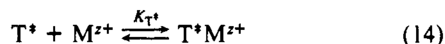
$$\frac{k_{obs}}{k_0} = \frac{1 + (k'_{cat}/k_0)K_{MeO}[M^{z+}]}{(1 + K_{AcOAr}[M^{z+}])(1 + K_{MeO}[M^{z+}])} \quad (12)$$

$$\frac{k_{obs}}{k_0} = \frac{1 + (k''_{cat}/k_0)K_{AcOAr}[M^{z+}]}{(1 + K_{AcOAr}[M^{z+}])(1 + K_{MeO}[M^{z+}])} \quad (13)$$

at constant ionic strength and relate $k_{obs}/k_0$ to $[M^{z+}]$ by means of expressions containing three unknown parameters. Clearly, the two mechanisms of eq 10 and 11 are operationally indistinguishable because the corresponding kinetic equations are. It is also clear that the existence of two distinct transition states containing the metal ion cannot be operationally verified. Hence, the most reasonable assumption, actually the only viable one, is that there is only one transition state $T^{\ddagger}M^{z+}$ attained through either step 10 or 11.[17] The latter are, in turn, mutually exclusive, in that only one of them is necessary to populate the transition state.[18]

Use of transition-state theory avoids the above ambiguities. The basic assumption of the existence of an equilibrium between reactants and transition state implies that the transition state $T^{\ddagger}$ is in equilibrium with the transition state $T^{\ddagger}M^{z+}$. This formal equilibrium is understood in the sense that $T^{\ddagger}$ and $T^{\ddagger}M^{z+}$ appear simultaneously and in proportions that are governed by $[M^{z+}]$ and by the equilibrium constant $K_{T^{\ddagger}}$ (eq 14).[19] Remembering that



$$T^{\ddagger} + M^{z+} \overset{K_{T^{\ddagger}}}{\rightleftharpoons} T^{\ddagger}M^{z+} \quad (14)$$

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$k_{obs}$ is operationally defined in terms of primitive concentrations of the reactants (eq 2), the transition-state theory expression of $k_{obs}$ is given by eq 15, where $c_{T^{\ddagger}} = [T^{\ddagger}] + [T^{\ddagger}M^{z+}]$ is the primitive

$$k_{obs} = \frac{kT}{h}\frac{c_{T^{\ddagger}}}{c_{AcOAr}c_{MeO}} \quad (15)$$

$$k_0 = \frac{kT}{h}\frac{[T^{\ddagger}]}{[AcOAr][MeO^-]} \quad (16)$$

$$\frac{k_{obs}}{k_0} = \frac{1 + K_{T^{\ddagger}}[M^{z+}]}{(1 + K_{AcOAr}[M^{z+}])(1 + K_{MeO}[M^{z+}])} \quad (17)$$

concentration of the transition state. When $[M^{z+}] = 0$, eq 15 reduces to eq 16. Combining eq 15 with eq 16 and with the expressions for the three equilibrium constants involved, eq 17 is obtained, which is clearly of the same form as eq 12 and 13. It is also apparent that $K_{T^{\ddagger}}$ is related to the relevant rate and equilibrium constants through eq 18 and 19. Our preference for

$$K_{T^{\ddagger}} = (k'_{cat}/k_0)K_{MeO} \quad (18)$$

$$K_{T^{\ddagger}} = (k''_{cat}/k_0)K_{AcOAr} \quad (19)$$

eq 17 is due to the fact that it bears no relation whatsoever to the microscopic or detailed mechanism by which the transition state is attained.[20] The quantity $K_{T^{\ddagger}}$ is a direct measure of transition-state stabilization brought about by the metal ion, to be compared with the corresponding stabilization of the reactant state, as measured by $K_{AcOAr}$ and $K_{MeO}$.

**Registry No. 1**, 122-79-2; **2**, 123903-47-9; **2** (alcohol), 57722-03-9; $Me_4NOMe$, 5568-08-1; KBr, 7758-02-3; $SrBr_2$, 10476-81-0; $BaBr_2$, 10553-31-8; NaBr, 7647-15-6; $Sr(OMe)_2$, 3214-53-7; $Ba(OMe)_2$, 2914-23-0.

(20) Reference 8, pp 116-117.

# Vanadate Tetramer as the Inhibiting Species in Enzyme Reactions in Vitro and in Vivo

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**Abstract:** Tetrameric vanadate polyanion inhibits 6-phosphogluconate dehydrogenases from human, mammalian, yeast, and bacterial sources. The inhibition by a vanadate mixture containing monomer, dimer, and tetramer was determined by measuring the rates of 6-phosphogluconate oxidation and NADP (or NAD) reduction catalyzed by 6-phosphogluconate dehydrogenase. The inhibition by vanadate is competitive with respect to 6-phosphogluconate and mixed or noncompetitive with respect to NADP or NAD. $^{51}$V NMR spectroscopy was used to directly correlate the inhibition of vanadate solutions to the vanadate tetramer. The measured inhibition constants with respect to 6-phosphogluconate for the tetramer are 0.078 mM for the human erythrocyte enzyme, 0.063 mM for the sheep liver enzyme, 0.013 mM for the yeast enzyme, and 0.24 mM for the *Leuconostoc mesenteroides*. The observed inhibition of 6-phosphogluconate dehydrogenase by vanadate tetramer is the *first enzymatic activity* observed of this polyanion. Our observations suggest the vanadate tetramer will be a potent inhibitor to other organic phosphate converting enzymes and preliminary results confirm this expectation. The vanadate tetramer may be an important species when considering the mechanism by which vanadium acts in biological systems in vitro and in vivo.

