Nature and Ligation of Vanadium within Whole Blood Cells and Henze Solution from the Tunicate *Ascidia ceratodes*, As Investigated by Using X-ray Absorption Spectroscopy

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Vanadium K-edge X-ray absorption spectroscopy (XAS) studies have been carried out at 10 K on packed whole blood cell samples and on Henze solution from the tunicate *Ascidia ceratodes* (*A. ceratodes*). High energy-resolution vanadium K-edge spectra exhibit pre-edge transitions at 5464.9 ± 0.1, 5466.8 ± 0.1, and 5468.8 ± 0.1 eV for both whole blood cell samples and Henze solution. The whole blood vanadium K-edge spectrum is very similar to that of vanadium(III) in aqueous sulfuric acid solution. Both spectra exhibit a feature at 5476 eV indicative of an endogenous vanadium(III)−sulfate interaction. This represents the first direct spectroscopic evidence for intracellular $[(VSO_4)(H_2O)_{4-5}]^+$. Absence of the vanadium(III)−sulfate feature in the vanadium X-ray absorption edge spectrum of Henze solution indicates loss of the sulfate ligand on dilution of the intravacuolar contents following whole cell lysis. Vanadium K-edge EXAFS analysis of the whole blood samples revealed about six nearest neighbor oxygen (or nitrogen) atoms at 1.99 ± 0.02 Å. No evidence either for the more distant carbon shells of an intracellular vanadium chelate or for $(VOV)^{4+}$ dimers, was found in the EXAFS spectra of whole blood cells. Since the XAS spectra were taken at 10 K, the likelihood that significant amounts of such species remained undetected is remote. Taken together, the results are consistent with the proposition that blood cell vanadium, at least within the tunicate *A. ceratodes*, is >90% monomeric, existing as a mixture of the hexaaquovanadium(III) ion and the $[(VSO_4)(H_2O)_{4-5}]^+$ complex ion. The possible presence of up to 10% of, *e.g.*, a tris-chelated tunichrome−vanadium(III) complex is not excluded. Within Henze solution, vanadium K-edge and EXAFS spectral analysis likewise indicated unoxidized, monomeric V(III) with six to seven first shell oxygens at 1.99 ± 0.02 Å but with no indication of a sulfate interaction.

Introduction

The chemical speciation of vanadium in the blood cells of ascidians has been the subject of considerable interest and intense discussion for some time.[1−27] Phlebobranch ascidians are sessile, filter-feeding marine animals[28] which remove vanadate from seawater and concentrate it 10^3−10^6-fold within blood cells, primarily in the air-sensitive oxidation state vanadium(III).[4,6,10,16,18−20,26] The question as to whether this trivalent metal is chelated *in vivo*[1,29−33] or alternatively exists as the aquo ion, possibly in a low-pH endogenous solution[11,13,14,17,19−21,27,34,35] has been central to proposed mechanisms of vanadium uptake and storage within blood cells.[1,2,26,36] In

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addition, the nature of vanadium in lysed blood cells, so-called Henze solution, also remains in question.

X-ray absorption spectroscopy (XAS) has enjoyed extensive use as an element-specific probe of the *in situ* nature of biologically important metal ions.[37] A previous study of blood cells from the tunicate *Ascidia ceratodes* (*A. ceratodes*),[13] using both X-ray absorption edge spectroscopy and extended X-ray absorption fine structure (EXAFS) spectroscopy at the vanadium K-edge, found no evidence for endogenous chelated vanadium. However, this study was carried out at room temperature, and thus the possibility remained open that the EXAFS signal from any chelating ligand was diminished as a result of thermal disorder. In addition, since these prior experiments, very significant improvements have accrued in the quality and flux of synchrotron X-ray sources and in data collection methods. These advances have significantly enhanced the quality of the XAS data that can be collected. An examination of blood cell vanadium EXAFS, acquired at 10 K, thus offered the likely resolution of persistent questions regarding the dominant storage mode of endogenous vanadium(III). In addition, newly acquired high energy-resolution XAS edge spectra are shown to reveal novel aspects of the electronic state, symmetry, and ligation of endogenous V(III).

Since packed whole blood cell samples from *A. ceratodes* were investigated, the results reported here do not discriminate among the different blood cell types[38] that accumulate vanadium.[20,23,27,34,39] The advantage of this approach is that all possible variations in vanadium environment within the blood cells of this organism were queried. In addition, systematic errors which might arise from nonrandom losses within a specific blood cell type (*e.g.*, signet ring cells) during blood-cell-sorting experiments[20,39] were thereby avoided. Extension of this research to blood cell samples of known cell count is anticipated.

Carrying out XAS experiments at 10 K, we have thus examined the question of the state of vanadium as it exists both in intact ascidian blood cells and in freshly lysed blood cells (Henze solution). Both high energy-resolution V K edge and EXAFS spectra have been acquired and are evaluated in detail. The question which remains outstanding of the *in vivo* ligation of vanadium is specifically addressed. The results of comparisons of blood cell vanadium with vanadium in structurally known chelating environments consistent with proposed biological models are also given. The vanadium K-edge XAS experiments reported herein have bearing on the valence, chemical status, and endogenous environment of this metal ion within whole blood from the tunicate *A. ceratodes*.

Materials and Methods

Sample Preparation. Specimens of *A. ceratodes* were collected from the Monterey Yacht Harbor in Monterey, CA, transported to Stanford in cool seawater, and acclimated overnight in an aerated, 4 °C seawater aquarium. Whole blood was obtained by heart puncture using sterile 1 cm^3 plastic syringes or (sample KR2) by pipetting from the body cavity, following removal of a portion of the ventral tunic using a scalpel. Blood samples were temporarily kept on ice in sterile plastic tubes prior to final workup.

In order to minimize ice formation on XAS sample freezing, blood plasma was exchanged for 0.5 M NaCl in 30% aqueous ethylene glycol. Prior room temperature microscopic examination of blood cells suspended in this solution revealed no sign of discoloration or lysis. Thus, the blood cells were layered onto cold 0.5 M NaCl in 30% aqueous ethylene glycol and centrifuged directly into a Lucite XAS sample cell, held within a specially designed receptacle and submerged within the same solution. The centrifugation was carried out at 4 °C for 10 min at 250*g* using a swinging bucket rotor. The resulting densely packed sample was inspected through the 38 μm thick Kapton window of the Lucite sample cell and exhibited no evidence of lysis, such as discoloration. In a typical XAS sample the blood cells from about 30 animals were pooled. XAS samples were immediately frozen by submersion in liquid nitrogen, and stored at 77 K at all times prior to acquisition of the XAS data. The Lucite cells had an internal volume of 140 μL (samples AB2, PF3, Henze solution) or 80 μL (sample KR2).

