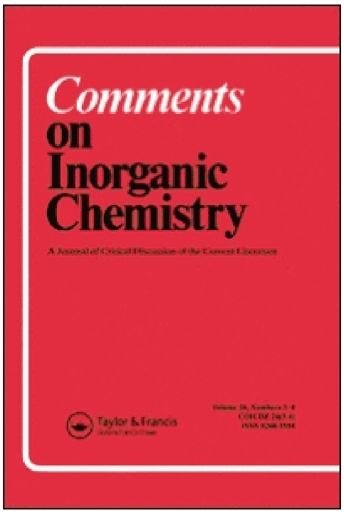

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Aqueous Chemistry of Labile Oxovanadates: Relevance to Biological Studies

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Aqueous Chemistry of Labile Oxovanadates: Relevance to Biological Studies

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The aqueous chemistry of vanadate (vanadium(V)) is very complex since many protonation equilibria and oligomerization equilibria are occurring simultaneously. Vanadate monomer (V_1), dimer (V_2), tetramer (V_4) and pentamer (V_5) are exchanging with each other on a millisecond time scale so that none of these species can be isolated for aqueous biological studies. Measuring the effects of each anion on an enzyme must be carried out in an equilibrium mixture containing the other vanadate oligomers. Defining conditions to measure the effects of oxovanadates is non-trivial, since vanadate interacts with buffers and other assay components. Information concerning the simple aqueous chemistry of vanadate with various ligands is thus necessary to ensure that the vanadate is free to interact with an enzyme or a protein. Experimental approaches, which take into account the aqueous chemistry of labile oxovanadates, to biological studies are described here. For this purpose, the interactions of oxovanadates with proteins and other protein-like ligands are considered briefly. The chemistry and approaches described here form the basis for an analysis of the interactions of vanadate oxoanions with proteins.

Key Words: *vanadium(V), vanadate, oxovanadates, aqueous solution, hydrolytic, oligomerization, redox chemistry, biological studies*

VANADIUM(V) IN BIOLOGY

The role of vanadium in biology extends from beneficial effects to be a required element. Vanadate acts as an insulin mimic and

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recent reports suggest that vanadium-based oral substitutes for insulin can be developed.[1-3] Vanadium acts as a growth factor[4] and affects cardiovascular function.[5] Vanadium-dependent bromoperoxidase in *Ascophyllum nodosum*[6-8] and the vanadium-dependent nitrogenase in *Azotobacter chroococcum* and *Azotobacter vinelandii*[9] employ vanadium as a cofactor. High levels of vanadium are found in tunicates[7,10,11] and a vanadium-containing natural product, amavadine, is found in toadstools.[12] The roles and activities of vanadium in all these cases remain ill defined.

Early studies of vanadate interaction with proteins focused on the development of vanadyl cation as a probe to study metal binding sites in proteins.[13,14] Vanadate is a potent enzyme inhibitor, and has been used to characterize enzymes catalyzing phosphoryl transfer (reviewed in Ref. 5). The potent inhibitory effects of vanadate were attributed to the transition state analogy between a pentacoordinate vanadate complex and the transition state of the enzyme reaction.[15] Organic vanadates can also act as enzyme substrates and even as enzyme cofactors.[16-19] In addition, the recent development of photolysis of protein–vanadate mixtures to probe specific anion binding sites in proteins[20,21] makes vanadate a potent probe to characterize proteins.

In 1985 we were stimulated by a ^{51}V NMR study by Gresser and Tracey describing labile vanadate esters[22] because of their potential as organic phosphate analogs.[23] Selected alkoxides and chloroalkoxides had already been reported by Rehder in organic solvents,[24,25] supporting the observation of vanadate esters in aqueous solutions. Since 1987 we have been exploring related vanadium alkoxides in organic solvents (see for example Refs. 26 and 27 and references therein) and examined the biological properties of various types of labile vanadate derivatives in aqueous solutions.[18,28-33] Studies of these labile oxovanadates are necessary because sodium vanadate (Na_3VO_4) upon dissolution in aqueous buffered solutions produces within milliseconds[34] several other species, all of which are enzyme inhibitors and/or activators.[18,28-33] Interactions of vanadate with proteins, including several phosphatases, dehydrogenases, isomerases, one aldolase, a superoxide dismutase and a kinase have all been studied by our group[17,18,28-33,35-37] and are reviewed in the accompanying paper.

During our enzyme studies it has become apparent that the aqueous vanadate chemistry taking place in the assay solutions could dictate the responses of the biological system under examination.[38] Formation of complexes between vanadate and commonly used buffers will tie up the vanadium in vanadate-buffer complexes and prevent it from interacting with an enzyme.[39,40] Other assay components could similarly mask the results obtained in studies with vanadate.[32,38] The focus of this review is to describe the aqueous chemistry of vanadium(V).

OXOVANADATE SPECIES IN AQUEOUS SOLUTIONS

Vanadate is the name commonly used to describe the form of vanadium(V) that exists in aqueous solution. Solutions containing vanadate are complicated; not only do several protonation equilibria exist, but oligomerization reactions and redox chemistry can also take place depending on the other compounds present. Species present under various conditions have been detailed elsewhere,[41-45] and only a brief summary of the speciation of the oxovanadates of particular relevance to biological studies will be given here.

Vanadate Monomer, V_1

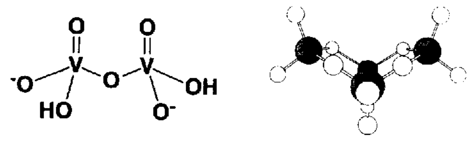
Vanadate in the form of VO_4^{3-} is structurally and electronically related to PO_4^{3-}.[46] Accordingly, HVO_4^{2-}, $H_2VO_4^-$ and H_3VO_4 that form upon protonation of VO_4^{3-} are presumed to be analogs of the corresponding phosphate derivative. The structural characterization of KVO_3[47] and $KVO_3 \cdot H_2O$[47] demonstrates that another possibility exists, and that the phosphate analogy may not hold for all the protonated forms of vanadate monomer.[43,48] In any event, the actual structure of the V_1 in aqueous solution may not be the form that binds to the enzyme. Interaction of vanadate monomer with enzymes can thus be as a tetrahedral phosphate analog ("a ground state analog") or as a trigonal bipyramidal phosphate analog ("a transition state analog"). Precedents for vanadate acting as both ground state and transition state analogs have been reported.[15,49]

Vanadate Oligomers

The vanadate monomer oligomerizes to dimer (V_2), tetramer (V_4) and pentamer (V_5) on a millisecond time scale.[34] First formed the vanadate dimer, which (depending on the pH) also can exist in several protonated forms ($H_3V_2O_7^-$, $H_2V_2O_7^{2-}$, $HV_2O_7^{3-}$, and $V_2O_7^{4-}$).[43] The V_2 was described by a potentiometric study which can not distinguish structural possibilities differing only by molecules of water. Structural characterization of $Ca_2V_2O_7$ shows the presumed structure for V_2 (Fig. 1).[50] The structure of the $Sr(VO_3)_2 \cdot 4H_2O$ represents another alternative and contains a pentacoordinate vanadium atom and bridging oxygen atoms.[51] In the case of V_2, the analogy with pyrophosphate is commonly presumed despite the lack of evidence of the structure in aqueous solution.