As a trace element in plants and mammals, vanadium has beneficial properties at low concentrations.[1] At high concentrations vanadium becomes toxic.[1] The action of vanadium is likely to vary with the oxidation state of the metal, although little is currently understood about the mechanisms of action of vanadium in mammals or plants. The recent discoveries of vanadium-requiring enzymes illustrate the increasing interest in this element.[2]

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Vanadium(V) is the most stable form of vanadium at neutral pH in the presence of oxygen.[1] Reducing environments readily convert vanadate to vanadium(IV). In mammals vanadium is generally believed to be found in oxidation state IV intracellularly, and vanadate is gradually reduced by endogenous reducing agents after injection of a solution of vanadate into the blood stream. However, in view of the presence of some free oxygen in the blood vanadyl cation may in part be reoxidized to vanadate.[3a] The oxidation states of vanadium in blood have been the center of considerable controversy in the last decade.[3b] Tunicate blood cells are now believed to contain vanadium in oxidation state +3, +4, or both depending on the species.[3c] In-depth understanding of the biological activities of vanadium is likely to require an understanding of the chemistry and biochemistry of both vanadium(IV) and -(V).

We have embarked on a research program with the intent of examining possible mechanisms by which vanadium(V) can act.[4-6] Currently, we have evidence for a new, general mechanism by which spontaneously generated vanadate derivatives can act as substrates for enzymes converting organic phosphate substrates.[4] As part of these studies the effects of vanadate on the enzyme-catalyzed conversion of sugar phosphates to their respective products had to be determined. Enzymes converting sugar phosphates are often inhibited by phosphate and organic phosphate analogues in a competitive inhibition pattern with respect to the sugar phosphate substrate. Vanadate monomer acts as a phosphate analogue, but in addition, vanadate has the potential to act through oligomeric species and by oxidation-reduction reactions.[1] Most of the reported biological effects of vanadium are attributed to monomeric vanadium as vanadate (vanadium(V)) or as the vanadyl cation (vanadium(IV)). Although many researchers do not specify the active vanadate species, their studies are often conducted at such low vanadium concentrations that the major vanadate species in solution is monomeric vanadate. The oligomeric vanadate species that have been shown to have enzymatic activity include the vanadate dimer and decamer. The vanadate dimer has been shown to inhibit phosphoglycerate mutase,[7a] and the decameric vanadate has been shown to inhibit $Ca^{2+}$-ATPase,[7b] muscle phosphorylase,[7c] adenylate cyclase,[7d] hexokinase,[7e] and phosphofructokinase.[7e] The decamer is the only vanadate oligomer that is structurally well characterized because solution studies show the X-ray structures have the same characteristics as the solution structure.[8]

6-Phosphogluconate dehydrogenase (6-PGDH) catalyzes the oxidation of 6-phosphogluconate (6-PG) to D-ribulose 5-phosphate with the production of an NADPH.[9] 6-PGDH is the second enzyme in the pentose phosphate pathway (i.e., the phosphogluconate pathway or the hexose monophosphate shunt), and it has been suggested that 6-PGDH is involved in the regulation of the pentose phosphate pathway.[10] The pentose phosphate pathway is a multifunctional pathway specialized to carry out four main activities depending on the organism and the metabolic state of the cell. One function is the conversion of hexoses into pentoses, particularly D-ribose 5-phosphate, required in the synthesis of nucleic acids. A second function occurs in tissues such as liver and cells such as erythrocytes and involves the generation of NADPH in the extramitochondial cytoplasm.

Vanadate is known to affect glycolysis both in vitro and in vivo,[11] but the effects of vanadate on the pentose phosphate pathway or isolated enzymes in this metabolic pathway have not been examined. The 6-PGDH enzymes from *Leuconostoc mesenteroides*,[12] from *Torula* yeast,[13] from sheep liver,[14] and from human erythrocytes[15] have a wide array of similarities and differences in amino acid sequences, Michaelis-Menten constants, required cofactors, and reaction mechanisms.[16-19] The nature of the 6-PG and NADP binding site in the sheep liver 6-PGDH is known since an X-ray structure of this enzyme has been described to 2.6-Å resolution.[16] The active site in the sheep enzyme is expected to be very similar to the yeast enzyme since these two enzymes show a high homology of the active site amino acid sequence.[16] Despite more complex kinetics, the 6-PGDH in human erythrocytes[17] appears to be similar to the sheep enzyme or yeast,[19] whereas the 6-PGDH from *L. mesenteroides* is considerably different.[12] For example, the amino acid sequence homology is low and the *L. mesenteroides* 6-PGDH enzyme prefers NAD as a cofactor over NADP, which is the cofactor preferred by the other three enzymes. Because of their similarities the 6-PGDH enzymes from sheep liver, yeast, and human erythrocytes are expected to show similar activity toward vanadate, whereas the 6-PGDH from *L. mesenteroides* is considerably different and therefore may show pronounced changes when interacting with vanadate. We plan to examine whether the specific details in the enzyme reaction or enzyme properties are important for the interaction of the vanadate with the protein. Since the *L. mesenteroides* enzyme is considerably different, the interaction of this enzyme with vanadate will, if similar to the yeast, sheep liver, and human erythrocyte enzymes, suggest that this type of interaction may be of a general nature.

In this work we present the *first evidence for enzymatic activity of the vanadate tetramer*. We find that the vanadate tetramer is an inhibitor for the 6-PGDH enzymes isolated from yeast, *L. mesenteroides*, sheep liver, and human erythrocytes, and we have preliminary evidence that such activity is also found with other enzymes including glucose-6-phosphate dehydrogenase.[4] Tetrameric vanadate is usually thought of as a chemically inert and inactive form of vanadate. The tetramer is generally believed to have a cyclic structure,[8,20] although recent evidence was presented in favor of an adamantane-like structure.[21] Our results show the tetrameric vanadate species has a biological activity in vitro, but the tetramer could be, at high—possibly toxic—vanadium levels, an active form of vanadium in vivo.