Henze solution was prepared by thawing a blood cell sample and stirring the cell mass within the Lucite XAS sample cell at ambient temperature for about 1 min, using a thin stainless steel rod. The initially yellow-green blood cell sample became the typical red-brown Henze solution. The Lucite sample cell was recapped, and the Henze solution was frozen by immersion of the sample cell into liquid nitrogen.

Blood cell vanadium was analyzed by digestion of each sample, including the Lucite XAS sample cell, in 10 mL of refluxing Fisher Optima (trace metal grade) HNO_3. Upon evaporation to dryness, residues were redissolved in 10 mL of 20% HNO_3. Vanadium concentration was measured in appropriately diluted samples using graphite furnace atomic absorption spectrometry, with Zeeman background correction. Analytical precision was checked by periodically analyzing diluted vanadium standards and NBS 1643 (trace elements in water) standard reference material. Accuracy was checked by reanalysis and by performing standard additions on selected samples. Control analyses on three empty Lucite XAS sample cells revealed negligible vanadium (26.2 ± 5.7 ng).

Potassium tris(catecholato)vanadium(III) ($K_3V(cat.)_3$) and triethylammonium tris(catecholato)vanadium(IV) ($((Et_3NH)_2V(cat)_3$) were prepared by the literature method.[40] Vanadium(III) tris(acetylacetonate) ($V(acac)_3$) was purchased from Aldrich Chemical Co. and recrystallized from methanol/water using standard anaerobic Schlenk techniques. Appropriate amounts of these compounds were finely ground and mixed with boron nitride powder under anaerobic conditions. Each solid sample was then mounted in a 0.5 mm thick aluminum spacer and sealed with 38 μm thick Kapton tape windows. The solid samples were stored under a dinitrogen atmosphere before use. Stock 0.5 M $V_2(SO_4)_3$ solution in 15 mM sulfuric acid was prepared as described earlier.[35] Preparation of the inorganic solution samples was carried out under a dinitrogen atmosphere within a PlasLabs (Lansing, MI) Plexiglas glovebox. The 0.1 M V(III) solution sample was prepared in 9 M sulfuric acid by appropriate dilution using Hamilton gas-tight micro-syringes. The Lucite XAS solution sample cell was then filled, and sealed, and the sample was frozen into a glass by rapid immersion in liquid nitrogen.

XAS Data Collection. XAS experiments were carried out at the Stanford Synchrotron Radiation Laboratory (SSRL) on wiggler beam line 7-3, using an 18 kG wiggler field, under dedicated operating conditions of 3 GeV and 50−90 mA of current, and using a Si(220) double-crystal monochromator, which was detuned 50% at 6336 eV to minimize harmonic contamination. Fluorescence excitation X-ray spectra (blood cells and solution samples) were collected using an Ar-filled fluorescence ionization chamber detector (Stern-Heald-Lytle detector) with Ti-filter and Soller slits, set at 90° from the incident X-ray beam. Transmission X-ray spectra (solid model complexes) were obtained using N_2-filled ionization chambers. Vanadium foil calibrations were collected after every three to four data scans during fluorescence data collection or were acquired simultaneously with transmission data using a third N_2-filled ionization chamber placed after

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the foil. All XAS samples were held at 10 K using an Oxford Instruments CF1208 continuous flow liquid helium cryostat.

XAS Data Processing and EXAFS Fitting Procedures. Raw XAS data were processed into XAS edge spectra and EXAFS spectra, as described earlier in detail.[37a,41] Spectra were calibrated to the first inflection point on the rising edge of the spectrum of the vanadium foil standard, which was assigned to 5464.0 eV. The normalized, background-subtracted data were converted into k-space by assuming a threshold energy of 5489.0 eV. All EXAFS fits and Fourier transforms (FT) were k^3 weighted, where "k" is the photoelectron wave vector in units of Å^{-1}. EXAFS data were truncated at $k = 13$ Å^{-1} to avoid ice crystal diffraction spikes in the blood cell and aquovanadium(III) EXAFS spectra. Data were analyzed over the range of $k = 4-13$ Å^{-1}, or $k = 4-11.5$ Å^{-1} (sample KR2).

For fits to the EXAFS spectra, a total of four independent sets of empirical oxygen atom parameters were extracted[37a,41] from the Fourier-filtered EXAFS spectra of $V(acac)_3$ and $K_3V(cat.)_3$. Each filtered spectrum representing the V–O interaction was obtained by back-transformation of data windowed from the $R \sim 1.6$ Å peak (see Results and Discussion) of the respective Fourier transform spectrum of each model compound. All FT spectra were back-transformed with a Gaussian line shape of a 0.1 Å half-Gaussian half-width applied to the end points. Wide window parameter sets were obtained using a back transform range of $R = 0.6-2.1$ Å. Narrow window parameter sets were obtained from data by the equivalent back-transform over the range $R = 1.1-2.1$ Å. Vanadium–oxygen distances and coordination numbers were obtained from the known crystal structures of the above model compounds.[40,42]

Empirical second shell carbon parameters were obtained from the vanadium K-edge FT spectrum of $K_3V(cat.)_3$. The Fourier peak at about 2.5 Å was extracted over a range of 2.1–2.8 Å. The first shell V–C bond length of 2.96 Å was obtained from the crystal structure of the complex.[40]

Each of the two empirical oxygen parameter sets (wide and narrow) obtained from $V(acac)_3$ were validated by fitting both the equivalently Fourier-filtered EXAFS spectrum of $K_3V(cat.)_3$ and the unfiltered spectrum. Likewise, those obtained from $K_3V(cat.)_3$ were validated by fits to both Fourier-filtered and unfiltered EXAFS spectra of $V(acac)_3$. The wide parameter set obtained from the $V(acac)_3$ data yielded the most accurate coordination number (CN) and bond length relative to the requisite crystal structure standard and were used in the subsequent data analyses. However, the results of all of the fits to a given data set, using the empirical parameter sets obtained either from $V(acac)_3$ or from $K_3V(cat.)_3$, were the same within the limits of error of the method (±0.02 Å in bond length; ±25% in CN).

