception of the higher vanadate concentration. Integrations of these spectra yield concentrations of various vanadate derivatives (Table III).

buffer, acetate, showed no evidence for interaction with vanadate as judged by the single resonance at −555 ppm in the NMR spectrum and the high $V_0/V_i$ ratio. MIDA (*N*-methyliminodiacetate), malate, citrate, and Tricine all complex vanadate strongly, since little free vanadate was observed in their NMR spectra. Accordingly low $V_0/V_i$ ratios were obtained in the acid phosphatase assay. TEA showed little interaction with vanadate at pH 5 and strong interaction at pH 8 according to both NMR and enzyme

(26) We have carried out $^{51}V$ NMR experiments on solutions containing TES and vanadate and find that the chemical shift for monomeric vanadate changes with increasing TES concentrations (pH 7.8). Our results are therefore consistent with the monomeric vanadate being in rapid exchange with the TES–vanadate complex.

assay data. These observations are in accord with the bell-curve stability dependence of the TEA–vanadate complex that has been previously described.[11] The results obtained with $^{51}V$ NMR and the acid phosphatase assay are in agreement since the deviations are within experimental error.

Quantitative determination of the $[V_i]$ in solutions of vanadate with ligands allows calculation of the vanadate–ligand complex concentration since the total vanadate and ligand concentrations are known. Such calculations are only appropriate if several assumptions are fulfilled. First, the experiments must be carried out when all solutions are at equilibrium. Second, only one major vanadate complex, in addition to the vanadate monomer, will be considered. If, however, several complexes form in significant concentrations, a plot of [complex] as a function of $[V_i]$ or $[V_i]^2$ should not be linear (assuming [ligand] $\gg$ [complex]). With the appropriate caution, this method can be applied to determine the formation constants of the vanadate–ligand complexes. The results shown in Table III for TEA give equilibrium constants of $1.8 \times 10^2$ L/min (pH 8.0), which agree with the results reported previously ($2.3 \times 10^2$ L/mol, pH 8.4).[11]

In this section we have demonstrated that the $V_0/V_i$ ratio is constant under variable conditions and can successfully be used to calculate equilibrium constants in agreement with the literature. In principle this approach is valid for any inhibitor. The relationships described in this paper are limited to competitive inhibitors, and as shown in Table III, studies were carried out with phosphate, arsenate, vanadate, and vanadyl cation. Focusing on monomeric vanadate as a specific inhibitor, the enzyme assay offers several advantages over other methods such as $^{51}V$ NMR spectroscopy. The enzyme assay is experimentally simple, rapid, cheap and instrumentally less demanding than $^{51}V$ NMR.[24] In addition, the enzyme assay will in some cases give results that are easier to interpret. Observation and differentation of complexes that accidentally have equivalent chemical shifts or complexes in rapid exchange with vanadate cannot be done by recording a single $^{51}V$ NMR spectrum. However, such complexes will be indicated by observing a low $V_0/V_i$ ratio. Molecules such as Tris,[27] DEA,[11] glucosides,[28] and glucose[29] form vanadate derivatives that fortuitously have their $^{51}V$ NMR signal superimposed on the $[V_i]$ signal. One $^{51}V$ NMR spectrum will not show evidence for such complexes whereas the enzyme assay will give a $V_0/V_i$ ratio that reflects such derivatives.

**Limitations in Quantitative Application of the Assays.** The applicability of this method requires fulfillment of the assumption that $K_m$, $K_i$, and $K_i/K_m$ are constant under various assay conditions. For many enzymes such assumptions are trivial; however, for acid and alkaline phosphatases such assumptions restrict the applicability of this method. Both acid and alkaline phosphatases have shown large variations of Michaelis–Menten parameters in different buffers and at different pH and ionic strengths. Detailed discussion describing the assay conditions under which the above assumptions break down is beyond the scope of this paper. However, we will describe when the quantitative results obtained by the assay are reliable. The experimental evidence for these conclusions will be published elsewhere.[24]

Variation in Michaelis–Menten parameters ($K_m$, $K_i$, and $K_i/K_m$) is followed by variation in reaction rates of enzyme reactions.[23] Conditions must therefore be chosen such that $V_{0,ref}$ is indistinguishable from $V_{0,compd}$, where $V_{0,ref}$ is the hydrolysis rate in the reference buffer and $V_{0,compd}$ is the hydrolysis rate measured when the compound under examination is added to the solution. If $V_{0,ref}$ is indistinguishable from $V_{0,compd}$, little change in the Michaelis–Menten parameters is observed. The assay will yield reliable quantitative results. If significant differences in the reaction rates are observed, large changes in the Michaelis–Menten parameters will also be observed. The assay is likely to yield less reliable results. We recommend an allowed maximum of 20% deviation in the hydrolysis rates between $V_{0,ref}$ and $V_{0,compd}$ in order to use this method quantitatively. If larger rate reductions are observed, we recommend decreasing the concentration of compound so that the rate reductions may be smaller and acceptable. Alternatively, the analysis could be conducted in a qualitative manner as follows.