## Experimental Section

**Reagents and Enzymes.** The reagents used in this work were all reagent grade. The water was distilled and further deionized on an anion-exchange column. Vanadium pentoxide was purchased from Fisher

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**Table I.** Summary of Equilibrium Constants for Vanadate Monomer, Dimer, and Tetramer Formation

| enzyme assay | $10^{-2}K_{12}$, $M^{-1}$ | $10^{-9}K_{14}$, $M^{-1}$ | $10^{-4}K_{24}$, $M^{-1}$ |
|---|---|---|---|
| *Torula* yeast[a] | 2.6 (±0.2) | 0.93 (±0.09) | 1.4 (±0.1) |
| sheep liver[b] | 1.7 (±0.2) | 0.65 (±0.06) | 2.3 (±0.2) |
| human erythrocytes[c] | 1.7 (±0.2) | 0.65 (±0.06) | 2.3 (±0.2) |
| *L. mesenteroides*[d] | 3.8 (±0.4) | 1.7 (±0.2) | 1.2 (±0.1) |

[a] 0.1 M imidazole, 3.3 mM magnesium chloride, pH 7.1. [b] 0.1 M *N*-ethylmorpholine, 0.1 M potassium chloride, 3.3 mM magnesium chloride, pH 7.8. [c] 0.1 M *N*-ethylmorpholine, 0.1 M potassium chloride, 3.3 mM magnesium chloride, pH 7.8. [d] 0.1 M imidazole, 0.05 M potassium chloride, 2.0 mM magnesium chloride, pH 7.1.

Scientific Co. The enzymes and biochemicals were purchased from Sigma.

**Kinetic Measurements.** Spectrophotometric determinations of rates of oxidation were obtained at 25 °C and 340 nm on a Lambda 4B Perkin-Elmer double-beam spectrophotometer equipped with a constant-temperature cell. The rate determinations for the human erythrocyte 6-PGDH were obtained on a HP 8452A Diode Array UV/Vis spectrophotometer. The assays for each enzyme were modified from known literature procedures and are described below. At low substrate concentrations the rates were determined in duplicate or triplicate. Controls were run before and after each series of constant inhibitor concentration and the rates were corrected if necessary. After each enzyme series was completed, a series was performed in which the 6-PG concentration was held constant and the inhibitor concentration was varied. These runs served as controls to check that the enzyme activities were constant during the various kinetic runs. The protein concentrations were quantified by the method of Lowry.

**$^{51}$V NMR Spectroscopy.**[5] 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. The $^{51}$V NMR spectra were recorded on a $^1$H 200-MHz Bruker WPSY (4.7-T) spectrometer. We typically used spectrum widths of 8064 Hz, a 90° pulse angle, an accumulation time of 0.2 s, and no relaxation delay. No change in integration of various peaks was observed if the relaxation delay was increased; the $T_1$'s were approximately 5–10 ms and were measured by using the saturation recovery method. The chemical shifts are reported relative to the external reference standard $VOCl_3$ (0 ppm). In practice we used a solution (pH 7.5) containing the vanadate–diethanolamine complex as a reference (−488 ppm).[5] The NMR samples were prepared as described for the assay with the exception that NADP, NAD, or 6-PG were omitted. It was shown experimentally that omission of cofactors and substrate would not noticeably affect the distribution of vanadate species since the results were within a 5% experimental uncertainty. The $^{51}$V NMR spectra were recorded with external lock.

Vanadate solutions contain complex mixtures of mono- and oligovanadates that vary with ionic strength, concentration, pH, and temperature. The concentrations of vanadate species were determined in assay solutions that contained a known amount of total vanadium. These samples allowed the calculation of the concentration of each vanadate species from the mole fraction and the integrated NMR sectrum. Vanadate monomer (−546 ppm), dimer (−566 ppm), and tetramer (−572 ppm) are related as shown in reactions 1–3. The correlation coefficients

$$2V_1 \overset{K_{12}}{\rightleftharpoons} V_2 \quad (1)$$

$$4V_1 \overset{K_{14}}{\rightleftharpoons} V_4 \quad (2)$$

$$2V_2 \overset{K_{24}}{\rightleftharpoons} V_4 \quad (3)$$

were 0.99 or above unless noted otherwise. The results from the $^{51}$V NMR spectra were therefore used to calculate the apparent ($[H^+]$-dependent) equilibrium constants for the oligomerization reactions occurring under the conditions where the *Torula* yeast enzyme, the sheep liver enzyme, the human erythrocyte enzyme, and the *L. mesenteroides* enzyme were studied. These results are summarized in Table I.

**Data Analysis.** The rates were measured from linear portions of the rate profile, and doubling the enzyme concentration produced double rates. The kinetic data were analyzed by Cricket Graph, a program designed for statistical manipulations on the Apple computer. We used both Lineweaver–Burk and Eisenthal–Cornish-Bowden types of plots to determine the Michaelis–Menten parameters. For convenience only Lineweaver–Burk plots are illustrated in this paper. The $K_i$'s were determined by plotting the slopes obtained from the Lineweaver–Burk plots against the concentrations of vanadium atoms found as tetrameric vanadate. Dixon plots are used for illustrations in this paper and show the $K_i$'s as intercepting points. We estimate that there is no higher than a 30% deviation on our constants (they were reproducible within 10%), and the experimental uncertainty is on the order of 5–10%. The correlation coefficients were usually 0.99 or above, except for the human erythrocyte enzyme where the correlation coefficients were 0.98 or above.

