Defining Chemical Species in Complex Environments Using K-Edge X-ray Absorption Spectroscopy: Vanadium in Intact Blood Cells and Henze Solution from the Tunicate *Ascidia ceratodes*

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A K-edge X-ray absorption spectrum (XAS) fitting approach has been developed to speciate elements of interest in complex materials and used here to model the storage of biological vanadium within whole blood cells from the tunicate *Ascidia ceratodes*. The response of the K-edge XAS of solution-phase V(III) to increasing *c*(sulfate) at constant pH 1.8 produced specific and systematic effects in the preedge transition at 5468.8 eV (preedge transitions: $1s \rightarrow {}^4A_2$ at 5464.9 ± 0.1 eV, $1s \rightarrow {}^4T_2$ at 5466.9 ± 0.1 eV, and $1s \rightarrow {}^4T_1$ at 5468.8 ± 0.1 eV for 11 different V(III)/sulfate solutions). In contrast, variations in acidity (as pH) at constant *c*(sulfate) systematically modified the V(III) preedge XAS at 5466.9 eV. The energy position of the K-edge absorption maximum also serially shifted −0.32 eV/pH unit, from 5483.7 eV (pH 3.0) to 5484.7 eV (pH 0.3). Fits to the V−K XAS of two samples of *A. ceratodes* whole blood cells representing dozens of animals implied storage of V(III) ions in four predominant solution regimes: ∼10% high sulfate/pH 0 acid; ∼40% high sulfate/pH 1.8 acid; ∼40% moderate sulfate/pH 1.8 acid; ∼10% moderate sulfate/pH 3 acid. For lysed blood cells, the best fit represented 63% of the V(III) in a pH 1.6 sulfate-free environment and a further 16% in acidic sulfate solution. Nearly 18% of lysed cells vanadium(III) appeared in a tris(catecholate)-like environment. A detailed speciation of biological vanadium complex ions was calculated from these fits by application of the known equilibrium constants governing V(III) and sulfate in acidic aqueous solution. The utility of blood cell V(III) to ascidians is discussed. Fits to K-edge XAS spectra using the XAS spectra of appropriate models are suggested to be generally applicable to elucidating the state of metal ions in a wide variety of complex environments.

Introduction

The status of vanadium within the blood cells of the ascidians continues to be under active investigation.[1−8] To characterize blood cell vanadium in ascidians in a chemically explicit way, we have begun experiments to fit the vanadium K-edge X-ray absorption spectrum (XAS) of whole blood cells from the tunicate *Ascidia ceratodes* using the vanadium K-edge spectra of appropriate model solutions and complexes. For biotic systems containing possibly complex mixtures of metal sites, an XAS edge-fitting strategy can potentially reveal the biological ligation of metal ions and discriminate among various proposed chemical environments and oxidation states.[9−12] This XAS fitting approach has the advantage of probing the intact biological system. However, care must be taken to ensure the use of model systems which reflect as nearly as possible the chemical nature of the biological sites to be investigated. Although the choice of the vanadium model systems described herein integrated information from a variety of studies,[13−24] the fact that a perfect correspondence between model and data is

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impossible means that the fitting results will always include an element of ambiguity. The experimental charge is both to acknowledge the ambiguity and to minimize it.

Here we report the results of systematic investigations into the variation of the K-edge XAS spectrum of solution V(III) as a function of pH at constant *c*(sulfate) (the total sulfate concentration) and as a function of *c*(sulfate) at constant pH. These solution characteristics were chosen for detailed study because of compelling evidence that significant acid and sulfate concentrations characterize the blood cell vanadium(III) storage locale in *A. ceratodes*.[13,14,21,24−27]

Using these results, and employing the K-edge XAS spectra of other suitable vanadium model complexes, we have developed a chemical description of the state of intracellular vanadium within two whole blood cell samples from the tunicate *A. ceratodes*, each representing dozens of individuals. The vanadium K-edge XAS spectrum of a sample of fresh Henze solution (lysed whole blood cells) has likewise been modeled in terms of vanadium complexes. We describe in detail the fitting procedure used, describe the endogenous environments found to sustain vanadium within intact blood cells, and calculate the distribution of vanadium solution complexes governed by these environments. We show that fitting K-edge XAS spectra can produce a detailed model of the biological inorganic chemistry and disposition of vanadium in the blood cells of *A. ceratodes* and in Henze solution. The method is also applicable to metal ions in other complex chemical environments.

Materials and Methods

Specimens of *A. ceratodes* were collected from the floating docks of the Monterey Bay Yacht Harbor, Monterey, CA, on March 16, 1993. The animals were maintained in an aerated seawater aquarium at 4 °C, and blood was collected by cardiac puncture using sterile disposable 1 cm^3 syringes as has been reported in detail elsewhere.[17,24,25] The two whole blood cell samples described here included the combined blood from 25 individuals (sample 1) and about 37 individuals (sample 2). Henze solution representing blood cells from a second group of about 37 individuals was prepared as described earlier.[24]

Inorganic vanadium(III) solutions and samples were prepared using anaerobic methods, either as described below or within an inert atmosphere glovebox (Plas-Labs Inc., Lansing, MI) under a purified dinitrogen atmosphere. All sample solutions described below were dispensed and handled using Hamilton gastight microliter syringes.

Vanadium(III) tris(trifluoromethanesulfonate) $\{V(CF_3SO_3)_3\}$ was directly prepared as a 0.60 M solution, by hydrogen reduction of vanadium pentoxide over 10% Pd/C. Thus 1364 mg (7.50 mmol) of V_2O_5 (Aldrich Chemicals) was suspended in 22.5 mL of 2 M trifluoromethanesulfonic acid (45.0 mmol), and the reaction flask was vented through a water bubbler. The system was first flushed with dinitrogen gas, and 100 mg of 10% Pd/C was then added. Hydrogen gas was bubbled through the system for 17 h., after which an additional 50 mg of Pd/C was added. After a further 24 h of reduction, the Pd/C was removed by anaerobic centrifugation (3000*g*, 20 min). The pellet was rinsed with 0.5 cm^3 of anaerobic 2 M trifluoromethanesulfonic acid which was combined with the supernatant. The clear olive green solution was then diluted to 25.0 mL using anaerobic deionized water. This stock solution was finally passed through a 0.45 μm porosity PTFE filter, stored under a dinitrogen atmosphere in glass vials capped with butyl rubber septa and aluminum crimp-tops, and tightly covered with 4 layers of 50 μm Saran (poly(vinylidene dichloride)) film.

Vanadium(III) trichloride solution was prepared by means of a similar reductive method, using 90.94 mg of V_2O_5 (0.5 mmol) in 10.0 mL of 1.3 M hydrochloric acid (13.0 mmol). After removal of the 10% Pd/C by centrifugation, and dilution to volume with anaerobic 1 M HCl, the final 0.10 M solution of VCl_3 in 1 M HCl was a clear dusky blue. The XAS sample was prepared immediately by filling a 2 mm path length Lexan XAS sample cell which was then frozen by immersion into liquid nitrogen. The sample was stored until use in a liquid-nitrogen Dewar flask.