If the assay conditions affect the hydrolysis rate, they will do so when the rate is decreased both by vanadate and by phosphate. Since phosphate is an innocuous inhibitor, variations in the $V_0/V_i$ ratio are very minor (see above Table II and Figure 6). Variations in the $V_0/V_i$ (larger than 20%) for phosphate therefore reflect the changes in the assay environment. If we observe low reaction rates, the $V_0/V_i$ ratio for phosphate is therefore determined as a control for how large the resulting deviations are expected to be. This approach has been used when interpreting and discussing the results shown in Table V.

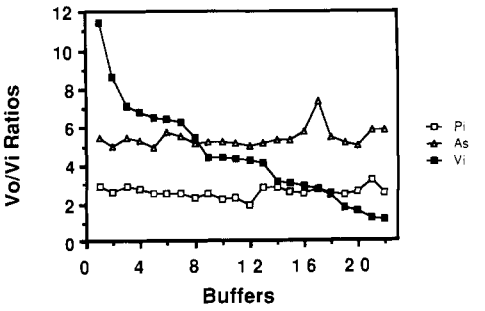


**Figure 6.** $V_0/V_i$ ratios are shown for the inhibition of phosphate (□), arsenate (△), and vanadate (■) in the alkaline phosphatase catalyzed hydrolysis of *p*-nitrophenyl phosphate in various buffers. The key to the buffers is as follows: 1 (Hepes), 2 (*N*-ethylmorpholine), 3 (Tris), 4-(Bis-Tris propane), 5 (EPPS); 6 (barbitol), 7 (TAPS), 8 (TES), 9 (PIPES), 10 (glycine), 11 (glycylglycine), 12 (pyridine), 13 (DEA), 14 (Bicine), 15 (MDEA), 16 (TEA), 17 (AMPSO), 18 (BES), 19 (DIPSO), 20 (IEA), 21 (TAPSO), 22 (Tricine). The assays contained[19] 0.0256 mM *p*-nitrophenyl phosphate, inhibitors [$P_i$, 0.30 mM; As, 0.075 mM; $V_i$, 0.010 7M], buffer (20 mM) at pH 8.0, 1 M KCl, and approximately 0.0063 mg/mL alkaline phosphatase. The rates were determined at 25 °C. The estimated error limit on each $V_0/V_i$ determination is 10% although the ratios are reproducible within 5%.

**Table IV.** $V_0/V_i$ Ratios Determined for Enzyme Substrates and Cofactors with Alkaline Phosphatase[a]

| compd | concn (mM) | buffer (mM) | $V_0/V_i$ | |
|---|---|---|---|---|
| | | | $V_i$ | $P_i$ |
| none | 0 | Hepes | 11.4 | 2.2 |
| enzyme substrates | | | | |
| glycerol | 50 | Hepes | 11.1 | 2.1 |
| glucose | 50 | Hepes | 11.4 | 2.0 |
| ribose | 50 | Hepes | 10.8 | 2.2 |
| citrate | 50 | Hepes | 7.5 | 1.6 |
| malate | 50 | Hepes | 6.0 | 1.8 |
| glycerate | 50 | Hepes | 6.5 | 1.7 |
| cofactors (or analogues) | | | | |
| NAD | 20 | Hepes | 11.6 | 1.8 |
| adenosine[b] | 20 | Hepes | >20 | 2.0 |

[a] The $V_0$ were determined at 25 °C at pH 8.0 in 20 mM Hepes, 0.025 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.0067–0.0072 mg/mL alkaline phosphatase. The $V_i$ were determined as above after adding 0.010 mM vanadate or 0.30 mM phosphate. We estimate an experimental uncertainty of 5% on the $V_0/V_i$ ratio. [b] Adenosine is used as a substitute for AMP, ADP, and ATP. Nucleotides cannot be studied in the alkaline phosphatase assay at these concentrations, since they are good substrates for the enzyme and therefore compete with the *p*-nitrophenyl phosphate as substrate.

**Vanadate Interactions with Buffers.** The rate of hydrolysis of 0.025 mM *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase (about 0.007 mg/mL) was measured in 22 buffers without inhibitor, with 0.30 mM phosphate, with 0.075 mM arsenate, or with 0.010 mM vanadate. The assay solutions contained 20 mM Hepes and 1 M KCl, and the pH was 8.0. The $V_0/V_i$ ratios calculated from these rates for phosphate, arsenate, and vanadate are shown in Figure 6.

Figure 6 shows the $V_0/V_i$ ratios for phosphate and arsenate are constant within experimental error with a few exceptions for

(27) Tracey, A. S.; Gresser, M. J. *Inorg. Chem.* **1988**, *27*, 1269–75.
(28) Tracey, A. S.; Gresser, M. J. *Inorg. Chem.*, in press.
(29) Crans, D. C.; Schelble, S., unpublished results.

