Interaction of Vanadium and Sulfate in Blood Cells from the Tunicate *Ascidia ceratodes*: Observations Using X-ray Absorption Edge Structure and EPR Spectroscopies

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Received December 3, 1993[⊗]

Sulfur K-edge X-ray absorption spectroscopy (S-K XAS) and EPR spectroscopy have been used to investigate the inorganic solution chemistry of vanadium, sulfate, and methanesulfonate, with application to blood cells from the tunicate *Ascidia ceratodes*. Three independent whole blood cell preparations (S85, S86, W87) collected over a period of 18 months were examined. Average blood cell vanadium concentrations were determined to be 0.099, 0.079, and 0.062 M, respectively. All three collections gave sulfur XAS spectra consistent with significant intracellular concentrations of low-valent sulfur, an alkanesulfonic acid, and sulfate. In model studies, the line width of the sulfate K-edge XAS spectrum was found to titrate with both pH and [V(III)]. Application of this finding to *A. ceratodes* blood cell sulfur XAS spectra provided evidence for direct interactions between endogenous dissolved sulfate and V(III) in two of the three collections. All three collections yielded sulfate XAS edge spectra consistent with low pH. Curve-fitting analysis of the S-K edge XAS spectra for the three whole blood cell collections yielded the ratios of intracellular sulfate:alkane sulfonate:low-valent sulfur to be as follows: S85, 1.0:0.9:0.36; S86, 1.0:0.5:1.5; W87, 1.0:0.44:0.24. Comparisons with models indicated that the low-valent blood cell sulfur included various disulfide-like compounds unlike cystine. This all implies a surprisingly rich and variable sulfur biochemistry in these marine organisms. EPR spectroscopy of whole blood cells from one animal from the W87 collection revealed an endogenous VO^{2+}–sulfate interaction. Thus both V(III) and VO^{2+} can sense an intracellular pool of sulfate, implying the biological colocation of these two metal ions. The variations in blood chemistry observed over time as described herein caution against definitive application of single point experiments.

Introduction

In a continuing study of the inorganic biochemistry of blood cells from the tunicate *Ascidia ceratodes* (a sessile, solitary, filter-feeding marine Urochordate[1-3]), we have focused on the intracellular status of vanadium,[4,5] acidity,[5,6] and, more recently, sulfur.[5,7,8] Previous vanadium K-edge X-ray absorption spectroscopic studies (EXAFS and edge) of whole blood cells revealed the valence state and first shell ligation of endogenous vanadium to be $V^{III}(O,N)_6$, with V–(O,N) distances averaging 1.99 Å. No sign of an ordered, chelate-derived second shell was detectable in analysis of the EXAFS data, which had been recorded at ambient temperature.

EPR spectroscopy was developed as an assay for the pH of the intracellular region(s) containing vanadyl ion within *A. ceratodes* whole blood preparations. Consistent with the acidity requirements implied by an aquated low-MW V(III) complex,[4,9] this pH was found to be 1.8.[5,6] Recent application of this method by Michibata *et al.*[10] yielded endogenous pH values of 1.86 and 2.05 for Ficoll-fractionated blood cell populations from *Ascidia gemmata*. In view of the differing opinions expressed in the literature[5,6,9-17] on the matter of intracellular pH, however, we emphasize that this finding characterizes only the vanadyl ion sequestering region or compartment within the pertinent blood cell(s) and is not general for the cellular cytoplasm. In addition, as we discuss below, there can be time-dependent variations in blood chemistry even within a single species.

The continuing interest of ourselves and others in the large quantities of intracellular sulfate[5,17] and sulfur[16-24] prompted X-ray absorption studies at the sulfur K-edge of *A. ceratodes* whole blood cell packs.[7,8] In addition to the expected[5] intense sulfate feature, a second, overlapping X-ray absorption transition

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1994.