Vanadium(III) in pH 1.8 solutions of known *c*(sulfate) were prepared by appropriate dilution of the 0.6 M $V(CF_3SO_3)_3$ stock solution (see above) with anaerobic 0.5 M aqueous $NaHSO_4$ and 1.1 M H_2SO_4 solutions. The pH was adjusted by addition of small amounts of powdered KOH. Acidity as pH was measured within the Plas-Labs inert-atmosphere glovebox using an external Beckman model 3500 pH meter connected by means of a long cable to a combination pH electrode resident in the glovebox, calibrated between pH 0.1 and pH 4.0. The final compositions of the two sample solutions were the following: 49.6 mM V(III), 446 mM sulfate, pH = 1.82; 50.0 mM V(III), 0.988 M sulfate, pH = 1.79. A third sample consisting of 50 mM $V_2(SO_4)_3$ in pH 1.8 sulfuric acid solution was prepared as described previously.[24]

Solutions of vanadium(III) in pH 0 sulfuric acid with varying *c*(sulfate) were prepared using 0.5 M $V_2(SO_4)_3$ in 15 mM H_2SO_4 stock solution.[17] The acidity and sulfate concentration were adjusted with 1 M $HClO_4$, 1.1 M H_2SO_4, or 0.5 M $(NH_4)_2SO_4$ in pH 1.8 solution and diluted to volume as necessary with deionized water. The three solutions prepared each contained 50 mM V(III) combined with one of the following: 75.8 mM sulfate, pH 0.04; 521 mM sulfate, pH 0.03; 779 mM sulfate, pH 0.13. Solution XAS samples were prepared immediately by filling a 2 mm path length Lexan XAS sample cell. Each sample was then frozen by plunging into a freezing isopentane slush (−159.9 °C) and stored until use in a liquid-nitrogen Dewar flask.

Vanadium(III) samples in solutions of varying pH in 40% methanolic deionized water solutions were prepared by appropriate dilution of the stock solution of 0.5 M $V_2(SO_4)_3$ in 15 mM H_2SO_4. Prior to dilution to a final volume of 1.0 mL with anaerobic methanol, acidity was adjusted using anaerobic aqueous 1 M $HClO_4$ or using anaerobic 2.7 M tetraethylammonium hydroxide in water solution (Aldrich Chemicals) or powdered KOH. The aqueous pH values were measured under dinitrogen as described above, using the same Beckman 3500 pH meter. The derived[28,29] pH values of the methanolic solutions were 0.3, 0.6, 1.2, 1.6, 2.1, and 2.7. An all-aqueous pH 3.0 solution was also prepared by making the final dilution with anaerobic deionized water only. The final V(III) concentration for all these solutions was 49.2 ± 0.8 mM. All the pH ≥ 2 methanolic solutions were clear green. The solutions were stored for 3 days at −20 °C in 1 mL glass vials under butyl rubber septa with aluminum crimp-tops and tightly covered with 4 layers of 50 μm Saran film. Samples for XAS were prepared under a dinitrogen atmosphere by filling a 2 mm path length Lexan XAS sample cell and then frozen by immersion in a freezing isopentane slush (−159.9 °C) and stored until use in a liquid-nitrogen Dewar flask. Crystalline $\{V(H_2O)_6H_5O_2(CF_3SO_3)_4\}$ was prepared using the method of Cotton et al.[30]

Vanadium K-edge XAS spectra of the solid inorganic vanadium model complexes $(Et_3NH)_2V(catecholate)_3$, $K_3V(catecholate)_3$, V(acetylacetonate)$_3$, $K_2VO(catecholate)_2$, and VO(acetylacetonate)$_2$, of 50 mM

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$V_2(SO_4)_3$ in pH 1.8 sulfuric acid solution, of vanadyl sulfate in 0.1 M sulfuric acid, of the two *A. ceratodes* whole blood cell samples, and of Henze solution from *A. ceratodes* were measured or obtained as has been reported in detail.[24]

Vanadium K-edge XAS spectra for the inorganic vanadium(III) solutions at the various pH values and sulfate concentrations and for crystalline $\{V(H_2O)_6H_5O_2(CF_3SO_3)_4\}$ (20 mg dispersed in 40 mg of BN) were measured on SSRL wiggler beamline 7-3, using a wiggler field of 18 kG. Data were collected under dedicated operating conditions of 3 GeV and 60−99 mA of current. The incident X-ray beam was energy-discriminated using a Si[220] double crystal monochromator which was detuned 50% at 6337 or 5861 eV, depending upon the energy range of the scan, so as to minimize harmonic contamination. Vanadium K-edge spectra were produced using X-ray fluorescence excitation and measured by means of an Ar-filled fluorescence ionization chamber detector (Stern−Heald−Lytle detector) set at 90° from the X-ray beam, equipped with a Ti filter and Soller slits.

Vanadium foil calibrations were measured as transmission spectra either after every three to four data scans for the solution samples or concurrently for the solid sample, using an in-line nitrogen-filled ionization chamber as detector. All XAS samples were maintained at 10 K using an Oxford Instruments CF1208 continuous-flow liquid-helium cryostat.

Raw vanadium K-edge data were processed as has been described in detail,[24,31] and the XAS edge spectra were calibrated to the first inflection point on the rising edge of the K-edge XAS spectrum of the vanadium foil standard, which was assigned to 5464.0 eV.

Fits to vanadium K-edge XAS spectra were carried out using the program DATFIT4 written by Dr. Graham George, Stanford Synchrotron Radiation Laboratory, Stanford, CA. The *A. ceratodes* vanadium K-edge spectra were fit over the range 5460−5500 eV, which covers the entire preedge and absorption edge XAS energy region. Four independent criteria were adopted to judge the acceptability of a final fit: (1) The goodness-of-fit parameter "*F*" was minimized, although the chosen final fit need not have exhibited the very lowest "*F*" value. (2) The final fit best reproduced the data as evaluated by detailed visual comparisons of the fit with the data over the small contiguous energy ranges 5462−5472, 5470−5482, and 5480−5500 eV. (3) The first derivative of the final fit was required to reproduce the first derivative of the experimental K-edge XAS spectrum. (4) The final fit always exhibited a (data minus fit) residuals spectrum of lowest maximal intensity.

Limits of precision in the fits were evaluated using numerically generated test spectra as described elsewhere.[11] In general, the precision of each model component reported for a given fit is about ±10% of the component percent. The energy reproducibility of vanadium K-edge spectra measured from time-to-time is about ±0.2 eV. Corrective energy shifts of up to ±0.2 eV were thus applied as necessary to model XAS spectra in order to achieve a fit. This shift is much smaller than those attending changes in ligation or oxidation state (see Results).

Results

K-edge X-ray absorption spectra involve the promotion of a 1s electron to higher energy bound states. These include 1s → 3d transitions in the preedge energy region, followed by transitions to higher energy "p"-symmetry states in the rising edge energy region out to energies near the ionization energy. The intensity of preedge transitions are especially sensitive to the point-group symmetry of the transition metal absorber, because the transition dipole intensity is strongly symmetry dependent.[9,32−34] Otherwise weak symmetry-forbidden 1s → 3d transitions can become intense following d−p mixing in noncentrosymmetric ligation environments (e.g., as in vanadyl ion). Transition metal K-edge XAS spectra have been analyzed in some theoretical detail.[9,33,35−37]