**Table V.** $V_0/V_i$ ratios Determined from Amino Acids and Peptides with Alkaline Phosphatase[a-c]

| compd | concn (mM) | buffer (20 mM) | $V_0/V_i$ | | |
|---|---|---|---|---|---|
| | | | $V_i$ | $P_i$ | As |
| none | | Hepes | 10.7 | 2.2 | 5.9 |
| L-proline | 200 | Hepes | 9.5 | 2.2 | 6.1 |
| DL-aspartate | 65 | Hepes | 7.1 | 2.2 | 5.4 |
| DL-alanine | 200 | Hepes | 7.9 | 2.0 | 5.0 |
| L-leucine | 38 | Hepes | 8.6 | 1.8 | 5.0 |
| L-lysine | 200 | Hepes | 7.7 | 1.8 | 4.5 |
| L-serine | 200 | Hepes | 8.3 | 1.9 | 4.7 |
| L-tyrosine | 2 | Hepes | 8.1 | 1.9 | 4.3 |
| DL-valine | 200 | Hepes | 7.2 | 1.8 | 4.3 |
| DL-valine methyl ester | 200 | Hepes | 7.7 | 1.8 | 4.5 |
| L-threonine | 200 | Hepes | 6.2 | 1.7 | 4.4 |
| L-glutamate[d] | 200 | Hepes | 5.3 | 1.6 | 3.3 |
| L-histidine[b,d] | 50 | Hepes | 3.6 | 1.4 | 2.5 |
| L-phenylalanine[b,d] | 30 | Hepes | 4.1 | 1.4 | 2.8 |
| L-glycine[d] | 200 | Hepes | 4.4 | 1.4 | 3.1 |
| glycylglycine[d] | 200 | Hepes | 4.0 | 1.5 | 3.2 |
| glycyl-L-leucine[b,d] | 200 | Hepes | 4.1 | 1.4 | 2.8 |
| L-methionylleucine[d] | 200 | Hepes | 3.4 | 1.5 | |
| glycylglycylglycine[d] | 200 | Hepes | 5.2 | 1.5 | 4.2 |
| L-leucylglycylglycine[b,d] | 200 | Hepes | 3.8 | 1.4 | 2.6 |

[a] The $V_0$ were determined at 25 °C at pH 8.0 in 20 mM Hepes, 0.025 mM *p*-nitrophenyl phosphate, 1 M KCl, and 0.0065–0.016 mg/mL alkaline phophatase. These high enzyme concentrations were used because the amino acids and peptides significantly decrease the enzyme activity. The $V_i$ were determined as above after addition of 0.010 mM vanadate, 0.30 mM phosphate, or 0.075 mM arsenate. We estimate an experimental uncertainty on the $V_0/V_i$ ratios of 5%. [b] Hydrolysis rates with L-histidine, L-leucylglycylglycine, L-phenylalanine, and glycyl-L-leucine were measured with 0.022–0.045 mg/mL alkaline phosphatase. [c] The rates in this table were determined under conditions where [S] = $K_m$, and to illustrate that similar results were obtained when [S] ≫ $K_m$, the assay described above was modified to contain 0.25 mM *p*-nitrophenyl phosphate. The $V_0/V_i$ ratios for vanadate and phosphate were as follows: Hepes, 2.5 and 1.2; L-proline, 2.6 and 1.2; tyrosine, 1.9 and 1.1; glycine, 1.7 and 1.1; L-glutamate, 1.7 and 1.1; glycylglycine, 1.4 and 1.04. Note, although the numerical value of the $V_0/V_i$ ratios changed, these results support a similar trend to that observed in the above table. [d] The observed $V_0/V_i$ ratios for vanadate and possibly arsenate may be lower than warranted by the peptide–vanadate interaction. However, the $V_0/V_i$ ratios for vanadate are so low that the interaction with vanadate is obvious (the control $V_0/V_i$ for phosphate are low, but not to the same extent as the $V_0/V_i$ for vanadate). These data should only be used qualitatively, since the assumptions for applying eq 3 were no longer valid.

arsenate. Arsenate has been reported to spontaneously form esters with alcohols. The rate constants of these reactions are on the order of $\sim 10^{-6}$ $M^{-1}$ $s^{-1}$ (this rate constant is $10^6$ times slower than the corresponding rate constant for the vanadate ester).[30] In the present studies arsenate was not allowed to preequilibrate with the buffer. It is therefore not surprising that Figure 6 shows little evidence for formation of arsenate complexes similar to the vanadate complexes. For selected buffers including Tricine, Hepes, and TEA, the buffer and arsenate were allowed to incubate 1 week before the rate studies were carried out. However, a maximum of 10–20% change in the $V_0/V_i$ ratios was observed for these buffers. We interpret our results to suggest that arsenate interactions are weak at these low arsenate concentrations and short reaction times.