The wide oxygen parameter set from $V(acac)_3$ yielded for $K_3V(cat.)_3$ (CN, $(V-O)_{av}$: crystal structure; EXAFS fit): 6.0 oxygens at 2.013 ± 0.009 Å ; 6.1 oxygens at 2.00 ± 0.02 Å. In this particular method of empirical EXAFS data analysis, non-integer coordination numbers arise primarily from imperfect transferability of the empirical parameters and differences in the thermal or static distribution of the ligands in a given shell.

Results

The XAS experiments described herein were designed to investigate the *in vivo* status of *A. ceratodes* blood cell vanadium. This metal is known not to participate in dioxygen transport[12] yet averages ~0.07–0.2 M in blood cells.[16,19,26,35] We report here vanadium K-edge XAS experiments performed on three whole blood cell packs and one sample of Henze solution. Vanadium analysis on these samples revealed the following (sample, $V_{\mu g}$: KR2, 304.6; AB2, 2128.3; PF3, 1476.7; Henze solution, 1899.9.

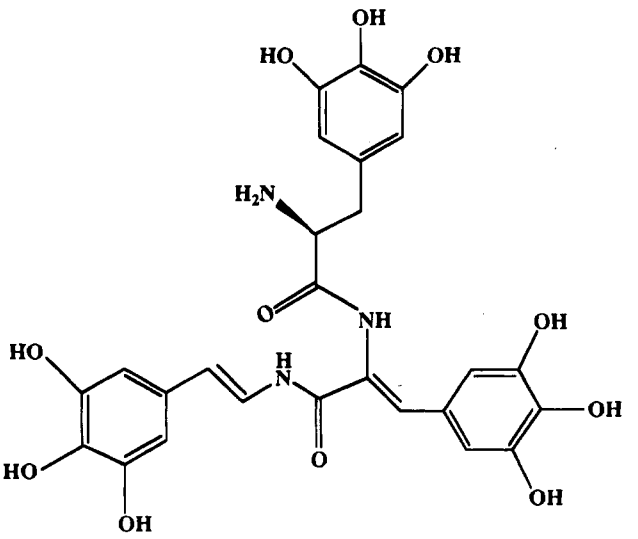

Figure 1. Structural formula for tunichrome An-1, as isolated from the blood cells of the tunicate *A. ceratodes*.[30] Complex formation with vanadium(III) ion would most likely involve chelation by the phenolic hydroxyls. Tunichrome An-1 is highly conjugated, and polymeric materials appear to be the major product following reaction with vanadium ion *in vitro*.

Possible storage modes of blood cell vanadium which have been discussed have included chelation within a biological ligand[1,16,25,30,31,32], which has been suggested to be tunichrome. The structure of tunichrome An-1, which can be isolated from *A. ceratodes* blood cells, is shown in Figure 1. Alternatively, V(III) has been suggested to be stored in blood cells as the aquo ion in an endogenous acidic solution.[11,13,14,17,19–21,24,27,34,35] The experiments described below address these questions.

Tunichrome and analogous blood cell materials may be ubiquitous within tunicates.[29–31,43,44] As a ligand, tunichrome An-1 would likely bind vanadium through the phenolic oxygens in a manner analogous to catechol (1,2-dihydroxybenzene) or pyrogallol (1,2,3-trihydroxybenzene). Thus, vanadium(III)–tris(catecholate) is likely to be a good model for a putative blood cell vanadium(III)–tunichrome chelate complex.[31,43,45–47]

Blood Cell Vanadium K-edge XAS Spectra. Discrete transitions imposed on the K-edge jump in X-ray absorption spectra typically derive from 1s → valence transitions and are subject to normal symmetry-related selection rule restrictions.[37] Thus, in first row transition metal complexes, 1s → 3d transitions are strongly forbidden in a centrosymmetric field (such as within an octahedral complex) and typically produce very weak K-edge XAS features occurring at energies just below the main rising edge. For recent, more thorough discussions of XAS edge spectra, see refs 37b,c.

In Figure 2 is shown a comparison of the vanadium K-edge XAS spectra of authentic vanadium(III)–tris(catecholate), of 0.1 M V(III) in a frozen 9 M sulfuric acid glass, and of a packed whole blood cell sample from *A. ceratodes*. The 1s → 3d region of each spectrum is shown in the inset to the figure. Three

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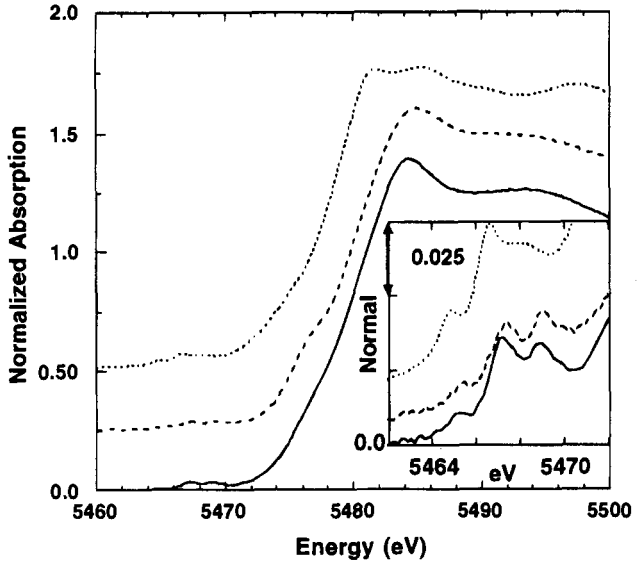

Figure 2. Vanadium K-edge XAS spectra of (—) a packed whole blood cell sample from *A. ceratodes*, (--) 0.1 M V(III) in 9 M sulfuric acid, and (•••) solid $K_3V(cat.)_3$ ground with boron nitride. The latter two spectra have been shifted vertically by 0.25 and 0.5 units, respectively, for clarity. Inset: Detail of the same spectra showing an expanded view of the 1s → 3d transitions. The double-headed arrow marks 0.025 intensity units. Zero intensity occurs at the abscissa. The clear and preferential similarity of the blood cell vanadium spectrum with that of V(III) in sulfuric acid is evident not only in the near superposition of the metal 1s → 3d transition features (inset) but also in the analogously smooth and featureless spectra above the edge and the energy position of the rising edges.