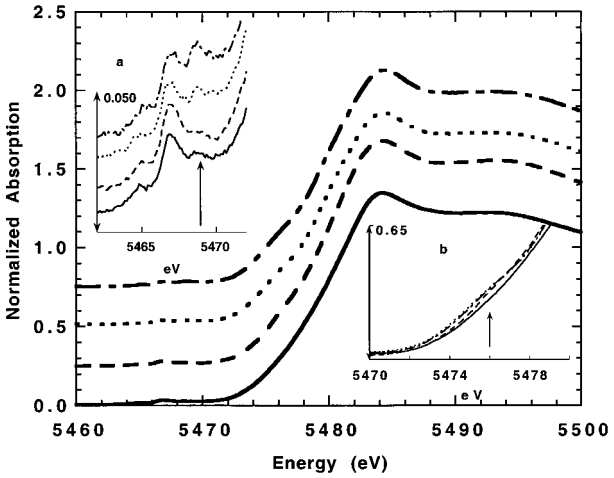

Figure 1. Vanadium K-edge XAS spectra of: (—) 100 mM VCl_3 in 1 M HCl solution; (− −) 100 mM V(III) with 150 mM sulfate; (· · ·) 49.6 mM V(III) with 446 mM sulfate; (− · −) 50 mM V(III) with 988 mM sulfate. Each of the latter three are in pH 1.8 solution. The upper three spectra have been progressively offset by 0.25 absorption unit for clarity. Inset a: Expansion of the preedge portion of the XAS spectra. The arrow indicates the feature at 5469 eV which responds to *c*(sulfate). The spectra have been offset for clarity. Inset b: Expansion of the rising edge portion of the XAS spectra. The arrow shows the 5476 eV shoulder serially intensifying with *c*(sulfate).

Vanadium K-edge XAS spectra are sensitive to details of metal ligation and oxidation state. Changes in ligand environment can modify the effective charge of the vanadium absorber and may produce shifts in K-edge energy of up to ±2.5 eV for oxygen ligation spheres. For vanadium within a constant O_6 coordination environment, a unit change in oxidation state can produce a K-edge energy shift of up to 5 eV.[24] In each case, a shift in K-edge energy is often accompanied by changes in the overall shape and intensity of a K-edge spectrum. Therefore, vanadium K-edge spectra are sensitive indicators of the nature of the ligand, the symmetry of the ligation environment, and the oxidation state of the absorbing metal ion.

These considerations are evident in Figure 1, which shows both the K-edge XAS spectrum of V(III) as $\{V(H_2O)_6\}^{3+}$ in 1 M HCl and the effect of progressively increasing *c*(sulfate) (the total sulfate concentration) on the K-edge XAS of V(III) ion in pH 1.8 solution. Because V(III) is a d^2 ion, the valence electron populations in the excited states resulting from promotion of a 1s electron into the 3d manifold are analogous to the optical ground and excited states of the $(Z + 1)d^{n+1}$ ion quartet d^3 species. Therefore, the appropriate spectroscopic comparisons are to the states of the d^3 ion $Cr(H_2O)_6^{3+}$. From the Tanabe−Sugano diagram for an octahedral d^3 ion, the three accessible 1s → 3d XAS transitions for d^2 V(III) (Figure 1, inset a) are, in order of increasing energy, 1s → $^4A_2$ (at 5464.9 ± 0.1 eV), 1s → $^4T_2(^4F)$ (at 5466.9 ± 0.1 eV), and 1s → $^4T_1(^4F)$ (at 5468.8 ± 0.1 eV). The energy positions are derived from the K-edge XAS spectra of 11 different acidic aqua V(III) solutions with or without added sulfate.

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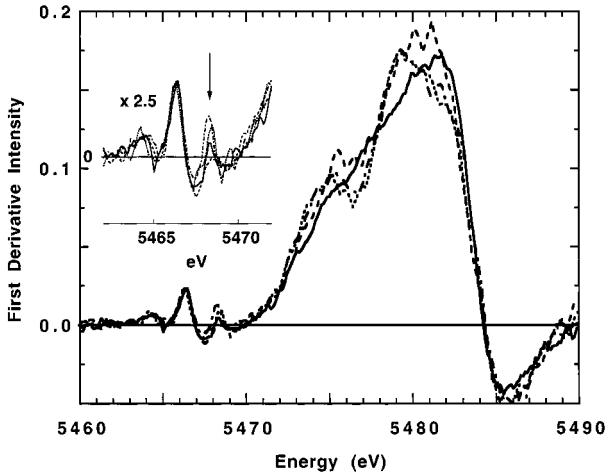

Figure 2. First derivatives of the vanadium K-edge XAS absorption spectra shown in Figure 1. The line types have the same significance. Note the increasing prominence with *c*(sulfate) of the inflection feature at 5475.5 eV. Inset: Expansion of the preedge portion of the first derivative XAS spectra. The arrow shows the feature at 5469 eV which uniquely responds to *c*(sulfate).

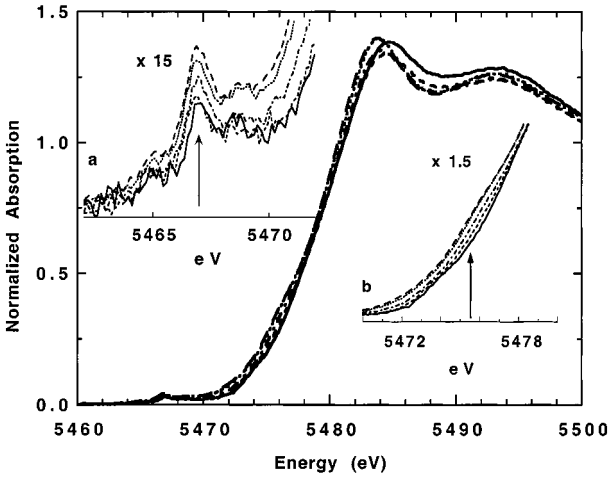

Figure 3. Vanadium K-edge XAS spectra of 49 ± 0.8 mM vanadium(III) sulfate in (—) pH 0.3, (- -) pH 1.2, (— · —) pH 2.1, (· · ·) pH 2.7, and (— —) pH 3.0 solutions. The details of preparation are given under Materials and Methods. The absorption maxima shift to higher eV with decreasing pH, an effect not observed with increasing *c*(sulfate). Inset a: Expansion of the preedge region of the XAS spectra showing the response to increasing acidity at 5467 eV (arrow), again in contrast with the effect of *c*(sulfate). Inset b: Expansion of the rising edge portion of the V−K XAS spectra showing the prominence of the shoulder near 5476 eV (arrow) increasing with pH.

On addition of sulfate ion to aqueous V(III), ligation to the metal center is governed by the relevant association constants,[38] and at higher *c*(sulfate) V(III)−sulfate complex ions dominate the composition. Electronic spectroscopy has indicated that sulfate ligation of $\{V(H_2O)_6\}^{3+}$ proceeds by substitution rather than addition.[38,39] Thus the pseudooctahedral disposition of six oxygen atoms in $\{V(H_2O)_5(SO_4)\}^{+}$ should retain the original transition multiplicity of $\{V(H_2O)_6\}^{3+}$ in the XAS preedge energy region. This is observed in Figure 1 (inset a), and V(III) sulfation is accompanied only by systematic modification the $1s \rightarrow {}^4T_1$ transition at 5468.8 eV.

Figure 1, inset b, shows that on addition of sulfate ion, significant vanadium K-edge XAS spectroscopic effects were also observed in the rising edge energy region at about 5476 eV. These spectral changes can be related to the calculated[38] composition of each vanadium(III) solution. A plot (not shown) of $([(V(SO_4)_{1,2})^{+,-}]_{total}/[V]_{total})$ versus absorption intensity at 5476 eV as obtained from Figure 1, inset b, yielded a linear least-squares (LLSQ) line with $r = 0.995$.