**Specific Enzyme Assays. (A) 6-Phosphogluconate Dehydrogenase from *Torula* Yeast.** The enzyme was assayed as described previously with minor modifications.[19,5] The assay solutions contained 0.1 M imidazole (pH 7.1), 3.3 mM magnesium chloride, 0.44 mM NADP, and from 0.040 to 0.20 mM 6-PG. The rates were measured at 25 °C. The assay solutions typically contained 0.0020 mg of 6-PGDH. Inhibition studies were carried out from 0.0 to 3.0 mM vanadate, from 0.0 to 10 mM phosphate, and from 0.0 to 10 mM pyrophosphate.

**(B) 6-Phosphogluconate Dehydrogenase from Sheep Liver.** The enzyme was assayed as described previously with minor modifications.[22,5] The assay solutions contained 0.1 M *N*-ethylmorpholine (pH 7.8), 0.10 M potassium chloride, 3.3 mM magnesium chloride, 0.10 mM NADP, and from 0.010 to 0.20 mM 6-PG. The rates were measured at 25 °C. The assay solutions typically contained 0.0015 mg of 6-PGDH. Inhibition studies were carried out from 0.0 to 3.0 mM vanadate, from 0.0 to 10 mM phosphate, and from 0.0 to 10 mM pyrophosphate.

**(C) 6-Phosphogluconate Dehydrogenase from Human Erythrocytes.** The enzyme was assayed as described previously with minor modifications.[23,5] The assay solutions contained 0.1 M *N*-ethylmorpholine (pH 7.8), 0.10 M potassium chloride, 3.3 mM magnesium chloride, 0.020 mM NADP, and from 0.0025 to 0.035 mM 6-PG. The rates were measured at ambient temperature. The assay solutions typically contained 0.0030 mg of 6-PGDH. Inhibition studies were carried out from 0.0 to 3.0 mM vanadate, from 0.0 to 10 mM phosphate, and from 0.0 to 10 mM pyrophosphate.

**(D) 6-Phosphogluconate Dehydrogenase from *L. mesenteroides*.** The enzyme was assayed as described previously with minor modifications.[12,5] The assay solutions contained 0.1 M imidazole (pH 7.1), 0.050 M potassium chloride, 2.0 mM magnesium chloride, 3.5 mM NAD, and from 0.050 to 1.5 mM 6-PG. The rates were measured at 25 °C. The assay solutions typically contained 0.0040 mg of 6-PGDH. Inhibition studies were carried out from 0.0 to 3.0 mM vanadate, from 0.0 to 10 mM phosphate, and from 0.0 to 10 mM pyrophosphate.

## Results and Discussion

**6-PG Oxidation Catalyzed by 6-PGDH from *Torula* Yeast.** The rate by which 6-PGDH catalyzes the oxidation of 6-PG to ribulose 5-phosphate and carbon dioxide while simultaneously reducing the NADP or NAD was determined by using UV spectroscopy. Enzyme reaction rates were measured by monitoring the production of NADPH at 340 nm in a previously described assay for 6-PGDH from yeast (*Candida utilis*)[19] that was modified to avoid vanadate–buffer complexation.[5] The buffers previously used for assaying yeast enzymes all complex vanadate, so we chose to use imidazole at pH 7.1. Imidazole neither affects biological reactions through addition reactions[5] nor through redox reactions[24] and is therefore an excellent choice as buffer. The reaction rates were determined at various 6-PG concentrations in the 0.040–0.20 mM range. $K_m$ for 6-PG was determined to 0.042 mM and the $K_m$ for NADP to 0.021 mM in agreement with those published previously.[13,19]

**Inhibition by Phosphate and Pyrophosphate of 6-PG Oxidation Catalyzed by 6-PGDH from *Torula* Yeast.** The rates of oxidation of 6-PG catalyzed by 6-PGDH were determined at 6-PG concentrations from 0.040 to 0.20 mM in the presence of various phosphate and pyrophosphate concentrations (up to 10 mM). Plotting the reciprocal rates obtained with inorganic phosphate as inhibitor as a function of the reciprocal concentration of 6-PG gives a competitive inhibition pattern. Competitive inhibition of substrate is commonly interpreted as phosphate binding in the active site of the enzyme thus preventing the substrate, 6-PG, from binding. The $K_i$'s for both phosphate and pyrophosphate were

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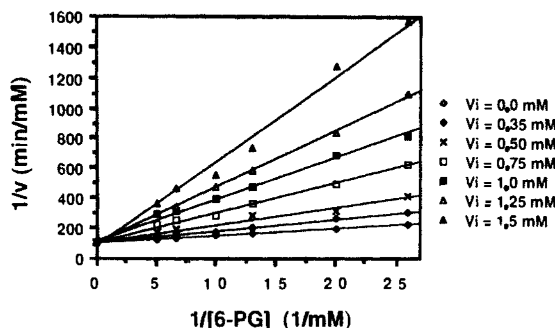



**Figure 1.** Kinetics of the 6-PG oxidation reaction catalyzed by 6-PGDH from *Torula* yeast shown in a Lineweaver–Burk plot. The reciprocal rate is plotted as a function of the reciprocal 6-PG concentration at various vanadate concentrations. The reactions were carried out in solutions containing 0.1 mM imidazole at pH 7.1, and 25 °C, 3.3 mM magnesium chloride, 0.44 mM NADP, approximately 0.0020 mg of 6-PGDH, and from 0.039 to 0.20 mM 6-PG. $K_m$ for 6-PG under these conditions were determined to 0.042 mM. The vanadate concentrations were as follows: 0.0 (◇), 0.35 (◆), 0.50 (×), 0.75 (□), 1.0 (■), 1.25 (△), and 1.5 (▲) mM.