The $V_0/V_i$ ratios for vanadate vary from 1 to 12. On the basis of the size of the $V_0/V_i$ ratios, we divide these buffers into three groups: the noninteracting or weakly interacting buffers ($V_0/V_i$ > 6), the interacting buffers (6 > $V_0/V_2$ > 2), and the strongly interacting buffers ($V_0/V_i$ < 2). Buffers in the first group include Hepes, *N*-ethylmorpholine, Bis-Tris propane, EPPS, barbitol, and TAPS. Buffers included in the second group are TES, PIPES, glycine, glycylglycine, pyridine, DEA, Bicine, MDEA, TEA,

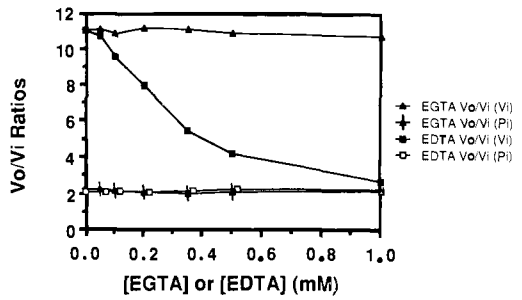


**Figure 7.** $V_0/V_i$ ratios for vanadate and phosphate are shown as a function of EDTA or EGTA concentration at pH 8.0. The rates of hydrolysis at pH 8.0, $V_0$, were determined at 25 °C containing 0.25 mM *p*-nitrophenyl phosphate, 20 mM Hepes, 1 M KCl, 0.025–0.050 mg/mL alkaline phosphatase, and various concentrations of EDTA or EGTA. The inhibited rates of hydrolysis at pH 8.0, $V_i$, were determined as above and in the presence of 0.025 mM vanadate or 0.30 mM phosphate and various concentrations of EDTA or EGTA. The $V_0/V_i$ ratios are shown as follows: EDTA–phosphate (□), EDTA–vanadate (■), EGTA–phosphate (▲), and EGTA–vanadate (▲).

AMPSO, and BES. Buffers included in the third group are DIPSO, IEA, TAPSO, and Tricine. We therefore recommend biological studies with vanadate to be carried out in Hepes, *N*-ethylmorpholine, Bis-Tris propane, EPPS, barbitol, or TAPS.

**Complexation of Vanadate by EDTA and EGTA.** The $V_0/V_i$ ratios for vanadate and phosphate were determined in the presence of various concentrations of EDTA and EGTA with the alkaline phosphatase assay. The results and experimental details are described in Figure 7. Figure 7 shows a plot of the $V_0/V_i$ ratios as a function of EDTA or EGTA concentrations at pH 8.0. The $V_0/V_i$ ratio for EDTA was found to decrease above 0.1 mM EDTA as illustrated by the $V_0/V_i$ ratios of 9.6, 7.9, and 2.5 at 0.1, 0.2, and 1.0 mM EDTA. These results suggest that approximately 95%, 90%, and 75% of the total vanadate was available to interact with alkaline phosphatase. The fact the $V_0/V_i$ ratio decreases more rapidly at pH 5 (data not shown)[24] than at pH 8 is presumably due to an increased stability of the EDTA–vanadate complex at low pH.[24] Using eq 3, we calculate a $K^{eff}_{pH\,8.0}$ = [complex]/[$V_{tot}$] × [EDTA] = 1.4 × $10^4$ L/mol, where [complex] is the various protonated forms of EDTA–vanadate complexes, [$V_{tot}$] is the total concentration of free vanadate in various protonated forms, and [EDTA] is the total concentration of free EDTA in various protonated forms. This terminology was previously used by Przyborowski et al., who determined $K^{eff}_{pH=1-3}$.[31] We used their results and projections to estimate $K^{eff}_{pH=8.0}$ to 6.3 × $10^4$ L/mol.[31] Considering the variations in temperature, ionic strength, and buffers used for the determination of this constant and the projection outside the pH range where the experimental determinations were carried out, we find our results agree favorably with the literature. The stability constant commonly cited for the strength of the EDTA–vanadate complex is the stability constant for the triply negatively charged complex, which by use of the results by Przyborowski and us corresponds to $K \cong 10^{15}$.[31]

We used eq 3 to calculate a $K^{eff}_{pH=8.0}$ for EGTA and vanadate of 1.1 × $10^2$ L/mol. This complex is 2 orders of magnitude less stable than the EDTA–vanadate complex. Both the EDTA- and EGTA–vanadate complexes were observed to be 2 orders of magnitude more stable at lower pH. Chelate agents are necessary for protection of enzymes and other biological systems and therefore commonly used in in vitro studies. Since the EGTA binds vanadate by a stability constant 2 orders of magnitude less than that of EDTA, EGTA is the reagent of choice when studies are conducted with vanadate. In addition the stability constants of both EDTA–vanadate and EGTA–vanadate are larger at lower pH; thus, neutral or higher pH is recommended when studies are

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(31) Przyborowski, L.; Schwarzenback, G.; Zimmermann, T. *Helv. Chim. Acta* **1965**, *48*, 1556–65.

(32) Van Etten, R. L.; Waymack, P. P.; Rehkop, D. M. *J. Am. Chem. Soc.* **1974**, *96*, 6782–5.

(33) Crans, D. C.; Gerhardt, K., unpublished results.

conducted in the presence of vanadate and metal chelator.