After formation of V_2, vanadate tetramer (V_4) forms. V_4 is the major species in concentrated solutions and is often referred to as "metavanadate." The X-ray structures for $V_4O_{12}^{4-}$ and $HV_4O_{12}^{3-}$ salts have been reported.[52,53] The structure is cyclical (Fig. 1). In aqueous solution the tetrameric species has the molecular formula $V_4O_{12}^{4-}$ and it is presumably also cyclical. An adamantane-like structure was also suggested.[54,55] No evidence for a species with the molecular formula $HV_4O_{12}^{3-}$ has been observed at any pH in aqueous solutions. The pentamer, $V_5O_{15}^{3-}$ (V_5) is the last species that forms, and it is also presumed to be cyclic even though no structural information is available for this anion. V_2, V_4 and V_5, in addition to V_1, are the major vanadate oxoanions present in aqueous solution (Fig. 2).

Each vanadate oligomer gives resolved resonances in the ^{51}V NMR spectrum as indicated in Fig. 3. The location of the reso-



V_2 V_4

FIGURE 1 A structural representation of vanadate dimer (V_2) and tetramer (V_4) are shown. The drawing of V_4 was obtained from the X-ray coordinates from Ref. 52.

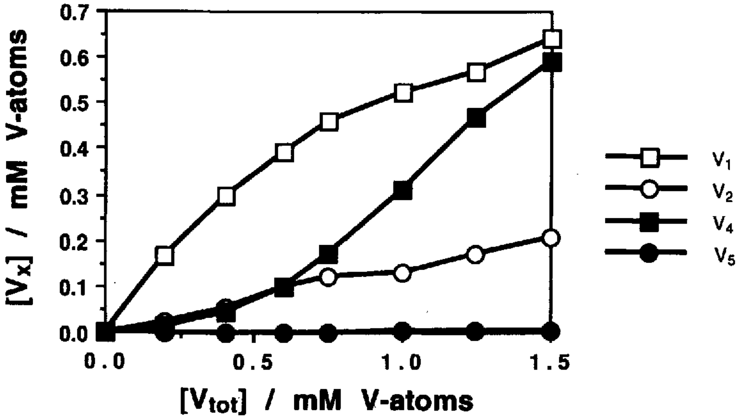


FIGURE 2 The distribution of oxovanadates as a function of total vanadate (as mM V-atoms). The data was adapted with permission (Copyright 1990 American Chemical Society) from Ref. 30, and the H^+-dependent formation constants for these experimental data are shown in Table I.

nances is sensitive to the protonation state of the solution; $H_2VO_4^-$ and HVO_4^{2-} are assigned to resonances around −560.4 and −538.8 ppm, respectively.[41] Since the vanadate species have similar relaxation time values,[34] integrating the ^{51}V NMR resonances provides mole fractions of each species that can be used to calculate the concentrations of each vanadate oligomer. The relationships between the oligomers are discussed in detail below. The UV spectra for each vanadate derivative have been assigned and can also be used for quantification.[56] ^{51}V NMR spectroscopy is less subject to data interpretation errors, since the resonances for each species are resolved in the NMR spectrum, in contrast to the significant overlap of the absorbance spectra for each isomer.

2D ^{51}V EXSY and variable temperature NMR spectroscopy can be used to measure the rates of interconversion of the vanadate oligomers in aqueous solution.[34] Figure 3 shows a series of 1D ^{51}V NMR spectra recorded at various temperatures. As the temperature increases the resonances approach coalescence, consistent with chemical exhange between the oxovanadates. A 2D EXSY spectrum of such a vanadate solution should provide additional details because the exchange processes between each of the isomers are specifically separated. A 2D EXSY spectrum of a 10 mM

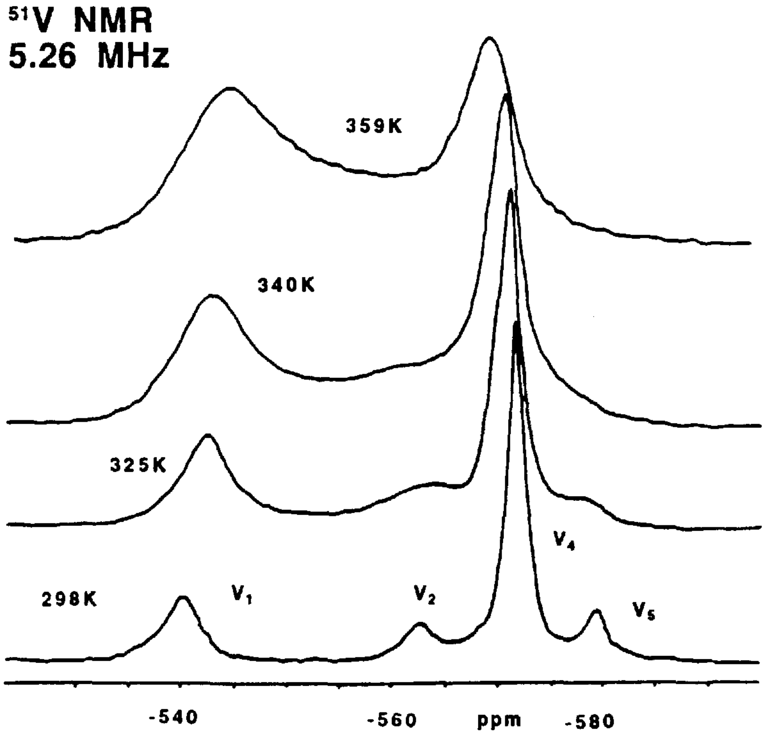


FIGURE 3 A series of 1D ^{51}V NMR spectra (52 MHz) at various temperatures of a solution containing 10 mM vanadate and 0.40 M KCl at pH 8.6 (±0.1). The assignments of the resonances are indicated. Adapted with permission from Ref. 34 (Copyright 1990 American Chemical Society).

vanadate solution at pH 8.6 containing 0.40 M KCl indeed shows the exchange between all the different isomers (Fig. 4). In any event, reaction equilibria are, for most practical purposes, established immediately since the labile oxovanadates (V_1, V_2, V_4 and V_5) exchange on a millisecond time scale.

Vanadate decamer (V_{10}) is a large oxovanadate that forms between pH 2 and 6. This species is structurally characterized, and the oxoanion present in solution has the same structure as that observed in the solid state.[58-61] Vanadate decamer is thermodynamically unstable at neutral and basic pH; but, the lability of this oxoanion[62] allows studies of limited duration (one to two days).