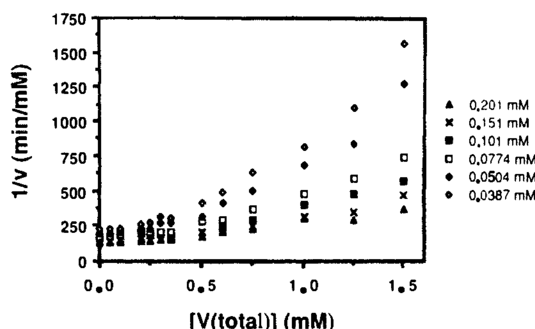



**Figure 2.** Dixon plot showing the reciprocal rate plotted as a function of total concentration of vanadium atoms. The 6-PGDH source was *Torula* yeast. $K_i$ cannot be obtained because of the nonlinear relationship between $1/v$ and $[V_{tot}]$. The experimental details are described in Figure 1. The 6-PG concentrations were as follows: 0.201 (▲), 0.151 (×), 0.101 (■), 0.0774 (□), 0.0504 (◆), and 0.0387 (◇) mM.

above 5 mM, which suggests the phosphate and pyrophosphate only bind weakly to the active site of the enzyme.

**Inhibition by Vanadate of 6-PG Oxidation Catalyzed by 6-PGDH from *Torula* Yeast.** The rates of 6-PG oxidation catalyzed by 6-PGDH were determined at 0.040–0.20 mM 6-PG in the presence of various vanadate concentrations from 0.0 to 1.5 mM vanadate. Plotting the reciprocal rate of 6-PG oxidation as a function of the reciprocal 6-PG concentration for various vanadate concentrations gives lines of increasing slopes with increased concentration of vanadate in a competitive inhibition pattern (Figure 1). However, when attempting to determine the $K_i$ of vanadate by plotting $1/v$ as a function of total vanadate concentration at various 6-PG concentrations (Dixon plot), a nonlinear relationship was obtained (Figure 2).

Figure 2 shows that no inhibition of the enzyme rates is observed below 0.4 mM vanadate, but above 0.4 mM vanadate the enzyme rates are rapidly decreased. Since aqueous solutions contain many species, the nonlinearity observed in Figure 2 could be a result of the oligomerization reactions. The concentrations of various vanadate species should be determined in order to examine if any specific vanadium species affects the enzyme reaction.

**Concentration of Vanadate Oligomers As Determined by $^{51}$V NMR.** $^{51}$V NMR was used to measure the distribution of vanadate species at various vanadate concentrations under the assay conditions used for the 6-PG oxidation reaction catalyzed by 6-PGDH from *Torula* yeast. Due to the oligomerization reaction equilibrium, the vanadate monomer, dimer, tetramer, and pentamer will all be present in solutions at any point in time. Low concentrations of various oligomers will however render several of these species undetectable by $^{51}$V NMR. In the following discussion we focus on the concentrations of vanadate species that are directly observable in the $^{51}$V NMR spectra. At vanadate concentrations below 0.1 mM, the vanadate monomer is the only species observed in aqueous solution. However, as the total vanadate concentration increases, both the concentration of vanadate dimer and tetramer increase slowly. At total vanadate concentrations from 0.1 to 0.6 mM, equivalent concentrations of vanadium atoms as dimer and tetramer are observed. Both the monomer and dimer concentrations are found to increase slowly above 0.6 mM total vanadate, whereas the tetramer increases rapidly at such high vanadate concentrations. The concentration of monomer, dimer, and tetramer are described by reactions 1–3, and the $[H^+]$-dependent equilibrium constants are summarized in Table I.

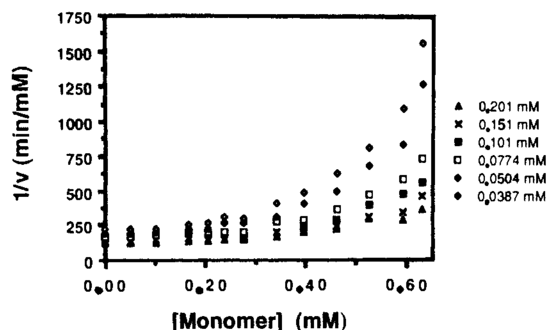



**Figure 3.** Dixon plot showing the reciprocal rate plotted as a function of the concentration of vanadium atoms found as monomeric vanadate. The 6-PGDH source was *Torula* yeast. $K_i$ cannot be obtained because of the nonlinear relationship between $1/v$ and $[V_1]$ ([monomer]). The experimental details are described in Figure 1. The 6-PG concentrations were as follows: 0.201 (▲), 0.151 (×), 0.101 (■), 0.0774 (□), 0.0504 (◆), and 0.0387 (◇) mM.

**Inhibition by Monomeric Vanadate of 6-PG Oxidation Catalyzed by 6-PGDH from *Torula* Yeast.** Vanadate is structurally and electronically an analogue of phosphate and behaves as a phosphate analogue in many biological systems.[1] On the basis of the similarity of monomeric vanadate with phosphate, one would presume that the phosphate analogue (monomeric vanadate) is binding in the active site of 6-PGDH. Combining the results obtained by $^{51}$V NMR and the enzyme kinetic data for *Torula* yeast, one can examine the effects of the specific vanadate species. Plotting the reciprocal rate as a function of monomeric vanadate concentration gives a nonlinear relationship (Figure 3). Figure 3 shows that no inhibition is observed below 0.3 mM monomer (slope = 0). Above 0.3 mM monomer inhibition increases exponentially. These results suggest that *monomeric vanadate is not producing the observed inhibition*! If monomeric vanadate significantly inhibited the enzyme reaction, such inhibition would have been observed in the concentration range of 0–0.3 mM monomer. When inhibition is observed, the concentrations of vanadate monomer are above 0.3 mM, and the solutions also contain significant concentrations of dimer and tetramer. The monomer is probably a weak inhibitor of the enzyme reaction, since it is a phosphate analogue; however, a higher vanadate oligomer is a much more potent inhibitor.