In Figure 2 the systematic modification with *c*(sulfate) of the first derivative XAS $1s \rightarrow {}^4T_1$ feature at 5468.8 eV (LLSQ fit, $r = 0.989$, plotted data not shown) is also evident. The other preedge features remained nearly unaffected by sulfate ion. The shoulder near 5476 eV (Figure 1) appears as an increasingly dominant dispersion-shaped first derivative feature at the same energy. Note also the systematic shift in the position of the rising edge inflection, from near 5482 eV for V(III) in 1 M HCl to near 5479 eV for V(III) in 0.45 and 0.99 M sulfate in pH 1.8 sulfuric acid. At the same time, the energy position of the *maximum* in absorption intensity remains constant at 5484 eV. These effects on vanadium XAS spectra are rigorously different from those accompanying changes in solution pH (see below).

When V(III) is titrated with acid at constant *c*(sulfate) (Figure 3), systematic modification of the $1s \rightarrow {}^4T_2$ preedge transition is observed at 5466.9 eV (Figure 3, inset a). Changes in the intensity of the rising edge shoulder near 5476 eV (Figure 3, inset b), and incremental shifts in energy of the absorption maximum near 5484 eV are also observed. The acid series was terminated at pH 3, because the $V(III)/VO^{2+}$ redox potential approaches zero volts vs NHE at this pH. It seemed unlikely that ascidian blood cells could sustain solution V(III) beyond this oxidation gradient.

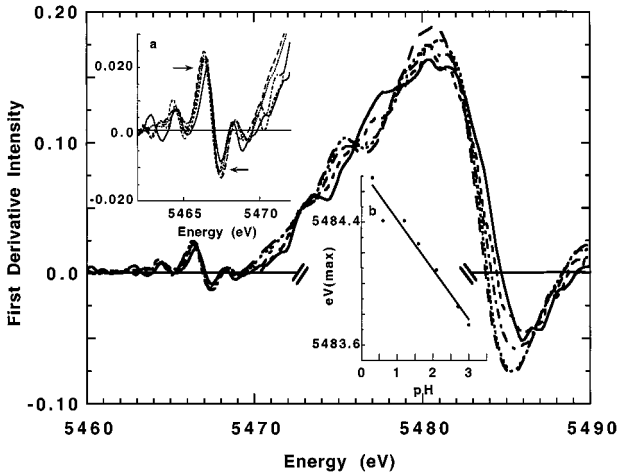

Figure 4. First derivative of the vanadium K-edge XAS spectra of Figure 3. The energy shift in absorption maximum with decreasing pH is seen near 5484 eV. Inset a: Arrows show the systematic changes with pH at 5467 eV. In inset b, the shift near 5484 eV is plotted against pH and fitted with a linear least-squares line ($y = 5484.7 - 0.32x$ ($r = 0.979$); slope = −0.32 eV/pH unit).

Comparison of Figure 4 with Figure 2 indicates that the effect of increasing pH on the $1s \rightarrow {}^4T_2$ transition feature at 5466.9 eV (Figure 4, inset a) is rigorously different from the effect of increasing sulfate on the $1s \rightarrow {}^4T_1$ transition at 5468.8 eV. Thus, in the former case, deprotonation dominates the changes in the XAS preedge energy region even in the presence of sulfate. In addition, a plot of the shift in the energy position of the absorption intensity maximum near 5484 eV (the first derivative zero) vs pH yields a good straight line of slope −0.32 eV/pH

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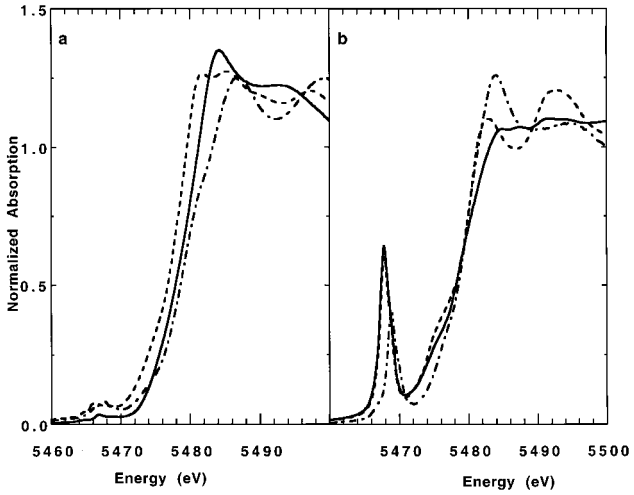

Figure 5. Vanadium K-edge XAS spectra of: (a) (—) 100 mM V(III) in 1 M HCl solution; (- -) solid K_3V^{III}(catecholate)$_3$; (— · —) solid $(Et_3NH)_2V^{IV}$(catecholate)$_3$. These spectra demonstrate the energy shift of the rising edge with change in ligation from water to catecholate and with change in oxidation state at constant ligation. (b) (—) solid $K_2V^{IV}O$(catecholate)$_2$; (- -) solid $V^{IV}O$(acetylacetonate)$_2$; (— · —) 50 mM vanadyl sulfate in 0.1 M sulfuric acid. These spectra show the energy shift of the V^{IV}=O preedge feature following chelation. All the solids were finely dispersed in BN.

unit (Figure 4, inset b). No such shift in solution V(III) K-edge absorption maximum is observed to follow *c*(sulfate) (Figure 2).

It is clear, therefore, that the composite details of the shape of V(III) K-edge XAS spectra are uniquely and separately sensitive to solution pH and to solution *c*(sulfate). These respective findings do not reflect simple incremental changes in deprotonation or sulfation of hexaaquaV(III), however, because several different V(III) species are present in solution.[38] Rather, the data show that K-edge XAS spectra of V(III) solutions are generally, systematically, and uniquely sensitive to overall variations in composition and pH. This point is expanded under Discussion.

The sensitivity of vanadium K-edge XAS spectra to oxidation state and ligation environment is shown in Figure 5. Here it is sufficient to note (Figure 5a) that the K-edge intensity maximum shifts about 2 eV to lower energy when V(III) exchanges 6 water oxygens for the 6 oxygens of tris(catecholate) chelation. Likewise, when V(III) tris(catecholate) is oxidized to the homologous V(IV) tris(catecholate) complex, the vanadium K-edge maximum shifts about 5 eV to higher energy.

In Figure 5b, comparison of the K-edges of pentaaquovanadyl ion (vanadyl ion is $\{V^{IV}=O\}^{2+}$) and bis-chelated vanadyl ion shows that complexation shifts the intense 5469 eV preedge feature of the former about 1.1 eV to lower energy,[7] accompanied by a ~50% increase in intensity. The specific identity of the chelating ligand, viz. catecholate vs acetylacetonate, does not appear to have an important effect on the energy position or intensity of the preedge absorption feature.[40] However, at energies *above* the rising edge, the overall shapes of the vanadium K-edge XAS spectra for the two vanadyl chelate complexes are very different, and each is also very different from that of pentaaquovanadyl ion. Thus, changes in ligation and differences in oxidation state can produce large variations both in the energy position of the rising edge and in the overall shape of the vanadium K-edge, even in the V(III,IV) catecholates which are similar in structure and symmetry.[41] Similar effects have been observed in the intense XAS preedge transitions of oxovanadate complexes.[40,42] Such differences provide critical justification for the detailed chemical speciation of heterogeneous samples, based upon an XAS fitting experiment.