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was observed,[8] consistent with an endogenous aliphatic sulfonic acid. These cell-derived S-K XAS features also proved broadened relative to those of inorganic model solutions containing sulfate and sulfonate. It was suggested[8] that this broadening could be due to complexation of these anions by endogenous V(III).

Herein we report the results from further sulfur K-edge XAS spectroscopic and other experiments designed to probe in greater detail any sulfate/sulfonate relationship and to investigate the environment and relative intracellular amounts of these ions. The question of a putative endogenous vanadium(III)–sulfate interaction is also addressed.[8] Finally, we describe other types of intracellular sulfur, including possibly novel low-valent species.

Materials and Methods

Specimens of *A. ceratodes* were collected from the Monterey, CA, Yacht Harbor and maintained in 4 °C sea water for up to 6 days. Blood was removed by heart puncture, using sterile disposable syringes, and was processed as described previously,[5,8] with modifications as noted below. The three independent collections discussed herein were obtained on June 1, 1985, May 27, 1986, and January 12, 1987, hereinafter designated samples S85, S86, and W87, respectively. Samples for vanadium analysis were prepared by digestion in nitric acid as described previously,[5] followed by appropriate quantitative dilution into 4 M HCl. For samples S86 and W87, vanadium was analyzed in triplicate using the pyridylazoresorcinol (PAR) method, as given by Marczenko, Z. (*Separation and Spectrophotometric Determination of Elements*; John Wiley & Sons: New York, 1986; p 627). The precision of the vanadium analyses was ±0.6%.

Inorganic Models. Solutions of 0.50 M $V_2(SO_4)_3$ in 15 mM sulfuric acid and 1.0 M $V(CH_3SO_3)_3$ in 15 mM CH_3SO_3H were prepared using the method described previously.[5] Solutions used to model the sulfate/sulfonate relationship within *A. ceratodes* blood cells were made to be relatively low (0.10 M) in total sulfur to minimize self-absorption induced amplitude distortions. All reagents, including the low-valent sulfur compounds discussed in the text, were used as received. Sodium hydrogen sulfide and $[(C_2H_5)_4N]_2[Fe_4S_4(SC_6H_5)_4]$ were prepared by literature methods.[24,25]

Titration of sulfate with vanadium(III) was carried out anaerobically. Appropriate amounts of 1.0 M sodium sulfate in pH 1.5 sulfuric acid were combined with 0.50 M $V_2(SO_4)_3$ in 15 mM sulfuric acid, so that the V(III):SO_4^{2-} ratio varied systematically between zero and 1.5.

Solutions made to model the cellular sulfate/sulfonate mixtures were prepared to contain sulfate:sulfonate ratios of 1:1, 2:1, and 3:2 with, or without, respectively, the additional presence of stoichiometric V(III). The V(III)-containing model solutions were prepared anaerobically, packed under dinitrogen in crimp-top serum vials and stored frozen at −20 °C before use. The vanadium(III)-containing stock solutions used to prepare the model solutions were prepared by appropriate dilution of 0.50 M $V_2(SO_4)_3$ solution or 1.0 M $V(CH_3SO_3)_3$ solution, each 15 mM in the corresponding acid. For sulfur K-edge XAS studies, the sample cells were loaded in a thoroughly purged argon-filled glovebag.

Model solutions which contained acidic sulfate and sulfonate only were prepared in air from stock solutions of sodium sulfate or sodium methanesulfonate, each 0.10 M in total sulfur. The latter was prepared by neutralizing a solution of methanesulfonic acid (Aldrich Chemical Co.) with solid sodium carbonate. All model solutions were prepared to be 0.1 M in total sulfur. Each solution was made to include 15 mM of the corresponding acid as part of the total sulfur. These were stored in crimp-top vials as specified above.

Anaerobic methods used included standard Schlenk and syringe techniques under an argon atmosphere and use of an argon-purged glovebag or a Vacuum Atmospheres glovebox operating with a dinitrogen atmosphere at typically less than 0.5 ppm ambient dioxygen.