**Inhibition by Dimeric Vanadate of 6-PG Oxidation Catalyzed by 6-PGDH from *Torula* Yeast.** Since monomeric vanadate does not inhibit the reaction catalyzed by 6-PGDH, we continued this analysis to determine which vanadate derivative was responsible for the rate inhibition. Plotting the reciprocal rate as a function of dimer concentration gave a nonlinear relationship. Below 0.08 mM the reaction rates are not significantly affected by dimer, but above 0.08 mM the reaction rates rapidly decline. These results indicate that the vanadate dimer does not affect the reaction rates significantly.

**Inhibition by Tetrameric Vanadate on 6-PG Oxidation Catalyzed by 6-PGDH from *Torula* Yeast.** Plotting the reciprocal rate as a function of tetramer concentration (Figure 4) shows a linear dependence! This linear relationship allows direct determination of a $K_i$ for the tetramer of 0.013 mM. These observations suggest the *vanadate tetrameric species alone can account for observed*

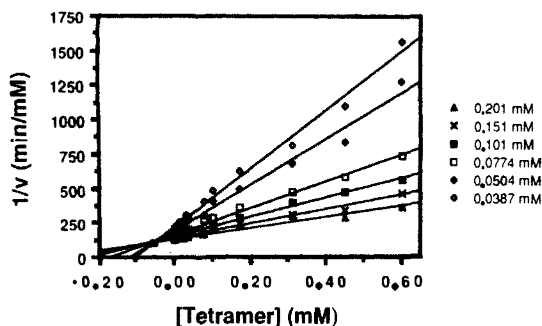



**Figure 4.** Dixon plot showing the reciprocal rate plotted as a function of the concentration of vanadium atoms found as tetrameric vanadate. The 6-PGDH source was *Torula* yeast. Since vanadate is a competitive inhibitor, $K_i$ was determined to 0.052 mM vanadium atoms found as vanadate tetramer or alternatively to 0.013 mM vanadate tetramer. The experimental details are described in Figure 1. The 6-PG concentrations were as follows: 0.201 (▲), 0.151 (×), 0.101 (■), 0.0774 (□), 0.0504 (◆), and 0.0387 (◇) mM.

*inhibition of the 6-PG oxidation catalyzed by 6-PGDH from Torula yeast* and represent the first observation of enzymatic activity of the tetramer. The vanadate tetramer has previously been found to bind[7,25-27] to or modify proteins, or both; however, it has not previously been shown to affect enzymatic activity under physiological conditions.

**Inhibition by Pentameric Vanadate of 6-PG Oxidation Catalyzed by 6-PGDH from *Torula* Yeast.** The effect of pentameric vanadate on enzyme rates was examined at a total vanadate concentration above 1.0 mM. The appearance of an additional inhibitor at these vanadate concentrations would affect the reaction kinetics such that the tetrameric vanadate no longer could account for the total observed inhibition. If the pentamer inhibits the enzyme reaction, the reaction rates will be affected by two inhibitors and the plots of reciprocal rates as a function of tetramer will not be linear in the concentration range where both tetramer and pentamer inhibit the enzyme reaction. Since the inhibition of the 6-PG oxidation at high vanadate concentrations was accounted for by the vanadate tetramer, the pentamer does not affect the enzyme reaction. These studies were carried out at low enzyme rates and large experimental uncertainties (up to ±30%), and only a potent inhibitory activity of the pentamer would have been observed under these conditions.

**Inhibition by Vanadate of the NADP Reduction Catalyzed by 6-PGDH from *Torula* Yeast.** The reaction rates were determined at NADP concentrations in the 0.0–0.080 mM range at vanadate concentrations from 0 to 1.0 mM. Plotting the reciprocal rates as a function of reciprocal NADP concentrations yields a plot showing a mixed inhibition pattern. If the vanadate tetramer were to bind to the NADP binding site in the enzyme, it would show a competitive inhibition pattern with respect to NADP. If, on the other hand, the vanadate tetramer binds to the 6-PG binding site, noncompetitive or mixed kinetics should be observed. Alternatively, the vanadate could potentially interact directly with the NADP and prevent the cofactor from binding to the enzyme. Such an explanation is unlikely, because a large excess of cofactor was used in the reactions, and even if some vanadate were interacting with the cofactor, excess NADP was present to saturate the enzyme. Since a mixed inhibition pattern of vanadate against NADP is observed, these results support our kinetic results obtained with 6-PG as substrate and suggest the tetrameric vanadate does indeed bind to the 6-PG binding site in the enzyme. The $K_i$ for NADP was determined to be 0.15 mM.

Inhibition by tetrameric vanadate represents a new mechanism by which vanadium could act both in in vivo and in vitro systems. It is therefore important to determine whether the tetramer also affects other sources of enzymes. Since biological effects of vanadate vary dramatically from species to species, we examined whether 6-PGDH enzymes from three additional sources, sheep liver, human erythrocytes, and *L. mesenteroides*, also are inhibited by tetrameric vanadate. These 6-PGDH enzymes represent an array of similarities and differences with respect to amino acid sequence, kinetics and enzyme mechanism, substrate activity, substrate rate, and coenzyme requirements. If indeed the tetrameric vanadate is found to inhibit all these enzymes, it reflects a general nature of this mechanism.