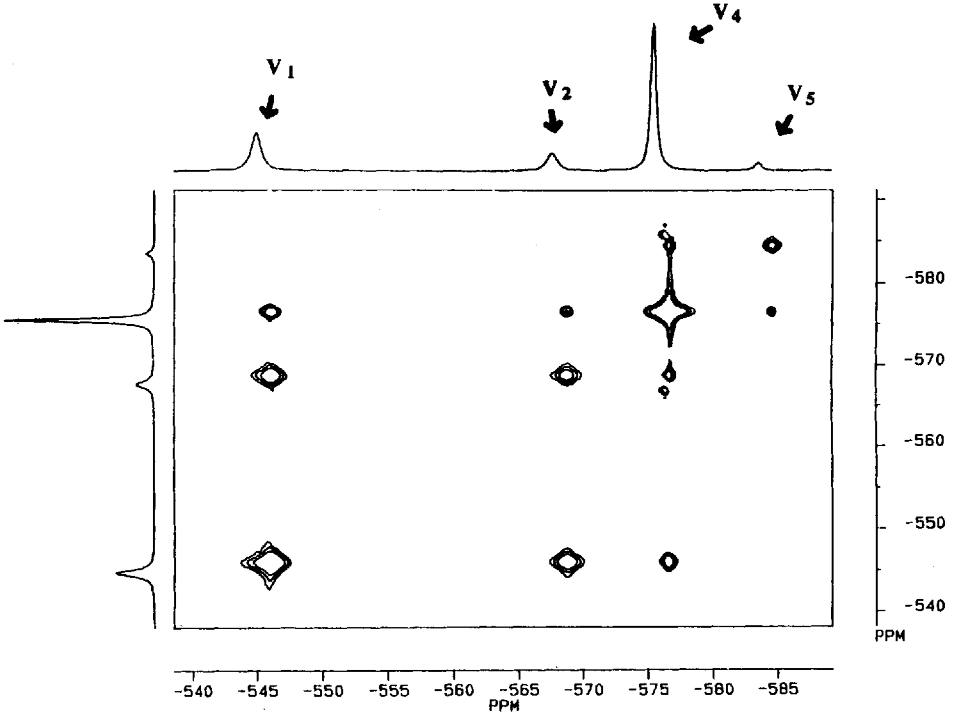


FIGURE 4 ^{51}V EXSY NMR spectrum (131.5 MHz) of solutions containing 10 mM vanadate and 0.40 M KCl at pH 8.6 (±0.1). Adapted from Ref. 57.

Other Vanadium(V) Species in Aqueous Solution

Below pH 2, VO_2^+ is the major species in solution.[41,43,44] This species is cationic and different from all the other oxovanadates. The VO_2^+ unit is found in many vanadium(V) complexes, which could exist under physiological conditions even though VO_2^+ is not likely to exist in cells (with the exception of vacuoles or similarly acidic compartments).

Other vanadate species, including a linear trimer and tetramer,[41] have been observed in the slightly alkaline pH range. Furthermore, a hexamer has been reported at high vanadate concentrations.[41] The latter species, as well as additional species not described here, are minor components of aqueous solutions.[41,43] Such species may be exceedingly important for the exchange reactions that take place in aqueous solution.[34,41] However, these species are not likely to contribute significantly to the observed interaction with enzymes and other cell components because they are present only at low concentrations. Thus they would have exceedingly high affinities if they interacted with proteins. Accordingly, the molecules responsible for the biological effects include V_1, V_2, V_4, V_5, V_{10} and potentially VO_2^+.

Reduction of Vanadate

The redox potential, $\mathscr{E}^o$, is reported as 1.0 V for vanadium(V) in acidic solution and −0.74 in basic solution (see (1) and (2)).[63] Vanadate reacts with reducing compounds, presumably to form vanadium(IV), in the presence of several compounds commonly found in the cell and assay solutions. Examples of such reactive cell components include L-ascorbic acid,[64] gluthathione,[65] cysteine[66] and possibly NADH.[67] The reaction of vanadate with gluthathione[65] secures most of the intracellular vanadium in the form of vanadium(IV).[68] Reaction of vanadate with ascorbic acid may also contribute to the conversion of vanadium(V) to (IV), although such a role for ascorbic acid is less appreciated for most metals.

$$VO_2^+ + 2H^+ + e^- \rightarrow VO^{2+} + H_2O \qquad (1)$$

$$V^{+5} + e^- \rightarrow V^{+4}\ (1\ \text{N NaOH}) \qquad (2)$$

The reaction of vanadate with thiols is of specific interest since enzymes contain thiol groups. Vanadate reacts rapidly with free cysteine to generate cystine and vanadyl cation in a pH-sensitive reaction.[66] Above pH 8 the reaction does not occur at observable rates.[66] Detailed kinetic studies have not been reported, although recently the reaction of vanadate with the thiol groups of fructose-1,6-bisphosphate aldolase has been described.[32]

In addition to the enzyme, enzyme substrates such as dihydroxyacetone and fructose will react with vanadate to generate the presumed vanadyl cation and oxidized organic substrates.[19,32] Careful mechanistic studies have been carried out with aromatic diols and suggest that the electron transfer occurs following a vanadium(V)–ligand complex.[69–71] Redox reactions will seriously complicate a biological study, since upon formation of vanadium(IV) the concentration of total vanadium(V) is altered; ^{51}V NMR spectroscopy can no longer be used to accurately quantitate labile oxovanadates. Redox reactions must thus be maintained at an insignificant level, unless methods designed to quantitate vanadium(IV) concentrations are employed.

BUFFERED AQUEOUS VANADATE SOLUTIONS

Most biological systems are very sensitive to pH, which accordingly must be carefully controlled.[72] The pH is often kept constant by addition of a buffer. In studies with vanadate the addition of a buffer can be a problem because vanadate has been shown to interact with many buffers (see Fig. 5).[38–40] Unless the reaction conditions are carefully controlled (including choice of buffer, pH, ionic strength and temperature), erratic and irreproducible results can be obtained. Unfortunately, the literature is replete with reports that upon closer examination show significant artifacts. The problem may be further compounded by subtle effects that CO_2 levels, countercations and hydrophobic compounds exert on the various equilibria. The latter are likely to be minor, but nevertheless add to the problems in the reproduction of even the "simple" aqueous oxovanadate equilibria under physiological conditions. Examinations of crude enzyme systems or complex membrane enzymes are particularly risky, because such preparations will often

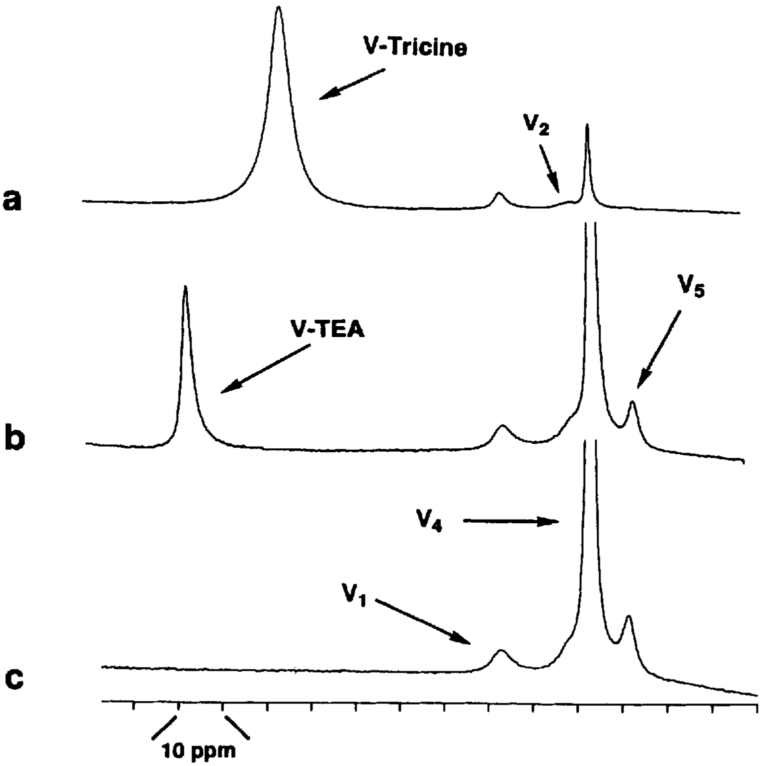


FIGURE 5 ^{51}V NMR spectra (52.6 MHz) of vanadate solutions at pH 7.5 containing 10 mM vanadate, 30 mM Tricine and 150 mM imidazole (a), 10 mM vanadate, 35 mM triethanolamine and 150 mM imidazole (b) and 10 mM vanadate and 150 mM imidazole (c).

contain several compounds that each have the potential to mask or enhance the effect of vanadate.