Removal of Extraneous Sulfate or Chloride from *A. ceratodes* Blood Cells. Blood cells from the 1985 and 1987 collections were washed free of plasma and surface sulfate using a sulfate-free pH 6.6 metal chloride buffer made to be isotonic with ascidian plasma, as described earlier.[8] Blood cells from the 1986 collection were also examined for the presence of chloride using XAS spectroscopy at the Cl K-edge. It was therefore necessary to wash these cells with a chloride-free medium. Several small-scale washing experiments were carried out to evaluate the best isotonic buffer solution.

The different isotonic pH 6.6 buffer systems evaluated included sodium phosphate, imidazolium trifluoroacetate, and metal formate buffered with imidazolium trifluoroacetate. In each evaluation experiment, blood-cell packs were subjected to three consecutive washes consisting of centrifugation (3000 × *g*, 10 min) followed by careful vortex resuspension into fresh cold buffer.

Criteria chosen for judgment of an acceptable washing regime were that the cells maintain their original healthy yellow-green color throughout the procedure and that the chloride-free buffer perform at least as well with regard to spontaneous cell lysis (*i.e.*, essentially zero lysis) as the previously used[8] isotonic chloride buffer used to wash surface sulfate from the S85 and W87 preparations.

These criteria were found to be best met using the following isotonic metal formate solution: $NaHCO_2$ (512 mM), $KHCO_2$ (9.05 mM), $Ca(HCO_2)_2$ (8.06 mM), and $Mg(HCO_2)_2$ (48.5 mM), buffered to pH 6.6 with 17 mM imidazolium trifluoroacetate. Cells washed with this solution generally maintained good color throughout and showed excellent resistance to spontaneous lysis. Chloride- and sulfate-free whole blood cell pellets were prepared by centrifuging the whole blood sample followed by removal of the plasma supernate. This was followed by three times resuspension and re-centrifugation (500 × *g*, 5 min) in consecutive fresh aliquots of cold (pH 6.6) buffered formate.

X-ray Absorption Measurements. Sulfur K-edge spectra were recorded at the Stanford Synchrotron Radiation Laboratory (SSRL) with the storage ring (SPEAR) operating at 3 GeV with about 50-mA average current. The spectra were measured on beamlines 6-2, 4-1, or 4-2, in all cases with a Si[111] two-crystal monochromator. Data were collected in the fluorescence mode using a N_2-filled ionization chamber (Lytle detector).[26] Data for blood cell samples S85 and W87, for the solid model compounds, and most of the solutions were measured as 3–4 scans, and subsequently averaged, on beamline 6-2 operating in either an undulator mode with a 1.45-kG field[7] or in a low magnetic field wiggler mode (5 kG). The S86 blood cell sample was measured (11 individual scans which were averaged) on wiggler beamline 4-2 in unfocused mode with the monochromator detuned 66% at 2740 eV to minimize harmonic contamination. Model solution spectra were measured on beamline 4-1 (4 scans recorded and averaged) again with the monochromator detuned 65% at 2740 eV. These data were somewhat noisier due to the lower flux of beamlines 4-1 and 4-2 as compared to beamline 6-2.

Blood-cell samples were maintained on ice until measurement, and spectra were recorded at ambient temperature. The blood cells were inspected visually for any sign of lysis following the X-ray experiment, as might be evidenced by blue or brown specks. No such evidence was ever observed in any of the samples.