**Interaction of Vanadate with Enzyme Substrates and Cofactors.** The $V_0/V_i$ ratios of rates of *p*-nitrophenyl phosphate hydrolysis catalyzed by alkaline phosphatase were determined in the presence of several enzyme substrates and cofactors. The enzyme substrates included polyalcohols and α-hydroxy carboxylic acids, and the enzyme cofactors were NAD and adenosine (Table IV).

Glycerol, ribose, and glucose at 50 mM concentrations were all found to have $V_0/V_i$ ratios for vanadate within a 5% range of the reference $V_0/V_i$ ratio. The $V_0/V_i$ ratios for phosphate were found to vary in a similar manner. These results suggest that carbohydrate-type substrates do not significantly interact with vanadate. More than 95% of vanadate will therefore be free to interact with proteins under these conditions. This suggests such interaction with carbohydrates would only occur in special cases in vivo. Interactions between substrates and vanadate may be stronger inside a protein, and the above conclusion is premature. In addition, it is not currently understood how proteins affect the reactivity and stability of vanadate complexes. However, if interactions of carbohydrates with vanadate are to be important in in vivo reactions, the vanadate interaction must be carefully stabilized and protected against side reactions with the environment. Interactions between vanadate and carbohydrates are more likely to occur in vitro because the higher concentrations of both vanadate and potential ligands increase the overall possibilities for interaction yielding vanadate derivatives. The $V_0/V_i$ ratios were not changed in the presence of NAD, and NAD-derived cofactors are therefore not expected to significantly interact with vanadate under these conditions. Adenosine on the other hand was found to increase the $V_0/V_i$ ratio, which suggests that vanadate in the presence of this molecule is a better inhibitor! This type of behavior has previously been observed with alkaline phosphatase for organic molecules such as phenol.[34]

Contrary to the carbohydrate enzyme substrates, citrate and malate generated $V_0/V_i$ ratios that at 50 mM substrates showed significant binding of vanadate. Substrates containing α-hydroxy carboxylic acids can potentially interact with vanadate even at a $10^{-6}$ M vanadate concentration, and this could either initiate the biological action of vanadate or prevent the vanadate interactions with other potential ligands. These types of substrates may indeed bind vanadate under in vivo conditions. Whether such interactions are important for the mechanisms by which vanadate acts remains to be determined.

**Interaction of Vanadyl Cation with Buffers.** Studies with vanadyl cation are commonly complicated by both redox and hydrolysis reactions in the physiological pH range. Furthermore, vanadium(IV) is an EPR-active nucleus, and quantitative determinations of vanadyl cation are difficult to carry out routinely. It is therefore desirable to measure the concentration of free vanadyl cation. Since vanadyl cation is a potent competitive inhibitor for both alkaline and acid phosphatase, the approach described in this paper is appropriate.[12] We have shown the $V_0/V_i$ ratio for vanadyl cation is proportional to the vanadyl cation concentration in barbitol-buffered solutions.[24] The relationships described by eq 2 and 3 and verified experimentally for vanadate in Figures 2 and 3 have also been carried out for the vanadyl cation.[24] The $V_0/V_i$ ratios for vanadyl cation at $10^{-6}$–$10^{-7}$ M concentration were determined in a variety of buffers including barbitol, *N*-ethylmorpholine, EPPS, Hepes, Tris, Tricine, TAPS, DIPSO, and glycylglycine (Table II). Barbitol and Hepes appeared to complex vanadyl the least, whereas Tris was found to interact fairly strongly with the vanadyl cation. TAPS and Tricine were also found to interact with vanadyl. However, only DIPSO and glycylglycine were found to interact strongly with vanadyl. This series of interaction deviates somewhat from the interaction of these buffers with vanadate. In general, the studies confirm that vanadyl cation forms more stable complexes than vanadate. For example, Tris will form a moderately strong complex with vanadyl[12] but only a weak complex with vanadate.[11,27] These observations are presumably a reflection of the greater affinity vanadyl cation has for ethanolamine and vicinal diols compared to vanadate.[12,11] Sulfate groups also will increase the stabilization of vanadyl complexes whereas no effect is observed for vanadate complexes.[12,11] A remarkable difference in stability is observed in the glycylglycine–vanadyl complex which is orders of magnitude more stable than the corresponding glycylglycine–vanadate complex. It is likely the presumed octahedral $VO^{2+}$ complex is more favorable than the presumed pentacoordinate complex of vanadium(V) in the glycylglycine–vanadate complex.

The Tricine–vanadate complex ($K^{eff}_{pH=8.0}$ = 1600 L/mol) is 8 times more stable than the corresponding Tricine–vanadyl complex ($K^{eff}_{pH=8.0}$ = 200 L/mol).[11] Presumably the reversed order of stability is caused by the strain induced in the ligand when spanning an octahedral vanadyl cation or a square-pyramidal vanadyl cation, compared to a bipyramidal, pentacoordinate vanadate. Tricine would therefore represent the first reported case in which the geometry of the ligand forces the reversal of the usual stability in vanadium complexes.