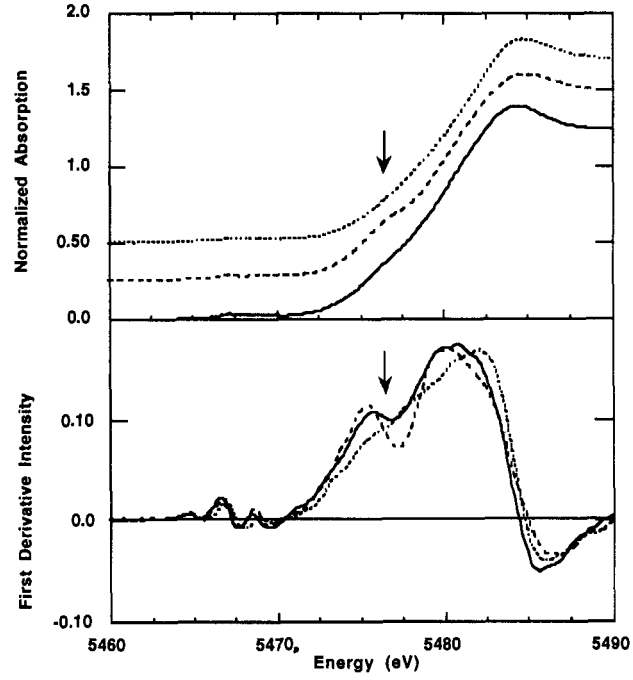

Figure 3. (a, top) Vanadium K-edge spectra of (—) the whole blood cell sample of Figure 2, (--) 0.1 M V(III) in a frozen 9 M sulfuric acid glass, and (•••) 0.1 M V(III) in 1 M HCl frozen solution. (b, bottom) First derivative of the same K-edge spectra. In b, the arrow at 5475.5 eV marks the position of the sulfate-related inflection corresponding to the shoulder (arrow in a) on the rising slope of both whole blood cells and the inorganic vanadium(III) sulfate. For V(III) in 1 M HCl both the K-edge shoulder and the K-edge first derivative feature are absent. This is evidence for an endogenous vanadium(III)—sulfate interaction within whole blood cells from *A. ceratodes*, in contrast with those of Henze solution shown in Figure 6.

transitions are seen in each spectrum, as has been described for the K-edge XAS spectra of other six-coordinate vanadium(III) centers.[48] The high signal-to-noise ratio of the spectra and the resolution of the relatively closely spaced 1s → 3d transitions indicate the high quality of X-ray spectra available, which now permit detailed comparative examinations.

Inspection of the edge spectra (Figure 2) reveals that the X-ray spectrum of V(III) in the 9 M sulfuric acid frozen glass is a very close match with that of blood cell vanadium. This is strong evidence that the ligand environments of the vanadium in these two systems are very similar. The blood cell X-ray spectrum is very similar to the known K-edge spectrum of $V(III)_{aq}$ (see refs 13 and 49, vide infra) and is similar in shape to the K-edge spectra of other first row aquo-transition metal ions.[50,51] In contrast, the position of the rising edge of vanadium(III)—tris(catecholate) occurs about 2 eV to lower energy than do those of blood cell vanadium and V(III) in the sulfuric acid frozen glass. Additionally, the overall shape and intensity of the X-ray spectrum of $K_3V(cat.)_3$ at energies above the edge (*e.g.*, features at 5482 and 5498 eV) are quite different from the blood cell or $V(III)_{aq}$ spectra.

The K-edge XAS spectra of complexes of vanadate and vanadyl ion (both of which contain the V=O unit) exhibit an intense pre-edge feature, observed at 5469 eV.[13,48,49] The intensity of these features arises from d—p mixing due to the strongly noncentric V=O group. The distinct absence of such a feature in the blood cell K-edge X-ray spectrum excludes the possibility that VO_2^+ (or, *e.g.*, $H_2VO_4^-$) or VO^{2+} can represent any more than about 2.5 or 5%, respectively, of the blood cell vanadium in this sample.

Extending this analysis, the 1s → 3d edge features arising from the vanadium(III)—tris(catecholate) complex (Figure 2, inset), are more intense and shifted to lower energies than are those corresponding to blood cells or aqueous acidic V(III). The near identity discussed above in the respective shapes, intensities, and energy positions of the features in the latter two spectra are here repeated. The comparatively low 1s → 3d intensities of all three spectra are consistent with the octahedral symmetry of the oxygen atom bonding array known to exist about vanadium in $K_3V(cat.)_3$.[40] This implies that a ligand field of similar symmetry characterizes V(III) within blood cells and in 9 M sulfuric acid.

In addition, the XAS edge spectra of both V(III) in sulfuric acid and blood cell vanadium exhibit a shoulder on the rising edge at 5475.5 eV, Figure 3a (top, arrow). This shoulder is absent from the K-edge spectrum of 0.1 M VCl_3 in 1 M HCl, as noted previously by Miyanaga *et al.*[51] A corresponding dispersion-shaped feature is clearly evident at 5475.5 eV in the first derivatives of the former two spectra, Figure 3b (bottom, arrow), but is absent in the first derivative K-edge spectrum of VCl_3 in 1 M HCl. Therefore, the 5475.5 eV shoulder appears to be characteristic of a specific solution interaction between hexaaquovanadium(III) and sulfate.

All of the *A. ceratodes* whole blood cell sample X-ray spectra obtained to date (six separate samples) have exhibited the shoulder and corresponding first derivative feature. The appearance of this feature in blood cell XAS spectra thus implies an endogenous vanadium(III)—sulfate interaction. Evidence for such an endocytic complex was reported previously following sulfur K-edge XAS[35] and ^1H NMR[11] studies on whole blood

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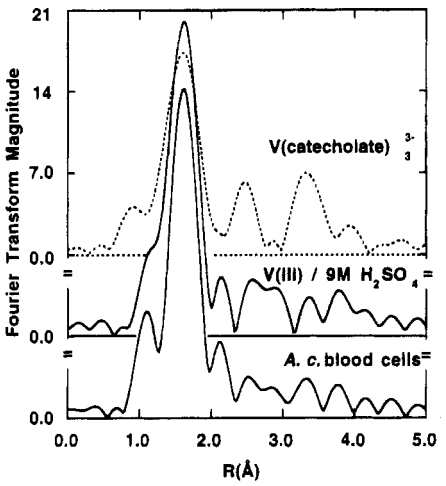

Figure 4. Fourier transforms of EXAFS spectra of (bottom) vanadium in *A. ceratodes* blood cells, (middle) V(III) in 9 M sulfuric acid, and (top) solid $K_3V(cat.)_3$ finely ground with boron nitride. All FT spectra are to the same scale. The former two have been offset by 7 and 14 units, respectively, for clarity. The intense peak at $R \approx 1.6$ Å in each spectrum is due to the V−O interaction. Since the spectra are uncorrected for phase shift,[37a] ~0.4 Å must be added to the value of R to obtain the approximate V−L bond length. The more distant carbon shells of vanadium catecholate yield readily observable peaks well above background near 2.4 and 3.4 Å, which are absent in the spectra of acidic $V(III)_{aq}$ and of *A. ceratodes* blood cells. There is also no sign of a peak in the blood cell spectrum consistent with an endogenous VOV dimer. See the text for a discussion of the limits of these conclusions.

cells. However, the data reported here mark the first unambiguous evidence, from the point of view of endogenous V(III), of the specific biological complex ion $[(VSO_4)(H_2O)_{4-5}]^+$ and lends credence to the notion that vanadium(III) and sulfate are colocated within intact blood cells.