Sulfur K-edge spectra were calibrated relative to the 2472.02-eV peak of thiosulfate as described previously.[27,28] Calibration spectra were collected at least twice during every beam "fill". Reproducibility in peak position of 0.1 eV or better for comparable solutions (*e.g.*, those containing sulfate) was obtained for the various runs over the time course of the experiments. Raw averaged spectra were normalized by fitting a smooth polynomial to the pre-edge region of the spectrum and subtracting this polynomial from the entire spectrum. A polynomial spline was then fit to the region above the edge. Spectrometer resolution was calculated to be 0.5 eV.[28]

Spectral Curve Fitting. Sulfur K-edge spectra were fit using the program Fitcur, written by Dr. Geoffrey S. Waldo and obtained from Prof. James E. Penner-Hahn, Department of Chemistry, University of Michigan. This program was locally modified by Mr. David Eliezer and especially Dr. Soichi Wakatsuki to permit inclusion of multiple arc tangent functions and to further permit the option of imposing separate optimization weightings for the fit, the first derivative, and/or the second derivative of the data within a fitting experiment. First and second derivative spectra of each fit were calculated analytically, using the functional form of the Gaussians used in the fit, rather than numerically. In the fitting experiments described herein, the weighting scheme, chosen empirically, was as follows: Fit, 0.75; first derivative, 0.0; second derivative,

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Table 1. Final Gaussian Parameters from Fits to Model Solutions

Gaussian[a]

	without V(III)		with V(III)	
G	fwhm	eV	fwhm	eV
sulfate	1.58	2482.3	1.70	2482.4
sulfonate	1.82	2481.1	1.90	2481.1
Rydberg(1)[b]	1.83 ± 0.01	2483.2	1.79 ± 0.01	2483.4
Rydberg(2)[b]	4.26 ± 0.13	2485.2	4.47 ± 0.32	2485.2

Empirical (I^s/A^s)[c]

	without V(III)	with V(III)
sulfate	0.108 ± 0.005	0.099 ± 0.003
sulfonate	0.074 ± 0.005	0.070 ± 0.006

Model Solution Sulfate:Sulfonate Ratios

	without V(III)			with V(III)		
as prepared	1.0:1.0	2.0:1.0	3.0:2.0	1.0:1.0	2.0:1.0	3.0:2.0
calcd[d]	1.1:1.0	1.8:1.0	3.2:2.0	1.1:1.0	1.8:1.0	3.0:2.0

[a] Full width at half-maximum (±0.01) and energy position (±0.01) in eV. [b] The Rydberg line widths were floated in each of the three final fits, yielding the average ± the standard deviation. [c] Gaussian area (A^s) = (fwhm × height). Empirical edge-jump intensities (I^s) were obtained directly from the model solution XAS spectra. Each value of (I^s/A^s) is the average of three independent determinations. [d] Calculated using the appropriate (I^s/A^s) ratio, illustrating the precision of the fit. Edge-jump height is independent of oxidation state.[30a]

0.25. These values were chosen to give greater significance to the data in the fit, while requiring a significant dependence of the goodness of fit on the second derivative. Any fit which reproduced the second derivative also reproduced the first derivative. Finally, the spectra were fit with a single Gaussian each for sulfonate and sulfate, along with the minimum number of additional Gaussian functions required to reproduce the spectral envelope.

Two criteria were set in order to evaluate the correctness of a fit. The first was that the fit should reproduce both the shape of the X-ray absorption edge spectrum and the salient regions of the second derivative of this spectrum. Several excellent fits to data were obtained which did not in turn match the second derivative. This marks the critical importance of this latter criterion in judging the adequacy of a fit. Second, the center position and half-height width of each Gaussian were required to be independent of the relative contributions of sulfate and sulfonate to a spectrum. Any chosen set of Gaussians (see below) was required to correctly reproduce the relative concentrations of sulfate and sulfonate within model solutions of known composition. In addition, independent parameter sets for mixtures with or without added V(III) were sought.

Gaussian parameters were obtained through fitting experiments on spectra from model solutions containing 1:1 sulfate:sulfonate, with and without stoichiometric V(III), and with the total [S] = 0.10 M. The utility and predictive power of these parameters were then tested on sulfate/sulfonate solutions of differing known composition, with and without added V(III). The final Gaussian sets obtained were unique with respect to the presence or absence of V(III). These were used in all subsequent fits on blood cell spectra and are given in Table 1. Attempts to cross-fit the acid-only model solution spectra using the Gaussians derived from the vanadium-containing model solution or vice-versa always resulted in poorer quality fits. The final parameter fits were able to successfully reproduce the sulfate:sulfonate ratio, to within ±10%, of all the model solutions of varying composition (Table 1).