Enzymes and other biological materials will show varying activities and properties in different buffers, and it is not possible to select one ideal buffer for all enzymes.[72] A suitable buffer involved in the least problematic interactions with vanadate for the particular study should be chosen.

Organic Buffers. Weakly Interacting Buffers

Hepes buffer ([4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]) interacts insignificantly with vanadate and does not appear to affect

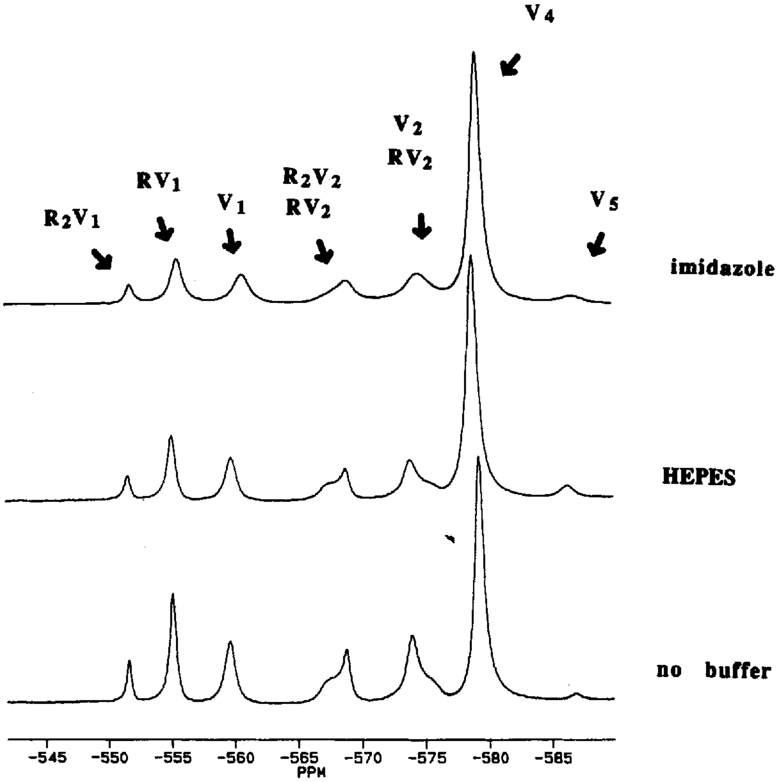


FIGURE 6 ^{51}V NMR spectra (131.5 MHz) of solutions containing 5.1 M ethanol and 10.0 mM vanadate (30% v/v D_2O) at pH 7.9 (±0.1) with no buffer, 50 mM HEPES and 50 mM imidazole. Adapted from Ref. 57.

the stabilities of complexes formed in aqueous solutions.[38,73–75] We have also found that the rates of vanadate exchange reactions and vanadate–ester formation are barely affected by the presence of low concentrations of Hepes.[57] The exchange rates between oxovanadates calculated from 2D ^{51}V EXSY NMR spectra are indistinguishable for solutions containing only vanadate (without buffer) and for solutions containing vanadate and 20 mM Hepes.[57] Solutions containing only vanadate and/or 5 M methanol, with or without buffer, show no significant change in the exchange rates and pathways as measured by 2D ^{51}V EXSY NMR spectroscopy.[57]

As illustrated in Fig. 6 this is not the case for solutions containing 5.1 M ethanol. A solution of 10.0 mM vanadate in 5.1 M ethanol at pH 7.9 (±0.1) was examined in the presence of no buffer, 50 mM Hepes and 50 mM imidazole by 1D and 2D ^{51}V EXSY NMR spectroscopy. The solutions containing Hepes show vanadium resonances with similar linewidths as those recorded in the absence of buffer (Fig. 6). The spectrum recorded in the presence of imidazole shows line broadening of all the resonances (Fig. 6). Since the line broadening is related to the exchange processes here,[76] Hepes is clearly affecting the oxovanadate system less than the imidazole.

Any functionality, including sulfonic acids, has the potential to form complexes with vanadate. Perhaps the best evidence for some type of complex formation between vanadate and Hepes is the slow reduction of vanadium(V) in the presence of Hepes and NADH.[77] Reduction of vanadium(V) by catechols and other derivatives requires prior complex formation,[70] and it seems reasonable to expect this is also the case for the vanadate–Hepes–NADH system.

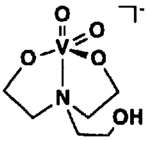
Tris (tris(hydroxymethyl)aminomethane, also abbreviated Trizma) was used extensively in early studies of vanadate–ester complexes and was then shown to form a vanadate–Tris complex.[40] The ^{51}V NMR spectra of protein–vanadate complexes will often have a signal that is attributed to the vanadate–Tris complex.[78,79] When Hepes is not compatible with the enzyme system at hand, Tris is a buffer that, when used in moderation, is appropriate for biological or chemical reactions. This conclusion is based on the observation that a solution containing 20 mM Tris and 1 mM vanadate at neutral pH will contain approximate 0.010 mM vanadate–Tris complex.[40] Assuming the Tris complex does not introduce interfering redox chemistry[77] or other problematic side reactions in the system at hand, the buffer complex can, for many practical purposes, be ignored.

Other buffers such as TAPS (N-tris[hydroxymethyl]methyl-3-aminopropane-sulfonic acid), Mes (2-[N-morpholino]ethanesulfonic acid), Pipes (piperazine-N,N'-bis[2-ethanesulfonic acid]) and acetate also are examples of buffers that can be used in studies with vanadate,[18,31,38,80] even though these buffers do form complexes with vanadate at high concentrations or under other special conditions.[18] The interactions between vanadate and these buffers have been examined less frequently, although the information available suggests that when used in modest concentrations these buffers would be appropriate.[38]

None of the complexes that form between vanadate and the above-mentioned buffers have been characterized structurally. The reaction between vanadate and Tris has been characterized with respect to stoichiometry and thermodynamics.[40] Several complexes were observed in this system, including mononuclear vanadium(V) mono- and diesters and dinuclear vanadium(V) complexes. The formation constant of the monoester (1:1 complex) is 0.51 M^{-1}, and for the 2:2 complex it is about 130 M^{-3}. In the 2:2 complex Tris is suggested to act as a tridentate ligand through two hydroxyl groups and the amine group.[40] The amine group is particularly important in the 2:2 complex, since tris(hydroxymethyl)ethane does not form an analogous 2:2 complex.[40]

Strongly Interacting Buffers

Both triethanolamine and Tricine (N-tris[hydroxymethyl]methylglycine) form stable complexes ((**1**) and (**2**)) in aqueous solution with vanadate (Fig. 5).[39,81,82] Addition to the diethanolamine unit of functionalities including hydroxyl, carboxylate, amine, amide, phosphonates, and sulfates produces various types of ligands; these are often used as buffers or other additives.[39] The series of buffers derived from ethanolamine (the buffers referred to as "good buffers"), which contains at a minimum of a second hydroxyethyl or acetic acid arm, and interacts strongly with vanadate. The affinity of vanadate for such ligands has been determined with respect to the 1:1 complex stoichiometry and its stability in aqueous solution.[39] In some cases, the kinetic properties of the complexes have been described.[81,82] These vanadium(V) complexes are kinetically less labile than oxovanadates and vanadate esters, since they exchange in the course of seconds.[83] Three commonly used buffers in this series include triethanolamine (Fig. 5), Tricine (Fig. 5) and Bicine (N,N-bis[2-hydroxyethyl]glycine), and these should be avoided in biological studies with vanadate.