Vanadium within blood cells from *A. ceratodes*,[13,14,17,21,24] as well as from some other ascidian species[15,23,43−46] has been shown to be stored principally as the trivalent ion, in the general presence of a high *c*(sulfate) and moderately low to low pH. There has been considerable discussion in the literature about the storage regimes for blood cell vanadium. Proposals have included acid sulfate solution as discussed above, storage within a catecholate-like chelation array,[16,18,20,22,47] and storage in apparently quasi-solid granules,[48−50] though other possibilities remain such as complexation in a weak-field chelate.[18,51]

Within this context, chelation of biological vanadium(III) can occur if this ion encounters either of two (di- or trihydroxyphenyl)alanine species found to be richly present in ascidian blood cells. Of these, tunichrome is a highly modified axially pseudosymmetric dimer or trimer of (3,4-dihydroxyphenyl)-alanine (DOPA) and/or (3,4,5-trihydroxyphenyl)alanine (TOPA) units.[20,22,47] Tunichrome molecules are extensively conjugated, planar, and rigid and are known to complex vanadium(III) in vitro most likely as an amorphous vanadium-cross-linked polymer.[11,20,22] Alternatively, a partially sequenced protein of low molecular weight which contains extensive regions of DOPA and TOPA units has been isolated from ascidian blood.[6,52] This protein is also an in-vivo candidate chelator of biological vanadium(III).[53] Both systems would provide a catecholate-like ligation environment to V(III).

With these considerations in mind, we have focused on modeling vanadium within the blood cells of *A. ceratodes*, using the array of inorganic vanadium species described above as representative of the major chemical and ligation environments which might reasonably be expected to occur. Possible environments for vanadium(III) as potentially solid granules within blood cells were modeled using the vanadium K-edge XAS spectra of crystalline $\{[V(H_2O)_6](H_5O_2)(CF_3SO_3)_4\}$ or of the catecholate or acetyacetonate complexes. The crystalline aqua complex was chosen because it produced a V−K XAS spectrum exhibiting an intense rising edge shoulder at 5476 eV which is absent from the XAS spectrum of $\{V(H_2O)_6\}^{3+}$ in 1 M HCl but present in the XAS spectrum of *A. ceratodes* blood cells. Recognizing the further possibility of, e.g., oxo-bridged multi-

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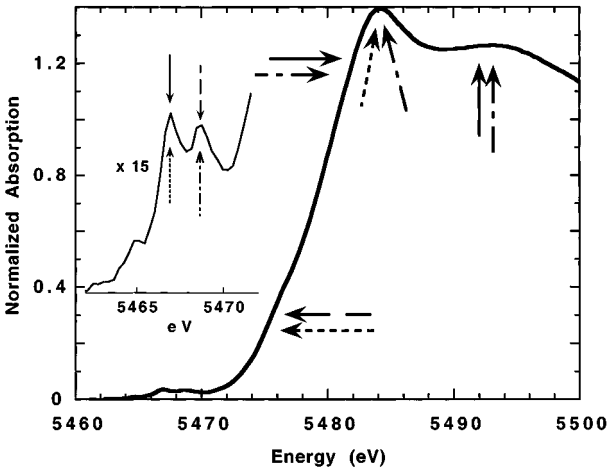

Figure 6. Vanadium K-edge spectrum of whole blood cell sample 1 from the tunicate *Ascidia ceratodes*. The inset shows the expanded preedge region of the spectrum. The overall shape of the spectrum is consistent with primarily aquavanadium(III) (see Figure 1). The arrows indicate the energy regions which are specifically impacted by the various important solution and ligation conditions discussed in the text. These are: (→), V(III) tris-chelation (as in K_3V^{III}(catecholate)$_3$); (— →) aquaV(III)−sulfate interactions; (- - →) effects of pH on aquaV(III); (—•→) vanadyl ion.

nuclear vanadium(III) complexes[51,52,54] in biological storage granules, we are currently extending our model series to include such complexes. However, because the K-edge EXAFS[14,24] of vanadium in *A. ceratodes* blood cells included no observable vanadium−vanadium vector, we considered it empirically reasonable to proceed without specifically including bridged binuclear vanadium.

In Figure 6, we show the vanadium K-edge spectrum from intact blood cell sample 1 (cf. Materials and Methods) from *A. ceratodes*. Following the discussion above, this spectrum is marked in energy regions where effects due to chelation, pH, sulfate complexation (if any), and oxidation state occur. Comparison of Figure 6 with any of the model vanadium K-edge XAS solution spectra in Figure 1 indicates that there is little to obviously distinguish the K-edge XAS spectrum of blood cell vanadium from that of the aqua V(III) ion in dilute sulfuric acid.[24] Vanadium K-edge XAS spectra for 9 discrete samples of intact whole blood cells from *A. ceratodes* have now been obtained, representing both individual organisms and the aggregate from numerous organisms, spanning approximately 18 years and including collections from two well-separated (ca. 200 km) locales. The XAS spectrum of Figure 6 is completely representative.

An extensive series of fits to the blood cell XAS spectra was made using the XAS spectra of V(III) solutions of varying pH or *c*(sulfate) and of solid V(III) complexes. In all tested fits, the XAS spectrum of crystalline $\{[V(H_2O)_6](H_5O_2)(CF_3SO_3)_4\}$ was rejected in favor of that of solution-state V(III)−sulfate. In the consideration of minority species (% ≤ 10), the decision for inclusion involved judgment of necessity as indicated by a decrease in "*F*" ≥ 10%. This cutoff value was not completely arbitrary because (data minus fit) difference spectra constructed from such fits were distinguishable. Additionally, the minimal number of model spectra needed to fit a blood cell spectrum was sought.

Figure 7 shows the final fit to the vanadium K-edge XAS spectrum of *A. ceratodes* whole blood cell sample 1. The first

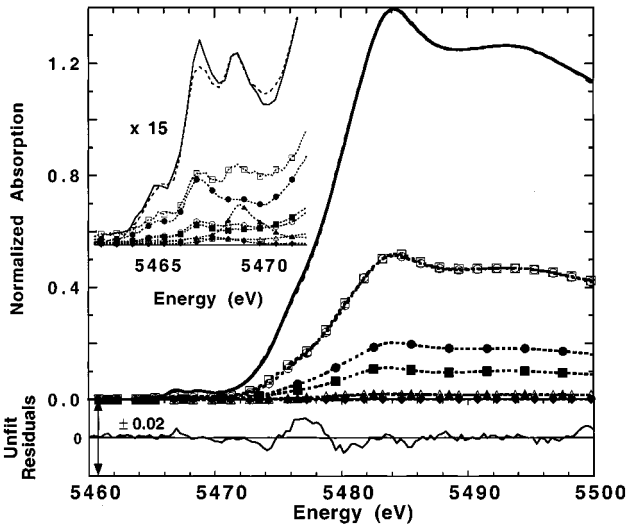

Figure 7. Vanadium K-edge spectrum of: (—) whole blood cell sample 1 from *A. ceratodes*; (- - -) the fit to the spectrum; (• • symbol • •) the components of the fit. Inset: Expansion of the preedge energy region of the spectrum, the fit to the spectrum, and the components of the fit. The unfit residuals (data minus fit) are shown in the plot at the bottom of the figure. The total intensity is ±2% of the normalized edge jump. The components to the fit are: (○) 25 mM $V_2(SO_4)_3$ in pH 1.8 sulfuric acid; (□) 25 mM $V_2(SO_4)_3$ in pH 1.8 sulfuric acid with 988 mM sulfate; (●) 25 mM $V_2(SO_4)_3$ in pH 0 sulfuric acid with 0.52 M sulfate; (■) 25 mM $V_2(SO_4)_3$ in pH 3.0 sulfuric acid; (△) K_3V^{III}(catecholate)$_3$; (▲) 50 mM vanadyl sulfate in 0.1 M sulfuric acid; (◆) $K_2V^{IV}O$(catecholate)$_2$.