EPR Spectroscopy. EPR experiments were carried out on single animal whole blood samples from the W87 collection using equipment and protocols as described in detail previously.[5,6]

Results and Discussion

Sulfur K-Edge XAS Experiments. In all, three independent washed whole blood-cell samples were examined. These were obtained as three separate collections across a period of 18 months: Sample S85 on June 1, 1985; sample S86 on May 27, 1986; and sample W87 on January 12, 1987. The sulfur K-edge spectra of all these samples are shown in Figure 1. In each case, the excellent signal-to-noise ratio of the cell sample spectra implied a high concentration of endocytic sulfur.[5]

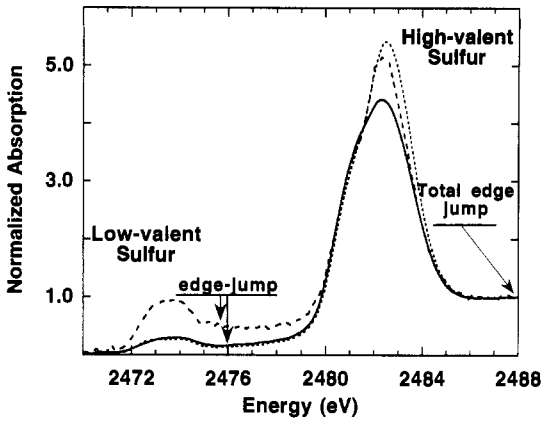

Figure 1. Sulfur K-edge XAS spectra of whole blood cell packs from three collections of *A. ceratodes*. These are (sample label, collection date) as follows: S85, June 1, 1985 (—); S86, May 27, 1986 (- - -); W87, January 12, 1987 (···). The spectra are normalized to reflect the same total concentration of sulfur. The features due to low-valent and high-valent sulfur are labeled, along with the respective edge-jump regions. Note the relative variation in low-valent edge-jumps heights, indicating large changes in the ratio of low- and high-valent sulfur.

Sulfur K-edge X-ray absorption spectral transitions involve the energy-dependent promotion of a sulfur 1s electron into the set of unfilled valence states below the ionization energy. Sulfur K-edge spectra are very sensitive to both the oxidation state and the chemical and structural environment of sulfur.[7,8,27] For example, a 13-eV spread in peak position has been noted for sulfur-containing compounds in which the formal oxidation state of sulfur varied from −2 to +6. The virtually monotonic shift in eV per unit of formal valence change of sulfur was 1.6 ± 0.25 eV. In addition, structurally dissimilar sulfur types of similar oxidation state, such as sulfate and alkyl sulfate ester, can be readily distinguished.

Transitions from reduced sulfur occur at the relatively low end of X-ray absorption energies in the sulfur domain. This is in keeping with the variation with oxidation state of 2p excitation energies in sulfur ESCA spectra.[29] At any oxidation state, the absorption edge-jump (the ionization threshold) occurs at higher energies than transitions to bound atomic and molecular electronic states. The intensity of the overall edge jump directly reflects the total sulfur present regardless of oxidation state.[30a] The spectra in Figure 1 have been labeled as to edge-jump region and relative sulfur valence state. The various regions defining a K-edge X-ray absorption spectrum are discussed in more detail in the section below describing the curve fitting experiments.