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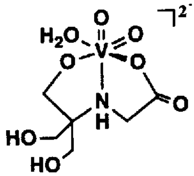

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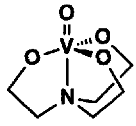


3

The complex formed between vanadate with triethanolamine contains a pentacoordinate vanadium atom with the ligand chelated through three of the four ligand functionalities (**1**) as determined by multinuclear NMR spectroscopy.[84] The pH stability curve of this complex is bell shaped.[39] The structure of **1** in aqueous solutions differs from the vanadium complex characterized by X-ray crystallography **3**. The latter complex was generated in methanol and contains a ligand chelated through all four functionalities to the vanadium in a distorted trigonal bipyramidal geometry.[84,85] Characterization in organic solvents by multinuclear NMR spectroscopy shows that this complex remains intact under these conditions. These studies establish the need to spectroscopically characterize even the simplest species in aqueous solution because the complex in the solid state (and in organic solutions) may be different.

The complex formed between vanadate and Tricine has been examined in detail using potentiometry and multinuclear spectroscopy.[81] Both the intra- and intermolecular reactions of vanadate with Tricine have been examined using 2D ^{13}C EXSY NMR spectroscopy (Fig. 7). No X-ray structure has been reported for this complex, and the initial report favored a 1:1 complex with a pentacoordinate vanadium atom coordinated by one hydroxyl group and the carboxylate group. Further spectroscopic studies of this and other complexes have suggested that the vanadium atom may be octahedral.[82] The formulation shown for **2** is based on the expectation that the electron density on the two ligand O-atoms are different; should the electron densities be identical, the formulation HO–V–OH would be expected. In any event, the studies of these buffers are directly relevant to the coordinational preferences of vanadium(V), given the ligands' structural relationship to the amino acid glycine.

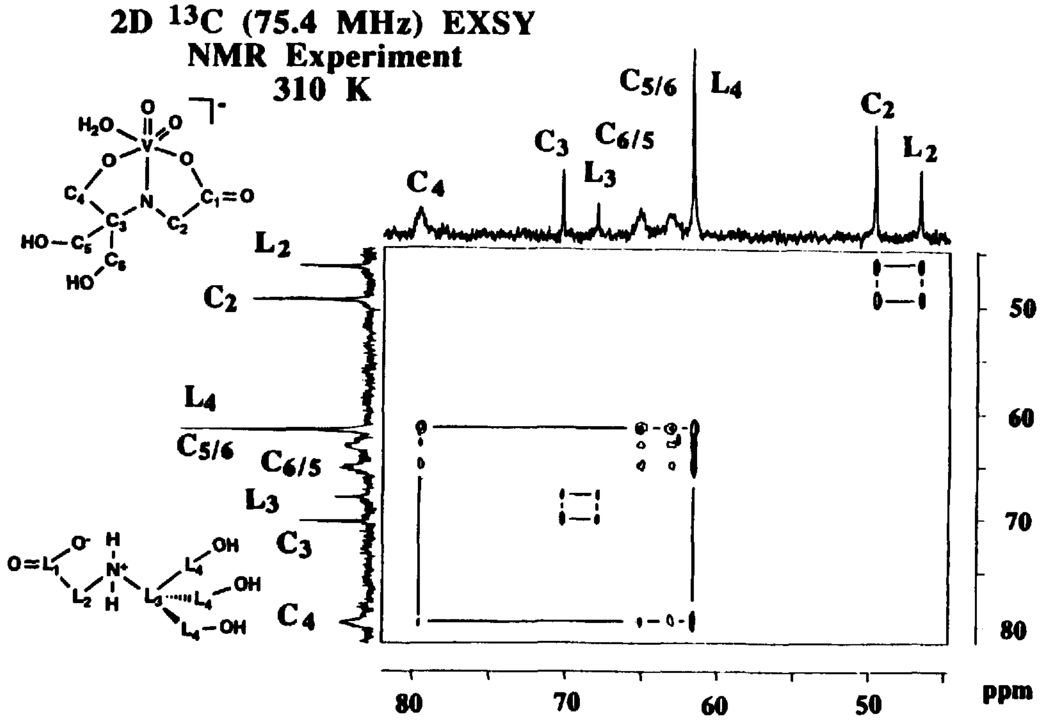


FIGURE 7 2D ^{13}C EXSY NMR spectrum recorded at 75.4 MHz (7.05 T) at 37°C (310 K) of a solution containing 398 mM vanadate–Tricine complex (412 mM total vanadate and 500 mM total Tricine). The intermolecular exchange paths are identified by solid lines. At this elevated temperature, the intramolecular exchange pathways are still visible by off-diagonal cross-resonances. Adapted with permission from Ref. 81 (Copyright 1991 American Chemical Society).

Vanadium(V) complexes with these types of ligands are rather stable so that vanadate solutions of such ligands (around the pK_a of the buffer) will only contain negligible concentrations of free vanadate available to interact with the protein (Fig. 5). Studies carried out in even low concentrations of these buffers (i.e., 20 mM) are likely to mask the true biological response to vanadate.

Naturally occurring ligands such as citrate,[86] glycine[38,87] and glycylglycine[38,87] are also used as buffers. These ligands do interact with vanadate and should be avoided since they form stable complexes with vanadate. The complexes that form between vanadate and citrate contain two vanadium atoms and one citrate ligand, whereas the complex that forms between vanadate and glycylglycine contains one vanadium atom and one ligand. The vanadate–glycylglycine complex is particularly troublesome[88] since, in addition to being thermodynamically stable, it is also much less labile than vanadate oligomers at neutral pH. Consequently, glycylglycine will remove the vanadate from the pool of rapidly converting vanadium derivatives.

Imidazole

Buffers such as Hepes and Tris support a chemical reaction between vanadate and reduced nicotinamide adenosine 5-diphosphate-cofactors (NAD or NADP).[77] Although the reaction is slow, and may be diminished by reducing the contact the sample has with direct light, the production of vanadium(IV) introduces one more vanadium derivative that can interact with the enzyme and introduces errors in ^{51}V NMR quantitation mentioned above. Studies monitoring the absorption of NAD or NADP (or the corresponding NADH or NADPH oxidation) can be carried out using imidazole as a buffer since such solutions show no evidence for the unwanted redox chemistry.[77]

Imidazole is known to bind many metal ions, and presumably the oxidation of substrates and buffers by vanadate is diminished by the complexation of vanadium(V) by imidazole. The ^{51}V NMR spectrum of a vanadate solution still shows the resonances for oligomers at the anticipated chemical shift and no new resonances are observed even at high imidazole concentrations (Fig. 5).[76] However, the concentrations of vanadate oligomers in the presence