In an effort to assess the sensitivity of these spectra to the possible presence within the blood cells of minority vanadium species in a catecholate-like chelating ligand array, linear combinations of the spectrum of $K_3V(cat.)_3$, or of $(Et_3NH)_2V(cat.)_3$, with that of V(III) in 9 M sulfuric acid were produced and examined. Comparisons indicated that a 10:90 ratio of the XAS spectrum of either of the inorganic vanadium−tris(catecholate) complexes with that of $V(III)_{aq}$ was detectably different from the XAS spectrum of pure $V(III)_{aq}$. These considerations provide an estimate that at most up to about 10% of blood cell vanadium may be chelated in a tris(catecholate)-like tunichrome complex. The XAS K-edge spectral data indicate in sum, therefore, that blood cell vanadium is ≥90% V(III), with either <5% vanadyl ion or <2.5% vanadate. Further, ≥90% of the V(III) is in a ligand array of symmetry and field strength similar to that provided by 9 M aqueous sulfuric acid. Finally, a specific interaction with sulfate ion appears to characterize the chemistry of endogenous vanadium(III).

Blood Cell Vanadium K-Edge EXAFS Spectra. Examination of the vanadium K-edge EXAFS data arising from whole blood cells can yield information about the type, number, and distance of the ligands around endogenous vanadium(III),[37,41] out to distances of around 4−5 Å from the vanadium absorber. Useful insights can also be obtained by comparative examination of the Fourier transforms of EXAFS data (Figure 4).

The intense peaks at $R \approx 1.6$ Å in the FT's originate from the first shell of ligand atoms. In the case of vanadium(III)−tris(catecholate), and V(III) in acidic aqueous solution, these ligands are oxygen atoms. In the FT spectrum of the catecholate complex, the more distant aromatic carbon shells are clearly evident as peaks at about 2.4 and 3.4 Å. These values are all uncorrected for phase shift, so ~0.4 Å must be added to the V−L distance observed in the FT spectra.[37a] The carbon peaks average about 38% of the intensity of the oxygen ligand peak at ~1.6 Å and are strongly visible because of the regularity of the $[V(cat.)_3]^{3-}$ structure. Strong second shell features are in general characteristic of the FT's of EXAFS data for transition metals within a well-ordered chelating ligand complex.

The 1.6 Å peaks in the FT spectra of V(III) in aqueous sulfuric acid solution and of blood cell vanadium are of nearly the same intensity and position. The major peak in the FT spectrum of vanadium(III)−tris(catecholate) is much weaker than, though positionally congruent with, the former two peaks. Within the noise level of the data neither of the former FT spectra exhibit any features which might reflect an ordered shell of more distant carbon atoms. This is as expected for the data from aqueous acidic V(III). However, for blood cell vanadium, this result argues against large amounts of an ordered endogenous biological V(III) chelate. Rather, the FT spectra in Figure 4 require that the respective ligand arrays for vanadium in aqueous sulfuric acid and within blood cells are quite similar. Both FT spectra also display the same overall features of FT spectra reflecting other aquo transition metal ions.[13,49,50,51]

In addition, no evidence of a more distant vanadium scatterer, as might be found in a vanadium dimer, can be observed in the blood cell FT spectrum. If vanadium(III) were stored under conditions which fostered the formation of large amounts of $(VOV)^{4+}$ dimers or complex polymers, an intense peak[51,52] near $R \approx 3.1$ Å, [53] indicative of second shell vanadium(III), would be expected in the Figure 4 blood cell FT spectrum. Therefore, the FT spectral data presented here are consistent with predominantly monomeric unchelated blood cell vanadium(III). Solution vanadium(III) begins to dimerize above pH ~ 2, especially at higher [V(III)],[54] but this reaction is suppressed by sulfate. This point is discussed further below.

The K-edge EXAFS data of V(III) in 9 M sulfuric acid along with the fit to these data are presented in Figure 5. This spectrum is predominantly characterized by a simple wave form. No complex frequency components indicative of distant or multiple shells of atoms are observed, as suggested by the observations in the FT's discussed above. The K-edge EXAFS data of blood cell vanadium along with the fit to the data, and the EXAFS data for $K_3V(cat.)_3$ are also presented in Figure 5. For this latter sample, the more distant carbon shells give rise to additional frequency components, which sum with the principle lower frequency from the V−O first shell to give the complicated line shape. Comparative examination reveals that the blood cell vanadium EXAFS spectrum is as equally simple and has the same frequency, as that of V(III) in sulfuric acid solution, with no evidence of any multiple frequency components. Therefore, the blood cell vanadium EXAFS spectrum is essentially identical to that of acidic aquovanadium(III) sulfate.

Fits to the EXAFS spectra, shown in Figure 5a,b, were carried out using empirical oxygen parameters obtained from $V(acac)_3$ (see Materials and Methods). In fitting EXAFS spectra using the empirical phase and amplitude approach, noninteger coordination numbers can be found. These arise from contributions

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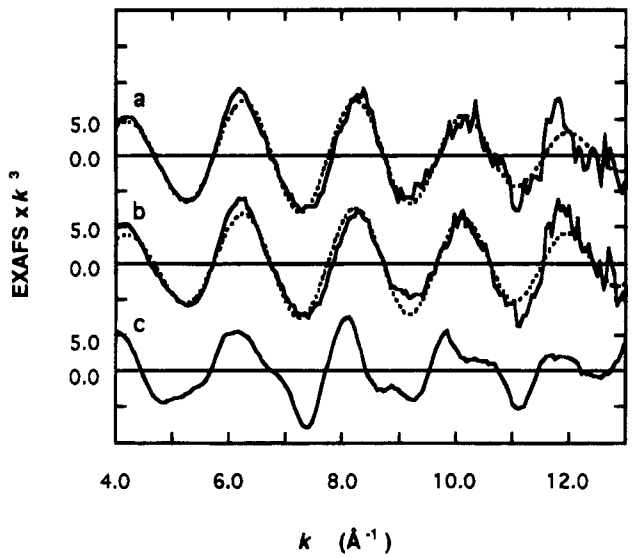