Application of Tricine as a buffer for some biological studies could potentially solve a problem often encountered in biological studies with vanadium. Vanadyl cation oxidizes easily, and vanadate reduces readily. It is therefore difficult to identify the actual oxidation state of vanadium that is responsible for a particular biological activity. If one uses a buffer that successfully complexes vanadate, the presence of vanadate in the vanadyl solution will not be responsible for biological activities, because the vanadate will immediately be complexed by the buffer. Oxidation of vanadyl cation to vanadate will therefore not complicate the observation of biological activities caused by the vanadyl cation. Tricine, although it interacts with vanadyl cation, binds vanadate much more strongly and will immediately remove the fraction of vanadyl cation that has oxidized to vanadium(V). Observation of biological activities in Tricine (at concentrations from 5 to 100 mM depending on the anticipated vanadate concentrations) would therefore suggest the biological activities are caused by the vanadyl cation. Application of TAPS as buffer also illustrates this point, because TAPS will interact only weakly with vanadate and therefore not affect the biological activity of vanadium(V). TAPS interacts strongly with the vanadyl cation and will therefore remove the vanadyl cation generated by the reduction of vanadate. Observation of biological activity of vanadium in TAPS would suggest the biological activity is caused by vanadate. The examination of the biological activity in these two buffers could therefore provide experimental evidence for which vanadium oxidation state is the active form of vanadium for a particular enzyme system.

**Interaction of Vanadate and Vanadyl Cation with Amino Acids, Peptides, and Proteins.** Recently it has been shown vanadate interacts with dipeptides and other amine derivatives at high amino acid concentration and vanadate concentrations containing mono- and oligovanadates.[10,11] These interactions could potentially occur under biological conditions and are therefore of interest at low vanadate concentrations. We determined the $V_0/V_i$ ratios of vanadate and phosphate for a series of amino acids and peptides using alkaline phosphatase under conditions described in Table V. Most of the amino acids and peptides were found to affect the hydrolysis rates considerably at high concentrations, presumably reflecting the changes that are taking place in the protein in such environments (which induces changes in the $K_m$ and $K_i$). Since the validity of eq 2 and 3 is based on the assumption that insignificant changes occur for $K_m$, $K_i$, and the $K_m/K_i$ ratio, quantitative interpretations should not be carried out without appropriate control experiments.[24] However, if the $V_0/V_i$ ratio for phosphate is determined as a reference, qualitative comparisons can be made. Surprisingly, we found the $V_0/V_i$ ratios for vanadate and 200 mM amino acids or peptides were considerably outside the experimental uncertainties for the $V_0/V_i$ ratio determination! The four amino acids showing least interaction with vanadate are proline with the highest $V_0/V_i$ ratio of 9.5 and leucine, serine, and tyrosine with $V_0/V_i$ ratios of 8.6, 8.3, and 8.1, respectively. Although we have included a greater error limit (±15%) on these

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Table VI. Formation Constants for Vanadate or Vanadyl Cation Complexes and Various Peptides and Proteins Determined with the $V_0/V_i$ Ratios Obtained by the Alkaline Phosphatase Assay[a,b]

| protein/biomolecule | vanadium species | formation constant ($\times 10^3$ $M^{-1}$)[c] |
|---|---|---|
| peptides | | |
| glycine | vanadate[a] | 0.07 |
| glycylglycine | vanadate[a] | 0.1 |
| glycylglycylglycine | vanadate[a] | 0.2 |
| glycine | vanadyl[b] | 5 |
| glycylglycine | vanadyl[b] | 20 |
| glycylglycylglycine | vanadyl[b] | 40 |
| proteins | | |
| bovine serum albumin | vanadate[a] | 1 |
| pepsin | vanadate[a] | 10 |
| chymotrypsin | vanadate[a] | 10 |
| acid phosphatase | vanadate[a] | 60 |
| aldolase | vanadate[a] | 50 |

[a] The $V_0$ were determined at 25 °C at pH 8.0 in 20 mM Hepes, 0.025 mM *p*-nitrophenyl phosphate, 1.0 M KCl, and 0.0070 mg/mL alkaline phosphatase. The $V_i$ were determined as above after addition of 0.005 mM vanadate. [b] The $V_0$ were determined at 25 °C at pH 8.0 in 67 mM barbitol, 0.025 mM *p*-nitrophenyl phosphate, 1.0 M KCl, and 0.0070 mg/mL alkaline phosphatase. The $V_i$ were determined as above after addition of 0.0001 mM vanadyl cation. All solutions were freshly purged with nitrogen. [c] The formation constants were calculated according to $K_{aff} = [complex]/([V_i(VO^{2+})][ligand])$. It is therefore assumed $[V_{tot}] = [V_i(VO^{2+})] + [complex]$ and the complex is the major vanadium–ligand derivative formed in solutions. An error limit of ±20% on the formation constant was estimated.