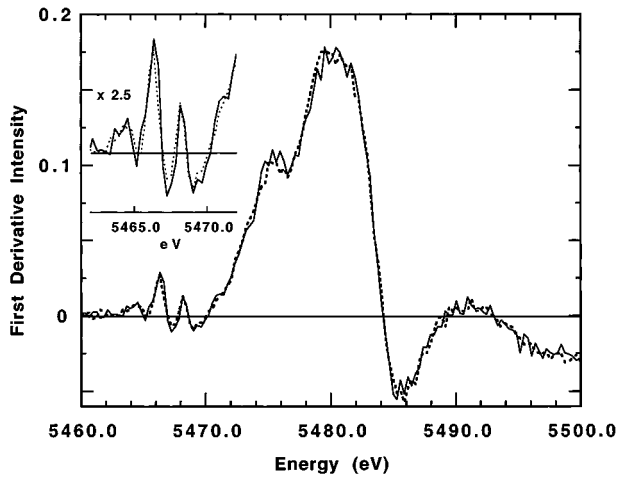

Figure 8. First derivatives of: (—) *A. ceratodes* whole blood cell sample 1 of Figure 7; (• • •) the fit to the spectrum. Inset: Expansion of the preedge energy region.

derivatives of the data and of the final fit, respectively, are shown in Figure 8. The percentages of the model spectra which produced the fit are listed in Table 1, which also includes the results of the fit to *A. ceratodes* whole blood cell sample 2.

In Figure 7 and inset, the vanadium K-edge XAS spectrum of whole blood cells is well fit. We suggest that the presence of the 5476 eV feature on the rising edge of the spectrum of *A. ceratodes* blood cells, obviously present in every single vanadium K-edge XAS spectrum of *A. ceratodes* blood cells obtained thus far, is necessary and perhaps sufficient to demonstrate their physical integrity, because this feature is lost directly upon lysis (see below).

At the bottom of Figure 7, the intensity of the unfit residuals (data minus fit) is small, and there is no remnant edge-jump

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Table 1. Fit-Modeled Environment of Blood Cell Vanadium

no.	model spectrum[a]	whole blood[b] sample 1 (%)	whole blood sample 2 (%)	Henze soln (%)
1	V(III)/pH 0.0, 10× sulfate	14.5	3.4	15.9
2	V(III)/pH 1.6, no sulfate[c]			63.2
3	V(III)/pH 1.8, 20× sulfate	37.8	36.6	
4	V(III)/pH 1.8, 1.5× sulfate	36.0	44.6	
5	V(III)/pH 3.0, 1.5× sulfate	8.2	8.2	
6	V(III) tris(catecholate)	1.5	3.6	17.6
7	vanadyl/0.1 M H_2SO_4	1.8	3.4	2.9
8	vanadyl bis-chelate[d]	0.2	0.3	0.3
	tot.	100.0[e]	100.0	100.0
	goodness of fit "*F*"	0.985E−5	1.656E−5	2.434E−5

[a] The compositions of the model solutions are specified in Materials and Methods. [b] The values refer to the percent of each component spectrum used in the fit. [c] The model solution contained sulfate ion, but the XAS spectrum showed no observable complexation features. [d] The preedge feature due to complexed vanadyl ion could be equally well fit using the vanadium K-edge XAS spectrum of vanadyl bis(catecholate) or vanadyl bis(acetyacetonate). [e] The fits were normalized to 100.0%. The sum of the components may not equal this value due to round-off error.

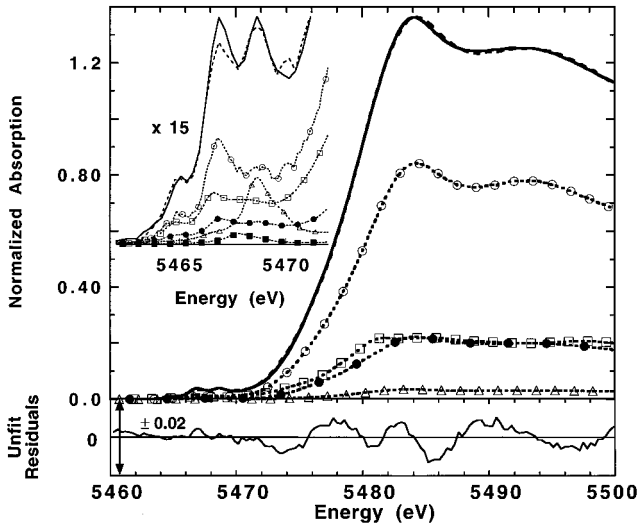

Figure 9. Vanadium K-edge spectrum of (—) freshly prepared Henze solution, (- - -) the fit to the spectrum, and (· · symbol · ·) the components of the fit. Inset: Expansion of the preedge energy region of the XAS spectrum, showing the fit and the fit components. The unfit residuals (data minus fit) are shown in the plot at the bottom of the Figure. The components to the fit are: (○) 25 mM $V_2(SO_4)_3$ in pH 1.6 40% methanolic perchloric acid; (●) 49.6 mM V(III) with 446 mM sulfate in pH 0 sulfuric acid; (□) $K_3V^{III}(catecholate)_3$; (△) 50 mM vanadyl sulfate in 0.1 M sulfuric acid; (■) $K_2V^{IV}O(catecholate)_2$.

which might demonstrate a systematically unmodeled XAS spectral component. The principal residual intensity is concentrated in the rising edge region near 5476 eV, which we ascribe to differences in detail between the certainly finely graded set of V(III) sulfate conditions[13] maintained by the millions of blood cells and the necessarily cruder approximation to this gradation approached by the use of a small number of model solution spectra. The correspondence between the first derivative of the preedge features and the first derivative of the fit (Figure 8) is shown to be excellent. This correspondence is very telling, because a fortuitous first-derivative conformity seems highly unlikely.

In Figures 9 and 10 are shown the final fit to the vanadium K-edge XAS spectrum of a sample of Henze solution (lysed blood cells) and the comparison of the first derivatives of these

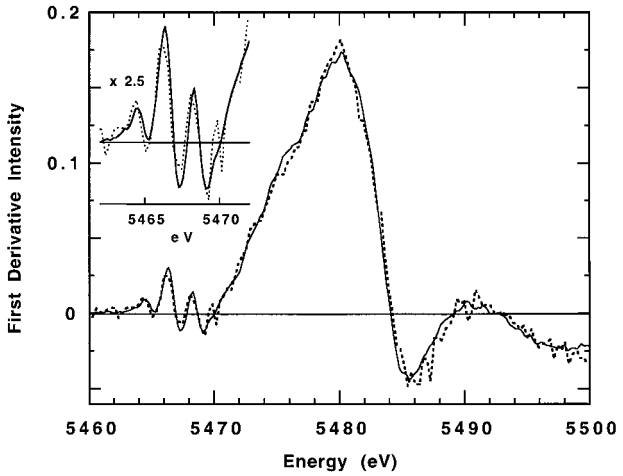

Figure 10. First derivative of (—) the vanadium K-edge XAS spectrum of *A. ceratodes* Henze solution of Figure 9 and (· · ·) the fit to the spectrum. Inset: Expansion of the preedge energy region. Note the virtually complete loss of the inflection feature near 5476 eV.

data, respectively. The fit to the Henze solution XAS spectrum is not quite as good as the fit to the blood cell spectra (cf. the respective *F* values in Table 1). Because vanadium binds to cell fragments on blood cell lysis,[17] the resulting microscopic heterogeneity of vanadium ligation environments is likely to be large. Nevertheless, the correspondence between data and fit is good.