Figure 5. Vanadium K-edge EXAFS spectra (solid lines) and the fits to the spectra (dotted lines) of (a) 0.1 M vanadium(III) in 9 M sulfuric acid, (b) vanadium within a packed blood cell sample from *A. ceratodes*, and (c) solid $K_3V(cat.)_3$ finely ground with boron nitride. The complex beat patterns in spectrum c represent contributions from the multiple shells of scatterers in the catecholate ligand. In spectra a and b, in contrast, no such beat patterns are observable; rather, only a single frequency is found. The frequency of the blood cell vanadium spectrum is the same as that of vanadium(III) in acidic solution, and both spectra are well fit by a single shell of oxygen scatterers. For blood cell V(III), 6.2 oxygens at 1.99 Å were found. For V(III) in 9 M sulfuric acid, the fit indicated 7.4 oxygens at 1.99 Å, suggesting an expanded coordination sphere for vanadium(III) in these conditions.

Table 1. Summary of EXAFS Curve-Fitting Results[a]

	CN_O[b]	R(V−O)[b] (Å)	CN_C[b]	R(V−C)[b] (Å)	F[c]
KR2					
1-shell (F)[d]	5.9	1.98			0.82
1-shell(U)[e]	6.0	1.98			1.45
2-shell (U)[e]	6.0	1.98	0.7	2.90	1.42
AB2					
1-shell (F)[d]	6.9	1.99			0.65
PF3					
1-shell(F)[d]	6.0	1.99			0.88
1-shell(U)[e]	6.2	1.99			1.52
2-shell (U)[e]	6.2	1.99	1.1	2.96	1.51
Henze solution					
1-shell(F)[d]	6.8	1.99			0.53
1-shell(U)[e]	6.9	1.99			0.93
2-shell(U)[e]	7.0	1.99	0.8	2.97	0.90
V(III) in sulfuric acid					
1-shell (F)[d]	7.4	1.98			0.52
1-shell (U)[e]	7.0	1.98			1.61
2-shell (U)[e]	7.0	1.98	2.2	2.93	1.59

[a] All fits were carried out over the photoelectron wave vector range $k = 4-13$ Å^{-1}, except for sample KR2 data which contained an ice-derived diffraction spike at $k = 12$ Å^{-1}. These data were analyzed over the range $k = 4-11.5$ Å^{-1}. [b] R is distance (±0.02 Å), and CN is coordination number (±25%). [c] F is a goodness-of-fit criterion defined by $F = [\sum k^6(\text{data} - \text{fit})^2/(\text{no. of points})]^{1/2}$. [d] Single shell fits of data which were Fourier-filtered (F), isolating the first-shell Fourier peak at 1.6 Å (see Materials and Methods). [e] Single shell or two-shell fits to unfiltered (U) data. Note that there is no significant improvement in "F" on addition of a carbon wave at 2.9 Å. In addition, about two second shell carbons were found at about 2.9 Å for V(III) in 9 M sulfuric acid, where surely none exist. It is possible that the "carbons" found instead represent distant solvation shells of water molecules.

to the fit from spectral noise, from static disorder in the metal−ligand bond distances, and from imperfect transferability of the empirical fit parameters derived from the model compound. A typical EXAFS accuracy of ±25% in coordination number can be expected, whereas bond length accuracies of ±0.02 Å are routinely produced for first shell ligand atoms.[37a,41] In addition, although ligand atoms of different periodic table rows can readily be distinguished, discrimination of adjacent, same row, ligand atoms is difficult or impossible in most circumstances by EXAFS analysis alone.

The results of all the fitting experiments are summarized in Table 1. Attempts were made to fit any second shell carbon atoms about blood cell vanadium using empirical second shell carbon parameters extracted from the FT spectrum of $K_3V(cat.)_3$ (see Materials and Methods). Fits to blood cell EXAFS data including a second shell carbon wave did converge to yield about 1 second shell carbon atom at about 2.9 Å. However, similar fits to the EXAFS spectrum of inorganic V(III) in 9 M sulfuric acid also yielded about 2 second shell carbon atoms at about 2.9 Å. Inspection of Table 1 shows that inclusion of carbon waves did not significantly improve the goodness-of-fit parameter "F". Therefore, these second shell carbon atoms are most likely spurious and may instead represent a contribution from the distant oxygen atoms of water molecules in outer sphere solvation shells.

Both the blood cell and model solution vanadium EXAFS spectra given in Figure 5 are well fit by a single shell of oxygen scatterers. For V(III) in 9 M sulfuric acid, the best fit yielded 7.4 oxygen atoms at 1.98 Å. Coordination numbers of 7−8 oxygen ligands at 1.98−1.99 Å were consistently found for fitting experiments using four independent sets of empirical oxygen parameters. This is in contrast to about 6 oxygen ligands invariably found for vanadium from fits to the EXAFS spectra of $V(acac)_3$ and $K_3V(cat.)_3$ (see Materials and Methods) and also in contrast with the fits to blood cell vanadium (see below). Therefore, it seems possible that in strongly acidic solution V(III) may command an expanded coordination sphere. Experiments are currently in progress to test this proposition more closely. Seven coordinate V(III) is known to occur with oxygen liganded complexes[55,56] and in $K_4V(CN)_7$.[57]

EXAFS spectra were obtained from three blood cell samples. Two of the blood cell sample vanadium EXAFS spectra, those from samples KR2 and PF3, yielded about 6 oxygen atoms at 1.98−1.99 Å (*cf.* Table 1). In some contrast, sample AB2 (Table 1) was found to have about 7 oxygen atoms at 1.99 Å, but this difference is likely not significant. Again, no evidence for a more distant shell of carbon atoms was found for any blood cell vanadium. The coordination number and distance of 6 oxygen atoms at 1.992 ± 0.009 Å in crystalline hexaaquovanadium(III)[58,59] is essentially indistinguishable from the ligation pattern of vanadium(III) in the blood cell samples. This result is consistent with the vanadium K-edge and EXAFS data and the discussion pertaining thereto, presented above.