**6-PGDH from Sheep Liver.** The buffers previously used for assaying the sheep enzyme complex with vanadate under the assay conditions,[22] so we chose to use *N*-ethylmorpholine at pH 7.8. This buffer is preferred over imidazole because the sheep liver 6-PG is active at higher pH than the yeast enzyme and the $pK_a$ of *N*-ethylmorpholine (7.7) is higher than the $pK_a$ of imidazole (7.00). The distribution of mono- and oligovanadates under the assay conditions was determined by $^{51}$V NMR spectroscopy. The concentration of the monomer, dimer, and tetramer can be expressed by the equilibria shown in reactions 1–3, and the corresponding $[H^+]$-dependent equilibrium constants under these conditions are summarized in Table I. The kinetic mechanism for the sheep liver enzyme is dependent on the buffer, and accordingly, the Michaelis–Menten parameters vary considerably with the conditions under which they were determined.[14,18,22] We determined a $K_m$ for 6-PG to be 0.034 mM and a $K_m$ for NADP to be 0.010 mM, in agreement with previous observations.[14,18,22] Both phosphate and pyrophosphate were found to inhibit the sheep liver enzyme competitively with $K_i$'s above 5 mM.

Carrying out an analysis as described for the *Torula* yeast enzyme we conclude the monomer, dimer, and pentamer do not significantly affect the sheep liver enzyme whereas the tetramer inhibits the enzyme efficiently. The vanadate tetramer has a $K_i$ of 0.063 mM with enzyme.

**6-PGDH from Human Erythrocytes.** The buffers previously used for assaying the human erythrocyte enzyme form complexes with vanadate.[5,15,17,23,24] Since the enzyme has higher activity at high pH, this enzyme was assayed in *N*-ethylmorpholine at pH 7.8 under conditions analogous to the sheep liver enzyme assay. The distribution of mono- and oligovanadates under the assay conditions was determined by $^{51}$V NMR spectroscopy, and the $[H^+]$-dependent equilibrium constants are shown in Table I. The oxidation rates of 6-PG were measured at 6-PG concentrations from 0.0025 to 0.035 mM, resulting in a $K_m$ for 6-PG of 0.024 mM and for NADP of 0.0029 mM in accord with previous observations.[15,17,23] Both phosphate and pyrophosphate were found to inhibit the human erythrocyte enzyme competitively with $K_i$'s above 5 mM. Carrying out an analysis as described for the *Torula* yeast enzyme, we conclude that the vanadate monomer, dimer, and pentamer do not affect the human erythrocyte enzyme, whereas the tetramer inhibits the enzyme efficiently. The $K_i$ for the vanadate tetramer of the human erythrocyte enzyme is 0.078 mM.

6-PGDH from sheep liver has many similarities with 6-PGDH from yeast and human erythrocytes including amino acid content, amino acid sequence, molecular weight, and required cofactor. An X-ray structure of the sheep enzyme shows the 6-PG binds in a domain at a considerable distance from the domain where NADP binds. Since the tetramer inhibits both the *Torula* yeast 6-PGDH, the human erythrocyte 6-PGDH, and the sheep liver 6-PGDH with inhibition constants of similar magnitude, one can presume that the tetramer will bind similarly to the 6-PG binding pocket in all three enzymes.[16] It is therefore of interest to examine how a 6-PGDH enzyme considerably different from the above enzymes is affected by vanadate.

**6-PGDH from *L. mesenteroides*.** The buffers previously used for assaying this enzyme complex vanadate, so we chose to use imidazole at pH 7.1.[5,12,24] The distribution of mono- and oligovanadates under the assay conditions were determined by $^{51}$V NMR spectroscopy, and the equilibrium parameters are shown in Table I. The reaction rates were measured at various 6-PG concentrations in the 0.050–1.5 mM range. $K_m$ for 6-PG was

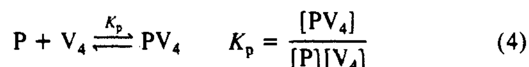
(25) Cremo, C.; Grammer, J.; Long, G. T.; Yount, R. G. *J. Cell. Biol.* **1988**, *107*, 257a; *Biophys. J.* **1989**, *155*, 81a (M-POS39).
(26) Vilter, H.; Rehder, D. *Inorg. Chim. Acta* **1987**, *136*, L7–L10.
(27) Butler, A.; Danzitz, M. J.; Eckert, H. *J. Am. Chem. Soc.* **1987**, *109*, 1864–5.

determined to be 0.45 mM and the $K_m$ for NAD was determined to be 0.15 mM under these conditions. Inorganic phosphate and pyrophosphate inhibit the 6-PGDH competitively with respect to 6-PG, and the inhibition constants were above 5 mM. Carrying out an analysis as described for the *Torula* yeast enzyme, we conclude that the vanadate monomer, dimer, and pentamer do not affect the *L. mesenteroides* enzyme, whereas the tetramer inhibits the enzyme. The $K_i$ for the tetramer of the *L. mesenteroides* is 0.24 mM.

Our work shows that the vanadate tetramer inhibits 6-PGDH from mammalian (human erythrocytes and sheep liver), yeast (*Torula* yeast) and bacterial (*L. mesenteroides*) sources. In addition, preliminary work with G6-PDH (*L. mesenteroides*) also shows that this enzyme is potently inhibited by the vanadate tetramer. Since the vanadate tetramer may inhibit a wide range of enzymes from various sources, we have searched for in vitro and in vivo studies that show precedence for the vanadate tetramer binding to enzymes or other cellular components.