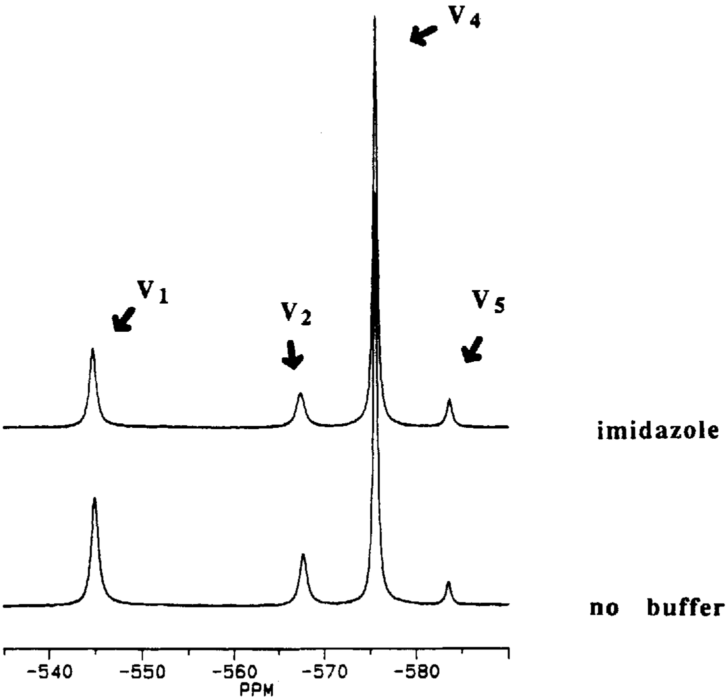


FIGURE 8 1D ^{51}V NMR spectra (131.5 MHz) of solutions containing 10 mM vanadate at pH 8.6 (±0.1) at ionic strength of 0.40 M (KCl) in 17% (V/V) D_2O and 25°C with and without 50 mM imidazole.

of imidazole change and this supports interactions between vanadate and imidazole. Potentiometric studies show that the vanadate–imidazole complex is very weak (Pettersson, unpublished). It is possible that the vanadium–imidazole complexes that do form have a chemical shift similar to that of V_1 or a very broad signal that is difficult to observe or is in rapid exchange with other oxovanadate signals. An observable but very broad signal with a chemical shift different from that of V_1 has been observed for the complex that forms between vanadate and pyridine.[80] The possibility that the vanadate–imidazole complex is in rapid exchange with V_1 is supported by the significant increase in the linewidths of the signals for the vanadate oxoanions in ethanolic solutions.[76] In solutions without high concentrations of alcohol, little change in the linewidths is observed (Fig. 8). Even the presence of up to 5 M methanol does not induce similar changes as observed with ethanol (Fig. 6). Despite the complex formation, imidazole is recommended as a buffer in assays where the reduction of the vanadium must be kept to a minimum.

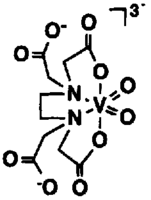
Solutions containing high concentrations of ligands in addition to imidazole (and Tris[24]) and vanadate can give rise to new resonances in the ^{51}V NMR spectrum. For example, the presence of a 200 mM nucleoside in a solution of 100 mM imidazole and 10 mM vanadate will give rise to a new resonance at -480 ppm. This resonance is attributed to a complex containing vanadate, nucleoside and imidazole.[89] Solutions of 10 mM vanadate and 100 mM imidazole in the presence of 50% (v/v) acetone will also exhibit a new signal in this ppm range; this signal is presumably due to a complex containing vanadate, imidazole and acetone (Crans and Shin, unpublished). New species of this type only form at high concentrations of imidazole and organic ligand and are not anticipated to give rise to problems in solutions containing modest concentrations of imidazole and other compounds. In any event, a greater knowledge of how vanadate interacts with imidazole is desirable, and some recent efforts have been successful in this area when the vanadium is stabilized by an additional ligand.[90,91]

Inorganic Buffers

Some enzymes function very well in inorganic buffers such as phosphate, borate and carbonate. Vanadate forms anhydride-like complexes with phosphate,[54,92] and presumably also with carbonate and borate. The interaction with phosphate is much stronger than the interaction with carbonate, although the stability of the vanadate–phosphate complex ($K_f = 64\ M^{-1}$)[92] cannot compare with the vanadate complexes with triethanolamine or Tricine. Given the 1:1 vanadate–phosphate complex that forms in such solutions, conducting vanadate studies in a phosphate buffer is not to be recommended. Indeed, the species formed between vanadate and the buffer may be the active component in the mixture. The vanadate-dependent NADH oxidation stimulated by plasma membranes is an example of a biological response that is *only* observed in phosphate buffers.[67,93] This plasma membrane reactivity is intriguing, and the dependence on the presence of phosphate is striking. Perhaps efforts in characterizing derivatives that form under these assay conditions would assist further studies in this area.

Other Assay Components

Enzyme preparations will often contain additives to increase protein stability.[72] Compounds such as EDTA and citrate are often added for protection against heavy metal ions and their destructive effects. Compounds such as DTT (1,4-dithio-L-threitol) are added to maintain a reducing environment and prevent the oxidation of the enzyme. Metal ions are often added to assay solutions to enhance enzyme activity or increase protein stability (by maintaining the protein in a stable conformation). Compounds such as glycerol, ethylene glycol or other proteins (bovine serum albumin) are often added to prevent either protein dissociation or association with glass or plastic containers.



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EDTA forms a very stable 1:1 complex **4** with vanadate even though both are anions. The complex is recognized as a complex between the anion EDTA and the cation VO_2^+ even though no observable concentrations of VO_2^+ exist in the neutral pH range where the complex is also observed. The solid state structure[94,95] corresponds to the solution structure of this compound, **4**.[96] The complex is extremely stable and will form even at micromolar concentrations of both ligand and vanadate. Formation and hydrolysis of this complex has furthermore been characterized kinetically using stopped flow[64] and 2D EXSY NMR spectroscopy.[83] Substitutes for EDTA such as EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid) are less effective in complexing vanadate, and if possible may serve as substitutes for

EDTA.[38] In any event, the presence of EDTA in the assay solution will reduce the free vanadate concentration and should be taken into consideration in the event EDTA is present in the protein preparation.

DTT rapidly reduces vanadate to vanadyl cation. 2-Mercaptoethanol (β-mercaptoethanol) is less effective in reducing vanadate but also readily accomplishes this task. The details of these reductions have not been examined; however, it is known that the reactions are very pH sensitive and slower at alkaline pH.[66] If possible thiols should be avoided as components in the assays. Low concentrations of thiols in the enzyme preparation will be sufficiently diluted in the enzyme assays to generate submicromolar concentrations in the enzyme assay. The total vanadate concentrations in the assay will therefore barely be affected. Unless these levels of vanadyl cation have potent effects on the enzyme system under study, such thiol levels can be ignored. The formation of low concentrations of vanadyl cation in the assay solution is of concern in such studies, not the quantization of oxovanadates.

Ethylene glycol,[97] glycerol (Crans, unpublished) and bovine serum albumin[38,98] interact with vanadate. However, the formation constants are several orders of magnitude smaller than those for the EDTA, Tricine or triethanolamine complexes. As long as additives are maintained at sufficiently low concentrations so that negligible concentrations of complexes will form in an assay solution, they will not significantly affect the concentrations of vanadium species. The major question in such studies is whether these additional vanadium complexes will affect enzyme activity. Although no detailed studies have yet emerged, preliminary results suggest that the EDTA complex may not be innocuous with all enzymes (Crans, unpublished).

In summary, no buffer or assay component can be characterized as not interacting with vanadate. A suitable assay will be one chosen to minimize the interactions of buffer and other assay components with vanadate and maximize the conditions for the enzyme activity.

EXPERIMENTAL APPROACHES TO STUDIES WITH LABILE VANADATE DERIVATIVES

Next, some pitfalls encountered when working with labile vanadate derivatives in the presence of enzymes and/or other biological systems are described. First, we will turn to experimental issues concerning preparation of the sample solutions.