Examination of the spectra in Figure 1 immediately reveals the presence of at least two general types of sulfur: low-valent sulfur exhibiting features near 2470 eV and high-valent sulfur with features near 2482 eV. The spectra have been normalized so as to reflect an equivalent sulfur content, that is, to show an equally intense total edge jump at 2488 eV.[27,28] The relative heights of the edge jump at 2476 eV and the total jump at 2488 eV within each spectrum reflect the ratio of low-valent to total (low- plus high-valent) sulfur. Low-valent sulfur, uniquely accounting for fully half the total sulfur edge jump in the S86 sample spectrum, provides only about 15% of the edge jump height in each of the other two blood cell collection spectra. Therefore,

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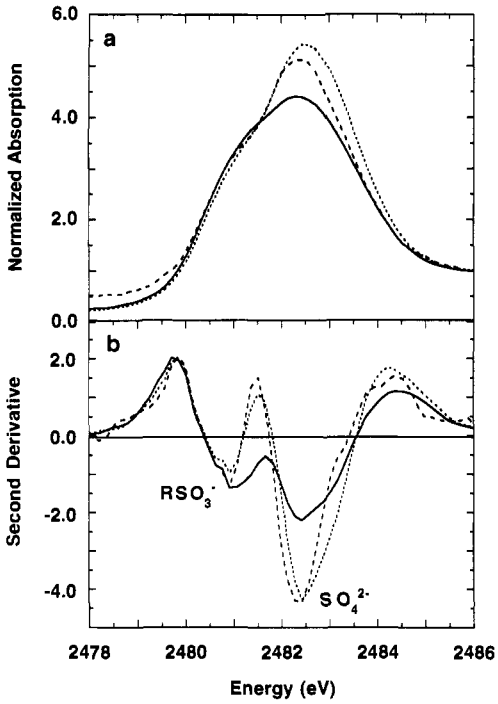

Figure 2. The high-valent region of each sulfur K-edge XAS spectrum shown in Figure 1: (a) X-ray absorption spectra; (b) second derivative X-ray spectra of blood cell sample S85 (—), blood cell sample S86 (- - -), and blood cell sample W87 (···). The main absorption envelope in (a) is revealed in (b) as containing two principal features, representing sulfonate at 2481.1 eV and sulfate at 2482.4 eV. The energy positions of the second derivative minima, and the line shapes, are sensitive to the chemical environment (*vide infra*).[8]

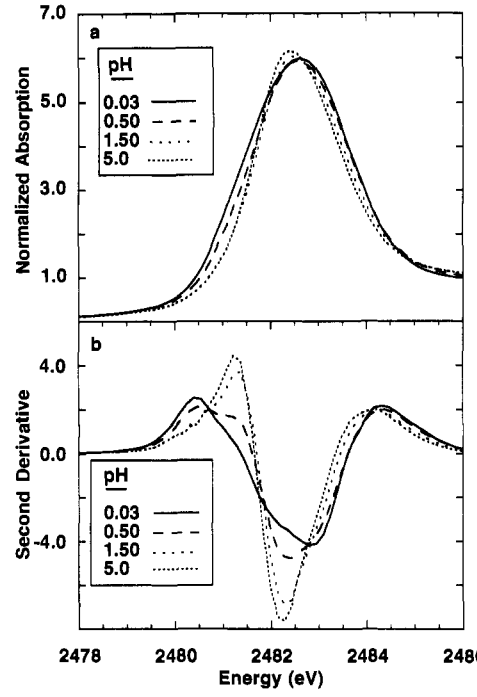

Figure 3. Sulfur K-edge XAS spectrum of 1.0 M aqueous sulfate on titration with acid. The pH is given in the inset of each part. (a) Absorption spectra: The shift in the energy position of the maximum and the width of the line clearly follow the trend in pH. (b) Second derivative spectra, plotted to the same relative scale as in (a): Protonation of sulfate is reflected in the appearance of a new minimum at 2482.8 eV. Note that the serially diminishing feature due to free sulfate remains stationary in energy.

it is evident that the relative amounts of low- and high-valent sulfur vary from sample-to-sample and, thus, from time-to-time.