results, this is a reflection of the experimental difficulties in these measurements. Repeated rate determinations give $V_0/V_i$ ratios within 5%. Both leucine and tyrosine are not very soluble in an aqueous environment, and their concentrations are considerably reduced, which probably explains the resulting high ratios. Serine has been shown to interact with vanadate at high concentrations, so it was therefore unexpected that serine had one of the highest $V_0/V_i$ ratios. However, since the $V_0/V_i$ ratio for serine was significantly lower than the reference ratio, weak interaction was confirmed between vanadate and serine. As seen from Table V the interactions between amino acids and vanadate were in most cases weak. Several amino acids including glutamate, histidine, phenylalanine, and glycine were all found to interact with vanadate resulting in $V_0/V_i$ ratios ranging from 4 to 6. This strong interaction seems somewhat exaggerated, and indeed, the $V_0/V_i$ ratios for phosphate were also found to be low, reflecting the fact these amino acids affect the catalytic activity of alkaline phosphatase. We therefore interpret the $V_0/V_i$ ratios for these amino acids as showing interaction occurs with vanadate; however, such interaction is less than reflected by the low $V_0/V_i$ ratio because the assumption that only insignificant changes in the $K_m$, $K_i$, and $K_m/K_i$ occur was not fulfilled.

All the dipeptides and tripeptides were found to have low $V_0/V_i$ ratios. The peptides also have low $V_0/V_i$ ratios for phosphate, which suggests the assumptions required for eq 1–3 were no longer valid. In order to determine the formation constants, we repeated these experiments at much lower concentrations of ligand such that $V_0$ varied only slightly from the reference $V_0$. $K_m$, $K_i$, and $K_m/K_i$ should then remain constant for both the vanadyl cation and vanadate measurements. The results from these experiments were used to calculate the equilibrium constants shown in Table VI. Vanadyl cation was found to complex more with glycylglycylglycine than with glycylglycine or with glycine. Vanadyl cation forms even stronger complexes with proteins. Vanadate shows a similar stability pattern and complexes least with glycine, more with glycylglycine and glycylglycylglycine, and most with the proteins we examined. The vanadyl complexes were found to be at least 2 orders of magnitude more stable than the vanadate complexes. It is interesting that the longer peptide residue such as glycylglycylglycine was found to interact more strongly with both vanadyl cation and vanadate, suggesting that both the free carboxylic acid group and a free amino group in glycylglycine or glycine may not be necessary for the peptide interaction. Rehder has recently observed interactions between vanadate and dipeptides using $^{51}V$ NMR, and the results were interpreted as formation of complexes containing at least one amide group complexing to the vanadium.[14] Rehder's studies therefore nicely support our observations, which suggest peptide amides and amines have comparable affinities for vanadate and vanadyl cation.[14] In summary, these results suggest that the amino acids and di- and tripeptides potentially interact with vanadium at $10^{-5}$–$10^{-7}$ M concentrations. These findings therefore suggest these types of vanadate interactions occur with proteins in vitro and potentially in in vivo systems.

It is of interest to examine if random protein–vanadate interactions at these low vanadate concentrations occur with similar affinity. Several proteins including bovine serum albumin, pepsin, chymotrypsin, acid phosphatase, and aldolase were used for such studies. The $V_0/V_i$ ratio for vanadate and phosphate were determined in the presence of bovine serum albumin and pepsin at protein concentrations varying from 0 to 33 mg/mL. The $V_0/V_i$ ratios were found to vary from 11.4 to 3.2 for vanadate and from 2.1 to 1.4 for phosphate in the presence of either pepsin or bovine serum albumin as the protein concentration increased to 33.3 mg/mL in the enzyme assay. High protein concentrations significantly reduce the hydrolysis rates measured in the assay and therefore affect the protein specificity (assumptions no longer fulfilled). Formation constants were therefore determined at much lower enzyme concentrations and lead to the results shown in Table VI. The formation constant for the vanadate–bovine serum albumin complex, $1 \times 10^3$ L/min, is in the vicinity of the constant previously determined with polarographic techniques and equilibrium dialysis.[13] Some variation was expected since the formation constants were determined under considerably different conditions (lower vanadate concentrations, higher ionic strength, higher pH, lower temperature).[13] The formation constant of the vanadate–pepsin complex was calculated to be $10 \times 10^3$ L/mol. Similar formation constant was obtained with chymotrypsin. The interactions of vanadate with aldolase or acid phosphatase were found to be a factor of 50 higher than the interaction with bovine serum albumin. The formation constant for acid phosphatase–vanadate was determined to be $6 \times 10^4$ L/mol, which is somewhat larger than the constants determined previously at lower pH.[32] This increased affinity of vanadate for such proteins may be a reflection of the high affinity of the charged polar substrates that are converted by these enzymes. We observed similar vanadate complexing behavior by myosin (in the presence of ADP) and ribonuclease (in the presence of uridine).