Vanadium K Edge and EXAFS Spectra of Henze Solution. Figure 6 shows the V-K edge spectrum of Henze solution, compared with that of the blood cell sample of Figure 2. Inspection of the Henze solution spectrum reveals that the vanadium has remained almost exclusively trivalent. However, the main inflection on the rising edge of the Henze solution

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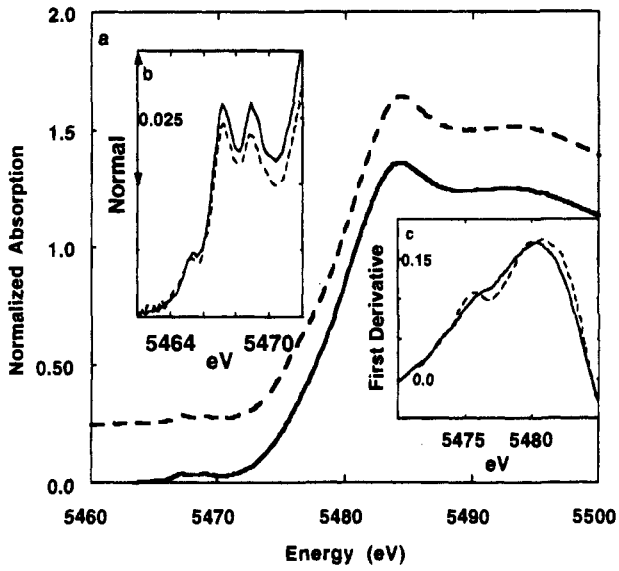

Figure 6. Vanadium K-edge XAS spectra of frozen (10 K) blood products from *A. ceratodes*: (a) (—) Henze solution; (--) whole blood cell sample PF3. Inset b: Y-expansion of the pre-edge region of the spectra in part a, showing the 1s → 3d transitions. The double-headed arrow along the ordinate marks 0.025 intensity units. Zero intensity occurs at the abscissa. Inset c: First derivative of the part a K-edge spectra of (—) Henze solution and (--) whole blood cell sample PF3. Note the 0.21 eV shift to lower energy of the rising edge of the Henze solution spectrum, evident near 5480 eV in the first derivative spectrum. The dispersion-shaped feature observable at 5475.5 eV in the first derivative of the blood cell spectrum, reflecting a shoulder on the rising edge of the XAS spectrum at 5476 eV, is absent in the first derivative of the spectrum of Henze solution. This indicates the apparent loss of the intracellular vanadium(III)−sulfate interaction on blood cell lysis (see text).

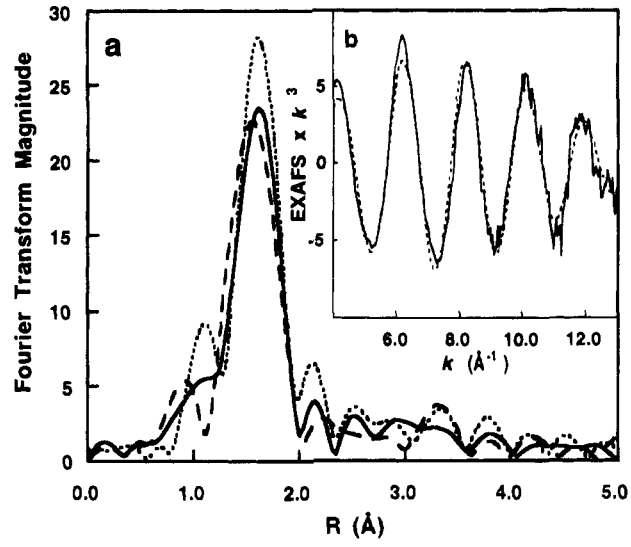

Figure 7. Vanadium K EXAFS data of frozen (10 K) blood cell products from *A. ceratodes*: (a) Fourier transform spectra of (—) Henze solution, (--) whole blood cell sample KR2, and (···) whole blood cell sample PF3. The intensity of the Henze solution peak is within the range observed for the XAS FT spectra of 6 whole blood cell samples. All three FT spectra uniformly reflect only a single shell at ~2.0 Å. Inset b: (—) EXAFS spectrum of Henze solution, (--) the single shell oxygen wave fit to the EXAFS spectrum, indicating about 7 first shell oxygen scatterers at 1.99 ± 0.02 Å.

spectrum is shifted about 0.2 eV to lower energy, relative to the same inflection on the rising edge of the blood cell sample spectra. This shift represents the limits of reproducibility expected[60] from the experimental setup used for these XAS K-edge spectroscopic experiments.

However, detailed comparison of the respective positions of each of the three 1s → 3d transitions (Figure 6, inset b) of blood cell and Henze solution V(III) reveals that the maxima of these transitions are essentially coincident in energy. Thus, the relative 0.2 eV shift in energy in the main rising edge of the Henze solution spectrum appears real. This implies a subtle change in ligation status for vanadium(III) following blood cell lysis. Although the 1s → 3d transition at 5469 eV (Figure 6, inset b) in the Henze solution spectrum is slightly more intense than the corresponding transition in the blood cell spectrum, it is within the range of variation observed in spectra of intact blood cells. The positions and intensities of 1s → 3d transitions are sensitive to the strength and symmetry of the ligand field.[37bc,48,61] Thus, V(III) in Henze solution remains in a ligand environment very similar to that provided by intact blood cells. Nevertheless, the edge shift in the Henze solution X-ray edge spectrum may reflect a change in the local environment of V(III) on blood cell lysis (see below).

Some of the intensity of the 5469 eV transition may reflect a contribution from a small [VO^{2+}], equivalent to an upper limit of *ca.* 0.5%[13,48] of the total vanadium in the Henze solution sample. Indeed, EPR spectra (not shown) indicated that the Henze solution sample contained about 1.5 and 5 times more pentaaquovanadyl ion respectively than blood cell samples AB2 and PF3. The $-7/2_{||}$ EPR spectral line width of $[VO(H_2O)_5]^{2+}$ is known to reflect solution pH.[19,21,34] For the three samples these values were as follows (sample, linewidth, pH): AB2, 22.4 G, 1.6; PF3, 26.0 G, 1.7; and Henze solution, 15.6 G, 1.25.