In previous work,[8] the high-valence region of the sulfur-K X-ray edge spectrum from a sample of *A. ceratodes* whole blood cells was found to have a relatively complex line shape, with a shoulder at about 2481.6 eV. This shoulder reflects the partially superposed contributions from sulfate (at 2482.4 eV) and an aliphatic sulfonic acid of unknown structure (at 2481.1 eV).[8] A similar feature also appears in all the spectra shown in Figure 1, indicating that the mixture of sulfate and sulfonate occurs in all the blood cell samples.

The set of three high-valent features in Figure 1 can be defined by the shape of the shoulder at 2481.6 eV. That is, the S85 spectrum is relatively smooth at 2481.6 eV, whereas the S86 and W87 spectra each have a well-defined shoulder. All these features are broad relative to spectra of neutral inorganic model solutions containing sodium sulfate and cysteate or methane sulfonate[8] (*vide infra*). Overall, each of the blood cell samples constitutes a class of one in the distribution and spectroscopic signatures of the endogenous sulfur types. These results, though rather qualitative, imply an unexpectedly rich variability in blood cell sulfur chemistry within *A. ceratodes*.

High-Valent Sulfur. The high-valent sulfur portion of the above three blood cell S-K XAS spectra, along with the respective second derivative spectra are shown in Figure 2. In the second derivative spectra, two components are clearly resolved: the sulfonic acid feature at 2481.1 eV and that of sulfate at 2482.4 eV [8] (see below).

Clear evidence that the endogenous chemical environment of sulfate and sulfonate varies with blood cell sample can be seen, especially in Figure 2b, in the line shape variation around 2481.6 eV and in the changes in the relative line widths and energy positions of the respective features. In summary, the line width distributions are (sample (sulfonate, sulfate)) as follows: S85 (broad, broad); S86 (narrow, narrow); W87 (narrow, broad).

Line-Width Determinants in Sulfate and Sulfonate K-Edge Spectra. Following these findings, possible sources of broadening in the sulfur K-edge spectra of sulfate and methanesulfonate were investigated. Maximum suppression, resulting in apparent and usually symmetrical line-width increases, can arise due to self-absorption.[31] Such effects can occur in the sulfur K-edge spectra of finely ground solids and in solution samples of concentration greater than about 100 mM.

However, the broadening observed in the S85 and W87 sample spectra is unsymmetric and skewed to high energy (Figure 2). Since these spectra arise from 1s-to-valence electronic transitions, it was thought that the enhanced line widths may reflect a perturbation due to complexation. In order to test this idea the sulfur K-edge spectra of sulfate titrated with acid and of sulfate in pH 1.5 sulfuric acid[5,8] titrated with V(III) or Mn(II) were measured. Manganese(II) is similar in Z to vanadium(III), but complexes sulfate weakly.[32] All the blood cell samples were found to contain significant amounts of vanadium (see below).

In Figure 3a the sulfur K-edge spectra obtained from 1 M sulfate in solutions ranging in pH from 0.0 to 5.0 are shown. The second derivative spectra are displayed in Figure 3b. The results from the analogous experiment of sulfate titrated with vanadium-(III), in 15 mM sulfuric acid throughout, are given in Figure 4a,b.

In 1 M sulfuric acid (pH 0.0) 99% of the sulfate is present as bisulfate ion (pK_a = 1.92 [33]). Examining Figure 3a, the sulfate X-ray absorption spectra show a clear broadening trend with acidity, including a concomitant shift of the peak maximum to higher energy. In the second derivative spectra (Figure 3b) the 2482.3-eV feature due to sulfate at pH 5.0 is virtually lost at pH

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(33) *Handbook of Chemistry and Physics*, 67th ed.; CRC Press Inc.: Boca Raton, FL, 1986; p D163.