A reasonably concentrated stock solution of colorless vanadate (such as 25 mM) will contain a mixture of labile oxovanadates in a composition dependent on pH, temperature and ionic strength.[99] The original concentrations of each vanadate oligomer in the stock solution are irrelevant because the new equilibrium is established within milliseconds of adding the vanadate to the assay solution (at $pH > 6.5$). In the presence of a buffer or other components the time required for establishing equilibrium is dependent upon the interaction of these other components with vanadate. We do not recommend addition of buffers or other compounds to the stock solution; such addition could introduce an additional complication because many ligands react more slowly with vanadate. Solutions with additional ligands may require longer periods to reach equilibrium (including formation of the slow complexes such as the vanadate–peptide derivatives). This added complication must be remembered. In most cases, such problems can be eliminated if a few control assays are conducted with solutions that have had longer incubation treatments.

In the event that the pH of the stock solution needs to be adjusted, the addition of both acid and base will change the concentrations of the oxovanadates. The addition of acid often produces the orange vanadate decamer. Since the vanadate decamer hydrolyzes only slowly above pH 7, the presence of this derivative will deplete the more labile oxovanadates. One should therefore either remake the solution or heat the material to facilitate the hydrolysis of the orange decamer. Heating the solution can cause problems if other compounds are present which react with vanadate slowly or in a temperature-dependent manner.[88]

Given the rapid exchange rates between the vanadate oxoanion and vanadate esters,[34,76] it is not possible to isolate each species for examination. Accordingly the activity measurements must be carried out on the equilibrium mixture of species. Enzyme reactions occur on a very fast time scale, and oxovanadates with millisecond lifetimes are likely to be distinguished by the enzyme. Experimental techniques that measure enzyme reaction progress include UV spectroscopy or fluorescence spectroscopy, but other techniques using radiolabeled substrates are appropriate. These measurements should be obtained preferentially in conjunction with ^{51}V NMR studies determining the populations of oxovanadates under the assay conditions (including the identical buffer, salt concentrations, assay additives and temperature).[30]

Binding studies of vanadate to proteins can be carried out using various techniques. Interaction of vanadate with enzymes such as 3-phosphoglyceratemutase,[100,101] phosphoglucomutase[102,103] and superoxide dismutase[36] has been characterized using multinuclear NMR spectroscopy. In all these cases the binding studies were conducted in the presence of excess vanadate recognizing the rapid exchange between the vanadate oligomers and the protein–vanadate complex. Some enzyme–vanadate complexes, such as the myosin-ADP-vanadate complex, do not dissociate rapidly.[104,105] In the myosin-ADP-vanadate complex a conformational change has occurred that prevents rapid release of the vanadate. Should the enzyme–vanadate complex be of this type, a vanadium-containing complex may indeed be isolated by the equilibrium dialysis method. However, the enzyme–vanadate complex is not likely to be isolated unless the off-rate for the vanadate is slow. Similar considerations must be satisfied if a covalent vanado-enzyme intermediate is to be isolated.

Careful quantification of changes in enzyme reaction rates with various populations of vanadate derivatives will allow correlation of the observed changes in rates with concentration of one or two labile vanadate derivatives. Although it is virtually impossible to prove which species in an exchanging mixture is actually the active form, a reasonable correlation can typically be made for one or two vanadate derivatives. The correlation is often based on the elimination of other reasonable possibilities.

QUANTIFICATION OF LABILE OXOVANADATES

Solutions containing millimolar vanadate are in a dynamic equilibrium, and each of the oligomers are related as shown in Eqs. (3) to (6).

$$2\,V_1 \overset{K_{12}}{\rightleftharpoons} V_2; \quad K_{12} = [V_2]/[V_1]^2, \tag{3}$$

$$4\,V_1 \overset{K_{14}}{\rightleftharpoons} V_4; \quad K_{14} = [V_4]/[V_1]^4, \tag{4}$$

$$5\,V_1 \underset{}{\overset{K_{15}}{\rightleftharpoons}} V_5; \quad K_{15} = [V_5]/[V_1]^5, \tag{5}$$

$$2\,V_2 \underset{}{\overset{K_{24}}{\rightleftharpoons}} V_4; \quad K_{24} = [V_4]/[V_2]^2. \tag{6}$$

The dimerization of vanadate monomer (3) is determined by the constant K_{12}. K_{12} is experimentally obtained from a series of ^{51}V NMR spectra recorded at different total vanadate concentrations and obtaining the slope from a plot of $[V_2]$ as a function of $[V_1]^2$. Since the structures of some of the oxovanadates are not known with certainty, the number of water molecules generated in the reaction is not known. Thus, we make no attempt to balance the equations with water molecules. Assuming the water content is in great excess and not changing significantly, this simplification is not likely to impact our calculations. Furthermore, the simplification provides a vehicle in which at constant pH all the different protonation states in the solutions do not have to be considered individually since the concentration of each species is related to the total oxoanion concentration. The constants obtained, however, should be treated as H^+-dependent equilibrium constants and not true formation constants. In this treatment we have also ignored the activity coefficients because in studies carried out at high ionic strength we assume small changes in vanadate, ligand, substrate or enzyme concentrations will not significantly affect the overall ionic strength. The impact of the activity coefficients on K_{12} will then be constant under the conditions used. All these assumptions and considerations are also made for the equilibria shown in (4) to (6).

Equations (3) to (6) define the H^+-dependent formation constants K_{12}, K_{14}, K_{15}, and K_{24} under the conditions of the assay. The experimentally obtained constants (Table I) are essential tools for analysis of the observed enzyme rates obtained in the kinetic studies, and their measurement should be repeated when variations in assay conditions are introduced. From Table I it is seen that the H^+-dependent formation constants will change by a factor of 2 to 3 by changing buffer, ionic strength and various assay additives. Differences up to a factor of 1000 are observed when varying pH. In any event, the constants shown in Table I can serve as a pre-

TABLE I

H^+-dependent formation constants of V_2, V_4 and V_5 under various assay conditions (298 K).

Conditions	$K_{12}{}^a/M^{-1}$	$K_{14}{}^b/M^{-3}$	$K_{15}{}^c/M^{-4}$	$K_{24}{}^d/M^{-1}$	Reference
20 mM Tris·Cl, pH 8.0, 1.0 M KCl	180	1.1×10^9		0.34×10^4	92
20 mM Tris·Cl, pH 8.0, 0.50 M KCl	260	1.0×10^{10}		1.5×10^4	92
20 mM Tris·Cl, pH 7.35, I(KCl) = 1.0 M	560	1.0×10^{10}		3.2×10^4	106
20 mM Hepes, pH 7.5, I(KCl) = 1.0 M	480	1.0×10^{10}		4.3×10^4	75
20 mM Hepes, pH 8.0, I(KCl) = 1.0 M	330	2.4×10^9		2.2×10^4	75
100 mM imidazole, pH 7.1, 3.3 mM $MgCl_2$	260	0.93×10^9		1.4×10^4	30
100 mM N-ethylmorpholine, pH 7.8 100 mM KCl, 3.3 mM $MgCl_2$	170	0.65×10^9		2.3×10^4	30
50 mM imidazole, pH 7.1, 400 mM KCl	460	6.0×10^9	9.9×10^{11}	2.8×10^4	32
50 mM TAPS, pH 9.0, 200 mM KCl, 5.0 mM semicarbazide	50	3.8×10^6	2.1×10^8	1.4×10^3	31
50 mM TAPS, pH 9.0, 200 mM KCl, 1.0 M ethanol	60	7.1×10^6	2.1×10^8	2.0×10^3	18
0.20 M acetate (Na^+) pH 5.5	110	6.5×10^8		5.5×10^4	107

[a] $K_{12} = [V_2]/[V_1]^2$.
[b] $K_{14} = [V_4]/[V_1]^4$.
[c] $K_{15} = [V_5]/[V_1]^5$.
[d] $K_{24} = [V_4]/[V_2]^2$.