In the Figure 6c inset, the first derivative of the V K-edge spectrum of Henze solution is compared to that of whole blood cells. The shift to lower energy of the Henze solution rising edge is again here evident near 5480 eV. The derivative spectrum of V(III) in whole blood cells exhibits the strong inflection at 5475.5 eV, as described previously. In contrast, this feature is lost from the Henze solution first derivative spectrum. This implies the loss of the endogenous vanadium(III)−sulfate interaction, likely due to dilution of the vacuolar contents into cytosolic and extracellular fluids. Thus, this feature may be diagnostic for intact *A. ceratodes* blood cells. The shift to lower energy of the Henze solution X-ray edge spectrum, then, may be a reflection of the concomitant loss of sulfate ligand and the 2.5-fold increase in acidity.

In Figure 7a the Fourier transform of the V−K EXAFS data from Henze solution is compared with those of whole blood cell samples KR2 and PF3. The inset to Figure 7 shows the Henze solution EXAFS spectrum and the fit to the spectrum.

The intensity of the main Fourier peak for Henze solution is within the range exhibited by all the whole blood cell sample spectra (FT magnitude = 22.5−28.2). There is no shift in the position of the main Fourier peak, nor is there a sign, for example, of a strong second shell carbon contribution near R = 2.5 Å. In addition, no feature at $R \approx 3.1$ Å, indicative of a second shell vanadium peak, such as might arise from a μ-oxo-bridged vanadium dimer,[52,53] is evident. Indeed, overlay plots of EXAFS data (not shown) revealed that the Henze solution EXAFS spectrum superimposed perfectly upon the whole cell V−K EXAFS data.

We note that although the blood cells were lysed in air, exposure time to oxygen was minimal. The color of the sample was the typical reddish-brown of Henze solution. Other

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methods of preparation could generate Henze solutions with different properties than those reported here. Nevertheless, in earlier room temperature XAS studies, even the oxygen-exposed blue hemolysate was found to contain primarily V(III) in an environment essentially indistinguishable from that of an unchelated, undimerized monomeric ion.[13]

The results of the single-wave fit to the Henze solution EXAFS data are given in Table 1. These show about 7 oxygen (or nitrogen) atoms at 1.99 ± 0.02 Å from the central vanadium. In this regard, Henze solution vanadium is nominally different from the vanadium found in two of the three whole blood cell packs. This difference again may be real, since the XAS edge data indicate loss of the intracellular sulfate interaction for V(III) in Henze solution following blood cell lysis. However, given the ±25% uncertainty in the EXAFS-derived ligand number, this finding is considered to be not significantly different from 6 oxygen ligands, pending more detailed analysis.

Discussion

All the vanadium K-edge XAS edge and EXAFS data obtained thus far on blood cells from the tunicate *A. ceratodes* are consistent with storage of this metal principally as the hexaaquovanadium(III) ion, with a V−O distance of 1.99 ± 0.02 Å. This aquo ion is likely in equilibrium with the sulfato complex ion[11,35,62,63] in acid solution.[14,17,19,21] No evidence was found for second shell carbon atom scatterers, the presence of which would indicate storage of blood cell vanadium(III) within a biological chelate. Neither was any evidence forthcoming for the presence of endogenous oxo-bridged vanadium(III) dimers, which might arise at pH > 2.

This latter result is, on the surface, incompatible with the finding by Anderson and Swinehart[26] who found about 15% $(VOV)^{4+}$ dimer in blood cells from *A. ceratodes*, collected from Bodega Bay, CA. However, as pointed out previously[21,35] variations in blood cell chemistry may occur within samples collected at different times or locales, even within a single species. Since sulfate suppresses V(III) dimerization,[54] a simple variation in blood cell [sulfate] may, at a given low pH (*e.g.*, pH 1.8), be sufficient to account for the presence or absence of endogenous $(VOV)^{4+}$ dimers. Thus, the detection, described above, of an endogenous vanadium(III)−sulfate interaction is pertinent to this question.

These differences in the inorganic chemistry of various blood cell collections may be due to variations in blood cell populations,[38] or to metabolic responses to environmental or seasonal cues. A blood cell sample from only one animal taken from a single locale may also be different from collections which include blood cells from many animals (*cf.* the data range in ref 38). These aspects of tunicate blood cell inorganic biochemistry remain relatively unexplored but are currently under investigation in our laboratories. For example, we have found significant variations in blood cell sulfur and sulfate contents in collections of blood cells taken 1 year apart.[35,64]

The vanadium K-edge XAS data described herein are not only consistent with solution storage of blood cell $V(III)_{aq}$ with sulfate but alternatively with storage of some of the endogenous hexaaquovanadium(III) in a hydrated solid form[58,59] (*e.g.*, as an alum: in $CsV(SO_4)_2 \cdot 12H_2O$ the shortest V−V distance is 8.80 Å and the shortest V−S distance is 5.09 Å,[58] both distances are unobservable using EXAFS). X-ray microprobe experiments[15,17,65,66] revealing vanadium-containing granules and experiments involving diffusion of complexing ligands into blood cells[25] are ambiguous on this point, since they do not reveal the detailed chemical nature of stored vanadium. Storage as a solid alum is incompatible with the results from ^1H NMR and sulfur K-edge XAS experiments,[11,21,35,62] however, since these experiments clearly implied a solution state for blood cell vanadium(III). It may thus be that alternative modes of vanadium(III) storage are available to ascidians from time to time as need or circumstance may dictate or to differing species of ascidians. We are currently engaged in exploration of these possibilities.

Finally, these experiments have been carried out on whole blood cell packs. This means that all the vanadium environments as they might occur in all the vanadium-carrying blood cells have been surveyed. The data are generally consistent with only one major type of endogenous vanadium: the trivalent monomeric aquo ion, in presumed equilibrium with the complex ion $[(VSO_4)(H_2O)_{4-5}]^+$. A tris-chelated form of vanadium(III) could be present only as a minority species (< 10%). In addition, vanadyl ion or vanadate would have been readily detectable if they constituted any more than about 5 or 2.5%, respectively, of the total blood cell vanadium. Recent ^{51}V NMR experiments on *A. ceratodes* blood cells, which were unable to detect any V(V)[26], are thus here corroborated. If there were any significant tris-chelated valence 4 vanadium minority species (*i.e.*, within a ligand environment which prevented formation of the short V=O bond), it could have amounted only to less than 10% of the total blood cell vanadium.

The results presented here are strictly applicable only to the blood cells of *A. ceratodes* as collected at Monterey Bay, CA. Some of us have suggested the possibility that variations in blood cell chemistry may exist among ascidians even of the same species.[21,35] Therefore, we are currently planning to extend these experiments to collections of *A. ceratodes* obtained from alternative locales. In addition, blood cells from ascidians of other species should also be examined. Experiments assessing vanadium within specific blood cell types are currently in progress.

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