The interaction of vanadate with proteins at high concentrations (33 mg/mL) was so strong that even bovine serum albumin would have been categorized as an interacting molecule. Although the concentrations of most individual proteins usually do not reach such high values in cells, the total protein concentration may become this high in certain regions of the cell. It is therefore possible that even random vanadate–protein interactions are important in the mechanism by which vanadium interacts in biological systems. Even specific interactions of vanadate with proteins could take place initiated by a random protein–vanadate association. We examined this possibility for acetylcholinesterase.[33] Vanadate was found to both stimulate and inhibit acetylcholinesterase-catalyzed reactions, and considering the reaction mechanism for this enzyme, the generation of a vanadate–protein complex that interferes with catalysis seems likely. However, kinetic evidence for such interactions was not observed with this enzyme.[33] Covalent vanadate complexes have previously been suggested between vanadate and insulin,[2] between vanadate and a histidine residue in phosphoglyceratemutase,[34] and between vanadate and a lysine residue in phosphoglucomutase.[35]

The results with acid phosphatase, pepsin, chymotrypsin, aldolase, myosin, and ribonuclease all suggest that vanadate could potentially bind to these enzymes under in vivo conditions since the interactions with these enzymes are 10–100-fold greater than the random interactions exhibited by bovine serum albumin. It

(35) Ray, W. J., Jr.; Peck, E. F., Jr. *Enzymes (3rd Ed.)* **1972**, *4*, 408–75.

is therefore possible the biological effects of not only vanadium(IV) but also vanadium(V) will depend on the compartmentalization of the vanadium and the particular proteins vanadium affects under these conditions.

## Conclusions

We have developed an acid or alkaline phosphatase assay to determine vanadium concentrations in the $10^{-5}$–$10^{-7}$ M range. The method is cheap, simple, and fast and has been applied to the interactions of vanadium(IV) and (V) with organic molecules. In general, we find that vanadium interactions are very important not only for in vitro studies of vanadyl cation but also for in vitro studies with vanadate. Many of the molecules present in biological studies such as TEA interact strongly with both vanadyl cation and vanadate, whereas others such as Tris interact only weakly at these low vanadium concentrations. The vanadyl cation was usually found to interact more strongly with a ligand than vanadate. However, one ligand, Tricine, was found to form vanadate complexes 8 times *more* stable than the vanadyl complex.

Carbohydrates or polyalcohols are enzyme substrates or cofactors not likely to interact with vanadate at low vanadate concentrations unless, for example, the carbohydrate and vanadate are enclosed in the active site of an enzyme or a hydrophobic pocket generated by a membrane and/or a protein. Other enzyme substrates such as $\alpha$-hydroxy carboxylic acids, including citrate, malate, and glycerate, complex vanadate even at very low vanadate concentrations. Most amino acids with the possible exception of proline are observed to interact weakly with vanadate and vanadyl cation even at $10^{-5}$ M vanadate concentrations. The high affinity of the amine group in lysine for interaction with vanadate has not previously been recognized. Random interaction with proteins such as bovine serum albumin was observed at $10^{-5}$ M vanadate concentrations. Enzymes including ribonuclease, myosin, acetylcholinesterase, acid phosphatase, aldolase, pepsin, and chymotrypsin were all found to interact with vanadate with greater affinity than bovine serum albumin.

The results presented in this paper suggest that both vanadyl and vanadate at $10^{-5}$ M concentrations can interact with many organic molecules including substrates, cofactors, amino acids, peptides, and proteins in in vitro biological studies. Such spontaneous interaction may be important for the mechanisms by which vanadate acts in vivo and must be considered in studies with vanadium.

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# Structure of Dinoflagellate Luciferin and Its Enzymatic and Nonenzymatic Air-Oxidation Products

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**Abstract:** Dinoflagellate luciferin (**1**) was isolated from cultured *Pyrocystis lunula*, and its structure was elucidated primarily by comparing the spectroscopic data of **1** with those of krill fluorescent substance F (**5**). From the crude extract of luciferin, air-oxidation product **3** with a characteristic blue color was isolated. Air oxidation of dinoflagellate luciferin in methanol in the absence of luciferase proceeded without emission of light to yield **2**, whereas air oxidation in water in the presence of luciferase proceeded with the emission of light at 474 nm to yield **4**.

Dinoflagellates have recently drawn much attention because they produce a variety of bioactive substances including neurotoxins and antineoplastic compounds.[1] They often reach abnormally high concentrations in the ocean, forming the so-called red tides. Some of them are toxic, often causing serious health and economic problems such as shellfish and fish poisoning.[2] Many species of dinoflagellates are bioluminescent; they are ubiquitous in the oceans of the world and are responsible for much of the sparkling luminescence at night elicited by disturbing surface waters, recognized as the "phosphorescence" of the sea.[3] Many factors are involved in the regulation of dinoflagellate bioluminescence, including the endogenous circadian clock.[4]

The chemical process of light emission involves air oxidation of dinoflagellate luciferin catalyzed by dinoflagellate luciferase.[5] Luciferin and luciferase from several different dinoflagellate species were proven to cross-react. Interestingly, the dinoflagellate bioluminescent system was shown also to cross-react with the krill bioluminescent system,[6] composed of a photoprotein and krill fluorescent substance F.[7] Krill fluorescent substance F appears to function both as a catalyst for air oxidation of the photoprotein and as the light emitter but not as a substrate for enzymatic air

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