The major component of the fit to the Henze solution vanadium K-edge XAS spectrum, accounting for 63% of the intensity, was the K-edge XAS spectrum of vanadium(III) in pH 1.6 solution and with no V(III)−sulfate interaction. A smaller quantity of V(III) (15.9%) was modeled by V(III) in a high acid, high-sulfate environment. However, fits that were nearly as good were found in which this latter component was represented by the XAS spectrum of V(III) in a high-acid, *low*-sulfate environment or in 1 M HCl. In these alternative fits, the percent of V(III) in pH 1.6 solution increased to about 76%, while the V(III) in strong acid decreased to about 7%. The other components of the fit remained nearly constant. Therefore, some ambiguity remains in the sulfation environment of V(III) in Henze solution. However, all these fits have in common a V(III) environment dominated by an acidic solution in which interaction with sulfate ion is low to absent. The total percent of V(III) in this circumstance was always about 80%. Most of the rest of the XAS intensity was modeled by the K-edge spectrum of vanadium(III) tris(catecholate) (Table 1).

Discussion

In this work we have shown that the sensitivity of K-edge XAS spectroscopy to variations in the chemical environment of transition metals such as vanadium ion can be used to successfully model the complex environments of such ions in naturally occurring materials. The K-edge XAS spectrum of V(III) in aqueous solution is a sensitive indicator of both solution *c*(sulfate) at constant pH and of solution pH at constant *c*(sulfate). Although the effects of pH or *c*(sulfate) upon the equilomeric composition of V(III)−sulfate solutions are complex,[38] the effects on the K-edge XAS spectrum of solution V(III) as discussed above systematically and rigorously reflect the response of aquaV(III) to pH and *c*(sulfate). Therefore, XAS spectroscopy can be used to describe blood cell vanadium within *A. ceratodes* in terms of inorganic models that can distinguish among proposed storage regimes and which are consistent with the intracellular environment as deduced by other methods.

The vanadium K-edge XAS spectra of model solutions comprised of V(III) in pH 1.8 sulfuric acid at various *c*(sulfate) were found to dominate the fits to blood cell XAS spectra. From Table 1, 96.5% of the vanadium in the intact whole blood cells in sample 1 and 92.8% in sample 2 corresponded to V(III) ion in acidic aqueous sulfate solution. This finding is fully consistent with the results of vanadium K-edge EXAFS analysis[14,24] and of ^{1}H NMR and EPR spectroscopies.[13,17,21] The environment of endogenous V(III) ion can be further divided into the four primary solution regimes as listed in Table 1 (i.e., 1, 3, 4, and 5). Following calculation of the distribution of vanadium(III) complex ions in the various model solutions, one can deduce a detailed chemical speciation of blood cell vanadium. This is discussed below.

The intracellular acidity represented by the small but significant contributions from the XAS spectra of a high-*c*(sulfate) pH 0 V(III) solution are relatively rare in biology but not unknown. For example, algal cells of the genus *Desmarestia* are known to contain 0.5 M sulfuric acid,[55,56] and the predatory snail *Casidaria echinophora* was found[57] to eject a defensive secretion consisting of pH 0.1 sulfuric acid. This acid is apparently resident within intracellular vacuoles of the buccal gland tissues. In Notaspid snails, sulfuric acid to pH 1−2 is secreted from skin cells in a defensive response[58] that is apparently widespread among marine gastropods.[59] As an interesting aside, the apparently exclusive use of sulfuric acid among marine organisms which make acid, in the ubiquitous presence of chloride, appears to demand an explanation. One possible rationale is that, in contrast with chloride, at any pH between 0 and 3 the buffering effect of sulfate can provide an available acidity far in excess of that indicated by pH.

The finding that an appreciable fraction of the blood cell vanadium K-edge XAS could be represented by a pH 3.0 model solution was unexpected. In this model solution 93.8% of the V(III) was calculated[38] to be resident in the complex ion $\{V(H_2O)_3(SO_4)(OH)_2\}^-$, with about 3.2% of the monodeprotonated aqua-ion $\{V(H_2O)_5(OH)\}^{2+}$ and about 1% percent each of the free hexaaqua ion and the monosulfate complex ion. The proportionate ligation of V(III) by sulfate increases with pH[38] because the concentration of free SO_4^{2-} also increases with pH. Thus the prominent feature at 5476 eV observed in the vanadium K-edge XAS data of this solution (cf. Figures 3, 4) is more intense than in data derived from 50 mM $V_2(SO_4)_3$ in pH 1.8 solution because only 52.7% of the V(III) is sulfated at pH 1.8. After taking this factor into account, the intensity of the 5476 eV XAS feature of the pH 3.0 model solution falls below the line which relates the three V(III)−sulfate pH 1.8 model solution XAS spectra (e.g., cf. Figure 3, inset b, and Figure 1, inset b). Therefore, the presence of hydroxide in the ligand sphere of sulfated V(III) apparently suppresses the intensity of the 5476 eV transition.

The blood cell fits also required only very minor percentages of the V(III) tris(catecholate) model. This corroborates the finding of no evidence for chelated V(III) in the vanadium K-edge EXAFS of whole blood cells from *A. ceratodes*.[14,24]

Following the successful fits, the equilibrium constants and pK_a's governing V(III) and sulfate in common aqueous solution[38] were used to speciate the sulfate-containing V(III) model solutions in terms of explicit V(III) complex ions. Then the proportions of these V(III) complex ions represented by each of the model solution percents in Table 1 were calculated. The results were combined to produce a complete chemical model for the equilibrium distribution of solution-phase biological V(III) for each of the blood cell samples. This result is shown in Table 2. The outcomes are compared in Table 2 with the vanadium(III) speciation for 0.05 M $V_2(SO_4)_3$ in pH 1.8 solution alone. This comparison is worthy because the concentration of V(III) in *A. ceratodes* blood cells averages 0.11 ± 0.06 M.[16,17,26,54]

Table 2. Comparative Speciation of Endogenous Vanadium in *A. ceratodes* Blood Cells

V(III,IV) species	blood cell sample 1 (%)	blood cell sample 2 (%)	pH 1.8 sulfate[a] (%)
$[V(H_2O)_6]^{3+}$	27.2	21.2	38.0
$[V(H_2O)_5SO_4]^+$	37.1	38.8	47.7
$[V(H_2O)_4(SO_4)_2]^-$	19.9	19.8	5.1
$[V(H_2O)_5(OH)]^{2+}$	2.6	3.2	6.0
$[V(H_2O)_4(SO_4)(OH)]$	0.7	0.7	0.2
$[V(H_2O)_3(SO_4)(OH)_2]^-$	7.7	7.7	0.0
$[V_2O(H_2O)_{10}]^{4+}$	0.6	0.7	1.5
$[V(catecholate)_3]^{3-}$	1.5	3.6	
VO^{2+}, aq ion	1.8	3.4	
VO(bis-chelate)	0.2	0.3	
sulfate/$V(III)_{aq}$	0.89	0.87	0.58

[a] 0.05 M $V_2(SO_4)_3$ in pH 1.8 sulfuric acid solution.