**Previous in Vitro and in Vivo Studies.** Several $^{51}V$ NMR studies on protein–vanadate interactions or vanadium metabolism have been reported.[7b,7c,25,26,27,28] Interaction of vanadate with protein components usually leads to signal broadening, and in most cases the signal disappears into the base line.[7b,7c,25,26] For example in vitro binding of oligovanadates to the sarcoplasmic reticulum was accompanied by the broadening and disappearance of $^{51}V$ NMR signals from the decamer, dimer, and tetramer, and the authors concluded the $Ca^{2+}$-ATPase from the sarcoplasmic reticulum membranes bound the vanadate decamer, dimer, and tetramer.[7b]

If the tetramer in general interacts strongly with intracellular proteins, the concentration of vanadate species will change. Expressing enzymes and cellular components that interact with the vanadate tetramer as a generic P and the complex as $PV_4$, the overall equilibrium is shown in (4). Although our studies



$$P + V_4 \underset{}{\overset{K_p}{\rightleftharpoons}} PV_4 \qquad K_p = \frac{[PV_4]}{[P][V_4]} \tag{4}$$

have been limited to two enzymes (6-PGDH and G-6-PDH), the tetramer has previously been suggested to bind to myosin,[25] transferrin,[26] $Ca^{2+}$-ATPase,[7b] and other cellular components. Monomeric vanadate[6] interacts with proteins with equilibrium quotients on the order of $10^3$ $M^{-1}$. Since $K_{p,6PGDH} = 1/K_{i,6PGDH}$, $K_p$ for the tetramer is a minimum of $10^3$ $M^{-1}$. A tetramer concentration of $10^{-4}$ M will, according to eq 4, yield $[PV_4]$ one-tenth of [P]. A tetramer concentration of $10^{-3}$ M yields a $[PV_4]$ equal to [P]. If on the other hand $K_p$ for the tetramer is around $10^{-5}$ $M^{-1}$, a concentration of $10^{-4}$ M gives $[PV_4]$ 10 times [P], and a tetramer concentration of $10^{-3}$ M gives $[PV_4]$ 100 times [P]. These considerations show that the vanadate tetramer can bind better or with similar affinity to proteins as the monomer.

In vivo metabolic studies of *Saccharomyces cerevisiae* by Willsky et al.[28] compared the vanadium metabolism in normal cells (normal growth) treated with a nontoxic vanadate concentration with cells treated with a toxic vanadate concentration (cells stopped growing) using $^{51}V$ NMR. Willsky et al. interpreted their results as suggesting the vanadate dimer and decamer are active species in vivo in yeast. Our studies suggest the vanadate tetramer may also be an active vanadium species. Willsky's results also can be interpreted in accord with the vanadate tetramer binding in vivo in yeast. This conclusion is based on the fact that the $^{51}V$ NMR spectrum of the medium treated with toxic levels of vanadate presumably contains signals for monomer (~−545 ppm; several peaks in this chemical shift range could be monomer), dimer (−563 ppm), tetramer (−575 ppm), pentamer (~−583 ppm; the chemical shift for the shoulder peak was not specified), and decamer (−427, −497, −512 ppm). The $^{51}V$ NMR spectra of the in vivo cells contain most of the signals above, except the presumed equilibrium between monomer, dimer, tetramer, and pentamer has shifted sufficiently such that only little tetramer is present in the spectrum. Although there are alternative explanations for such changes in the monomer, dimer, and tetramer pool, Willsky's observations are also consistent with the binding of the tetramer to the protein or cellular material.

(28) (a) Willsky, G. R.; White, D. A.; McCabe, B. C. *J. Biol. Chem.* **1984**, *259*, 13273–81. (b) Willsky, G. R.; Leung, J. O.; Offermann, P. V., Jr.; Plotnick, E. K.; Dosch, S. F. *J. Bactiol.* **1985**, *164*, 611–7. (c) Willsky, G. R.; Dosch, S. F. *Yeast* **1986**, *2*, 77–85.

## Conclusion

Vanadate solutions containing monomeric, dimeric, tetrameric, and pentameric vanadate competitively inhibit the oxidation of 6-PG catalyzed by 6-PGDH (6-phosphogluconate dehydrogenase). $^{51}V$ NMR spectroscopy was used to determine the concentration of the vanadate species in solution, such that the effects of vanadate monomer, dimer, tetramer, and pentamer as inhibitors could be accessed. Neither the monomer, dimer, nor pentamer was observed to affect the 6-PGDH from human erythrocytes, sheep liver, *Torula* yeast, or *L. mesenteroides*. The tetramer inhibited all the 6-PGDH enzymes, and the inhibition constants were determined to be 0.078 mM for the erythrocyte enzyme, 0.063 mM for the sheep liver enzyme, 0.013 mM for the *Torula* yeast enzyme, and 0.24 mM for the *L. mesenteroides* enzyme. The *Torula* yeast, sheep liver, and human erythrocyte enzymes are known to have similar physical properties and were indeed inhibited by vanadate with similar specificity. The *L. mesenteroides* enzyme has considerably different physical properties but was inhibited by vanadate with similar specificity. These results suggest the inhibition by the vanadate tetramer may be a general property of organic phosphate converting enzymes including dehydrogenases, oxidases, isomerases, aldolases, transferases, glycosidases, and epimerases.

This paper represents the first reported enzymatic activity exhibited by the vanadate tetramer. Preliminary results have been obtained with additional enzymes, including glucose-6-phosphate dehydrogenase, showing that the vanadate tetramer also inhibits other enzymes. Tetrameric vanadate accumulates in significant concentrations at millimolar total vanadate concentrations. The tetramer can therefore form at toxic vanadium levels but not in healthy tissue in mammals and plants. The vanadate tetramer may therefore directly be reponsible for some of the toxic effects observed by vanadate in humans, plants, yeasts, and bacteria and should be considered as a potentially active species in biological systems.

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**Registry No.** 6PGDH, 9073-95-4; 6PG, 921-62-0; NADP, 53-59-8; vanadate tetramer, 12379-27-0.

**Supplementary Material Available:** A figure showing the vanadate species distribution for the *Torula* yeast assay and four figures and one table describing the kinetics observed with the dimer and 6-PGDH from yeast, the tetramer with the 6-PGDH from sheep liver, human erythrocytes, and *L. mesenteroides* (8 pages). Ordering information is given on any current masthead page.