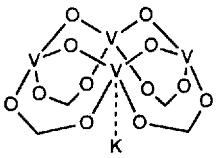
liminary estimate of the expected concentrations of vanadate oligomers at equilibrium.

STRUCTURAL CONSIDERATIONS: OXOVANADATE INTERACTIONS WITH ORGANIC LIGANDS AND ENZYMES

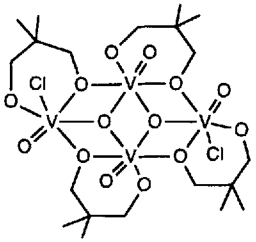
The X-ray structure of two organic vanadate derivatives, the vanadate–uridine complex and the vanadate–glucose-1-phosphate, with their respective enzymes, ribonuclease[108–110] and phosphoglucomutase,[111] are known. Currently, there are no high resolution X-ray data available describing the interaction of oxovanadates or other oxometalates with proteins. The early study of adenylate kinase with V_{10} was not refined to the point of examining the interactions of the anion with specific residues.[112,113] Studies probing the specific interactions in binding site by alternative techniques such as representative model systems are therefore desirable.

In the past five years, the characterizations of vanadium(V) complexes with amino acids and peptides have been of interest.[38,87,114] Most of these derivatives are difficult to crystallize and various stabilizing groups are added to obtain crystals that can be characterized.[90,91] The solution studies that have been carried out suggest the side group in the amino acid is actively involved in the complex formation. In aqueous solutions V_2 forms several derivatives with hydroxyl groups.[54,73] Structural characterizations of several dinuclear oxovanadium(V) derivatives with organic ligands have demonstrated several structural possibilities, including vanadium atom connectivity through a V–O–V bridge[26] or a V_2O_2 unit.[115–119] The V_{10} has also been found to form complexes in aqueous solution (Tracey, unpublished) and elsewhere.[120]

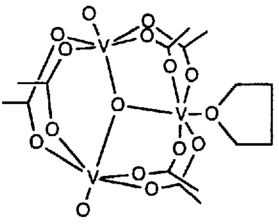
As described in the accompanying paper, the V_4 unit is of particular interest because of its reactivity and potential physiological significance. Although no precedents for structures related to the aqueous V_4 [52,53] or V_5 have yet been reported, new tetranuclear vanadium(V) complexes have been characterized by X-ray spectroscopy in the past few years.[121] In Fig. 9 two examples are shown,[27,122,123] one with carboxylates as the organic ligands (**9a** and **9b**), and the other with bidentate alkoxide ligands (**9b**). The



9a



9b



9c

FIGURE 9 Schematic drawings (Ref. 121) of tetrameric and trimeric oxovanadates and their interactions with organic ligands. $[V_4O_7(OH)(O_2CR)_4K]^+$ (Ref. 122) and $[V_4O_8(O_2CR^1)_4NO_3]$ (**9a**) (Ref. 124), $[VOCl(OCH_2C(CH_3)_2CH_2O)]$ (**9b**) (Ref. 27) and $[V_3O_3(CH_3COO)_6OC_4H_8]$ (**9c**) (Ref. 45).

organic ligands are incorporated in the framework of vanadium atoms coordinated in an octahedral arrangement.[123] Complex **9a** is shaped like a cup, but, in contrast to the aqueous V_4 (Fig. 1), all oxygen atoms in the eight-membered ring are in one plane and the vanadium atoms in another. The structure found for **9b** is different in the sense that two of the metal atoms have been pushed together, flattening the ring such that intramolecular bonds become stronger. These two geometric arrangements, combined with that shown for V_4 (Fig. 1), illustrate the molecular flexibility such systems exhibit and the types of interactions that may be important in vanadium–protein interactions. Although no examples have yet been reported of true peptide–oxovanadate complexes, the molecules described above illustrate that it would be premature not to consider inner-sphere complexes between the proteins and V_4.

In addition to the V_4 unit, other examples of oxovanadium alkoxides and carboxylates have been prepared and characterized recently. Rehder reported an interesting trimeric unit with bridging acetate molecules (**9c**), which demonstrates that oxometalates containing an uneven number of metal ions also can form.[45] Analogous complexes[125,126] suggest that this type of complex may not be as rare as expected even if a trimer is not a major species in aqueous solution. Hexameric structures supported by either alkoxides or carboxylates also form readily.[124,127] Hexamers can have very different types of oxoanion backbones depending on the method of preparation and isolation.

An example of an outer-sphere interaction between a simple peptide and an oxovanadate has also been characterized.[128] We were able to crystallographically describe the interactions between V_{10} and gly-gly, and find that the structure contains an intricate pattern of hydrogen bonding in the crystal. Disruption of this hydrogen bonding network is likely to result in significant changes in the interactions between peptide and oxoanions. Indeed, altering one glycine residue to a histidine residue (i.e., gly-his) resulted in a material with different stoichiometry! A network of hydrogen bonding interactions was also recently reported in the X-ray structure of a vanadium–histidine-N-(2-oxinaphthalidene) complex showing that hydrogen bonding plays an important role even in vanadium complexes of amino acids.[90] The latter complex is perhaps particularly interesting because the molecule is hydrogen-bonded in a tetrameric unit.

In summary, these studies show that the interactions between oxovanadates and simple peptides are very sensitive to the amino acid sequence. Such interactions may be essential to the crystallization of these materials.

CONCLUDING REMARKS

Vanadate is recognized as a structural and electronic analog of phosphate. It is less commonly recognized that vanadate can be both ground state analog and a transition state analog for phosphate. Structures of higher oxovanadates (V_2, V_4 and V_5) in aqueous solutions are not known with certainty since these compounds are exchanging with each other and with V_1 on a millisecond time scale. The rapid chemical exchange complicates biological studies since none of the oxovanadates can be isolated and tested separately. Enzyme activity and other biological properties must be measured in the presence of equilibrating mixtures, and the active oxovanadate is identified by correlating effects and oxovanadate concentrations. In general, Hepes is the best buffer for studying vanadate chemistry and the effects of vanadate on enzyme reactions not involving redox chemistry. Imidazole, however, is the best buffer when interactions of vanadate with enzymes are monitored by the redox reaction of NADH/NAD or NADPH/NADPH. Buffers that should be avoided include triethanolamine, Tricine, Bicine, phosphate, citrate and gly-gly. The complexes of the three former ligands with vanadate illustrate the need for spectroscopic studies in aqueous solutions since the solid state structure may very well be different from the solution structure. Assay additives such as EDTA, DTT and bovine serum albumin can mask and alter the biological effects of vanadate. The presence of DTT can be particularly problematic because vanadyl cation is introduced into the system. Quantitation of oxovanadates shows that pH is the major factor changing the oxovanadate populations, whereas buffer, ionic strength and other assay components typically affect the H^+-dependent formation constants by a factor of 2 to 3. Appropriately designed biological experiments allow the application of this powerful group of oxoanions to study enzyme structure, anion interactions, and mechanism. Protein–anion interactions are the topic of the accompanying *Comments* article.

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