Thus, relative to the pH 1.8 solution, the data in Table 2 indicate that within *A. ceratodes* blood cells, the hexaaquo ion, and the monosulfate complex ion have decreased to the benefit of the more highly sulfated complex ions and the dideprotonated sulfato homolog, $\{V(H_2O)_3(SO_4)(OH)_2\}^-$. The average number of sulfate ligands complexed to V(III) in blood cell sample 1 is 0.89 and is 0.87 in blood cell sample 2 but is 0.58 in the pH 1.8 model. That is, the complexation of blood cell V(III) by sulfate is about 1.5• that expected if it were purely 0.05 M $V_2(SO_4)_3$ dissolved in pH 1.8 solution.

Following lysis only two vanadium(III) environments were found to be significant in fresh unoxidized Henze solution (Table 1): acidic aqueous solution with little or no sulfate ligation, and chelation in a catecholate-like array. The vanadium K-edge XAS of Henze solution lacks the 5476 eV feature characteristic of sulfate ligation (Figure 9), and so the majority form of lysate vanadium is considered to be either aquated or possibly in a partially aquated weak-field complex such as carboxylate. The lack of any significant vanadyl/vanadate feature near 5469 eV in Figure 9 establishes beyond dispute that lysis of blood cells is not necessarily accompanied by any significant oxidation of liberated V(III). Therefore the acidity immediately produced in Henze solution need not be ascribed to a vanadium(III) oxidation process. The chelated V(III) amounted to nearly 18% of the released vanadium and was modeled by the tris(catecholate) complex. This result implies that some of the released V(III) ion was captured by DOPA/TOPA-like molecules. From the concentration range of blood cell V(III) in *A. ceratodes* (see above), and the percent of tris-chelated V(III) in Henze solution, one can calculate that the acid produced by liberation of six protons on substitution of six water ligands by, e.g., tris((−6H)-tunichrome) ligation, would result in values of 1.6−2.1 for solution pH. This estimate ignores cytosolic buffering and is both in addition to, and approximately equal to, the average intracellular acidity marking vanadium storage as

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deduced by a variety of methods.[15−17,21,23,43,60] It is likely that the distribution of V(III) among the various lysate environments will depend strongly on the manner of lysis and the treatment history. Liberated blood cell vanadium is noninnocent and has open reaction pathways. Thus, the results presented here bring into sharp focus the ascidian blood cell chemistry generally implied by other methods.

Overall, blood cell vanadium in *A. ceratodes* was found to closely follow the model proposed in 1966 by Bielig et al.[45] following their work on the chemistry of blood cells from *Phallusia mamillata*, that is, "an equilibrium, determined by the pH value (<4), between different sulfatovanadium(III) ions, proteinium- and hydrogen sulfate ions."

The chelating ligand 2,2′-bipyridine has been found to invariably stain signet-ring and other vacuolated ascidian blood cells due to intracellular formation of the $[V_2O(bipy)_4]^{4+}$ complex ion,[8,51,61] indicating the presence of available V(III). However, morula cells, which are also known to contain vanadium, at times do not stain with this ligand.[8,51] Bipyridine is known to be incapable of displacing catecholates from a complex with V(III) in acetonitrile solution, and this chemistry has been adduced to explain the lack of cytochemical staining of morula cells.[62] It would be worthwhile testing whether this order of ligand displacement extends to aqueous solution. If so, then all vacuolated ascidian blood cells rapidly staining with bipyridine would likely contain endogenous aquated vanadium(III)−sulfate as described herein. Such a finding would make bipyridine staining an easy and general diagnostic test for this metallobiochemical regime.

The utility of vanadium(III) and acid to ascidians remains obscure. Vanadium- and sulfur-containing cells have been implicated in the immune response of some ascidians.[63,64] Stroecker has reported evidence that both acid and vanadium are repellent to generalized ascidian predators, such as fish and crabs,[65] as well as to fouling epibionts,[66] though these results have been disputed (see below). Blood cells are known to collect at the site of a wound and to lyse there releasing vanadium and other materials,[6] implying involvement in wound-healing or perhaps antisepsis. Admixture of aquaV(III) with tunichrome[6,11] or tunichrome models[67] is known to produce insoluble polymeric materials. The XAS results above indicate the relative stability of such a polymer even in acid.

The universal deterrance of predation by vanadium and acid is contradicted by the observation that several predatory gastropods including *Fusitriton oregonensis*, which is found on the Pacific north coast of North America, readily attack ascidians with vanadium- and acid-containing blood cells.[68,69] In addition, the defensive efficacy of vanadium and acid has been disputed on the basis of the observed clearance of acid- and vanadium-containing ascidian residents from noncryptic habitats.[69,70] Therefore the defensive utility of vanadium and acid to ascidians is problematic at best. However, the predatory Notaspid gastropod *P. californica* is strongly repelled by both acid and taurine.[58] Taurine may be the source of the intense aliphatic sulfonate signal we have detected in whole blood cells from *A. ceratodes* using sulfur K-edge XAS.[25−27] Definitive conclusions regarding chemical deterrance are also made problematic by the observation that the avoidance behavior of predatory snails following exposure to noxious chemicals is influenced by the hunger/satiation state of the snail.[71]

It may be worth observing from an evolutionary perspective that useful deterrence is not necessarily an all-or-none phenomenon. Any given prey species is likely to deploy a variety of defensive strategies to deter the variety of predators. The presence of high levels of vanadium among ascidians has been suggested to be an ancestral trait, lost through more recent evolutionary developments among, e.g., the Stolidobranchs.[72,73] Therefore, the existence of only some ascidian species still deploying acid and vanadium, along with the partial suite of predators that are not thus deterred, may comprise a snapshot of ongoing adaptive coevolution.

It is interesting to note that all five gastropods mentioned as specifically preying on ascidians which contain vanadium, and thus acid, i.e., *Lamellaria, Cypraea, Velutina, Pleurobranchus*,[58,69,70] and *Fusitriton*,[68] are themselves known to deploy defensive and/or predation-assisting sulfuric acid. It may be that these predators have learned to recruit the defenses of their prey, as in the case of the nudibranch *C. luteomarginata*.[74] We are not aware of any reports describing tissue assays for vanadium in the above predatory gastropods. It may be worthwhile investigating whether the vanadium they consume with ascidian prey is recruited and deployed into surface tissues to deter predation on themselves.

Finally, we have shown that with a proper library of model XAS spectra, X-ray absorption edge spectroscopy can be used to extract detailed information regarding the in-situ speciation of a metal ion within a manifold of complex biological milieus. This method is readily applicable to other areas which face the problem of multifarious metallic environments, such as in characterization of nuclear wastes[75,76] or in the chemical speciation of complex geological minerals.[77] Similar approaches to the speciation of iron and sulfur in coals,[78,79] sulfur in equine blood, asphaltenes, and soils,[12,80,81] and vanadium in tunichrome polymers[5,11] have also been useful. In future work, we will extend the XAS fitting approach to the study of intra- and interspecific differences in vanadium and sulfur storage among ascidians at the population level. Using K-edge XAS spectroscopy, we and others have recently shown[7] that endogenous

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vanadium in *Ascidia (Phallusia) nigra* blood cells is almost certainly extensively chelated. In addition, correlation of types and environments of blood cell vanadium with accurate blood cell counts should help illuminate the distribution of vanadium among specific blood cell types in different ascidian species or in the same species in different locales.

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