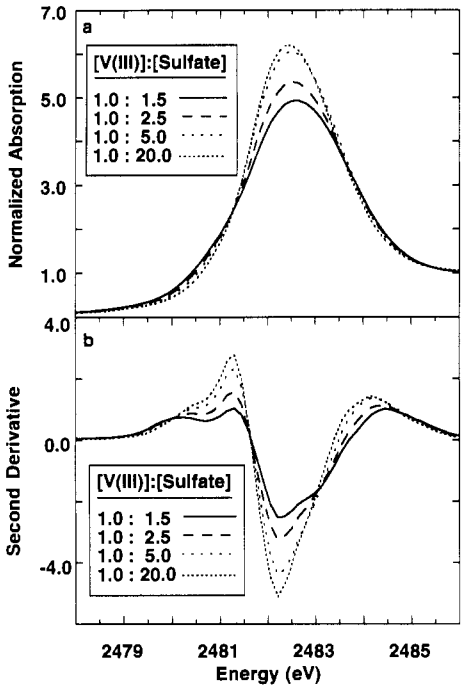

Figure 4. Sulfur K-edge XAS spectra of aqueous sulfate titrated with V(III) in 15 mM sulfuric acid. The ratio of [V(III)] to [sulfate] is given in the inset to each part. (a) Absorption spectra: As with protonation, the increase in line width and the shift in energy position of the maximum follows the increase in [V(III)]. (b) Second derivative spectra, plotted to the same relative scale as in (a): Note that while again the feature due to uncomplexed sulfate remains stationary in energy, an emergent feature at 2483.0 eV follows the [V(III)].

0.0 in favor of the new feature at 2482.8 eV almost certainly reflecting bisulfate.

A similar trend governs the absorption (Figure 4a) and second derivative (Figure 4b) spectra of pH 1.5 sulfate during the titration with V(III). In this case the new feature appears at *ca.* 2483.0 eV. Close comparative examination of Figures 3 and 4 indicates that the line shape changes induced by V(III) differ in detail from those produced by protonation. This point will have bearing below.

Isosbestic points at 2482.6 eV in Figure 3a and 2481.3 eV in Figure 4a indicate the interconversion of only two species in each titration experiment. Therefore, the complexation equilibrium in eq 1 applies, where M = H or V and $n = +1$ or $+3$, respectively.

$$SO_4^{2-} + M^{n+} \rightleftharpoons [MSO_4]^{(n-2)+} \qquad (1)$$

Kimura *et al.*[34] have measured the equilibrium constant of eq 1, where $M^{n+} = V^{3+}$, to be 29 M^{-1} (μ = 1 M and $[H_3O]^+ = 0.85$ M), demonstrating formation of the VSO_4^+ complex ion even under conditions of high acidity. In addition, recent resonance Raman experiments[35] have provided direct evidence for association of V(III) and sulfate in the VSO_4^+ complex ion in acidic (pH 1.2) solutions. In this work, the sulfur K-edge spectrum of sulfate is now also shown to be sensitive to the association state of this anion.

Because the concentrations of the titration solutions were on the order of 1 M, the count rate of the data was very high. Therefore the counting error is essentially within the thickness of the lines. However the concentration of sulfate in the vanadium titration experiment varied systematically between 1.0 M (at zero [V(III)]) and 1.5 M (for 0.5 M $V_2(SO_4)_3$), so that some of the observed broadening in the V(III) titration of sulfate instead reflects the maximum suppression due to self-absorption (see above).[31]

Quantitative comparison of the spectrum of 0.5 M $V_2(SO_4)_3$ with 1.0 M and 1.5 M pH 1.5 sulfate solutions nevertheless indicated that self-absorption could have accounted for no more than 41% of the peak broadening observed over the course of the titration. In addition, the second derivative spectrum of 1.5 M sulfate pH 1.5 was essentially superimposable over that of 1.0 M sulfate pH 1.5 (data not shown), in contrast to the effects shown in Figure 4b. Neutral 1 M solutions of sodium, ammonium, and manganous sulfate exhibited narrow and superimposable sulfate K-edge spectra (data not shown). In addition, titration with Mn(II) of 1 M sulfate in 15 mM sulfuric acid produced no detectable broadening of the sulfate K-edge line or shift in peak position (data not shown). Therefore the effect seen in the V(III) titration of sulfate can only be due to formation of the VSO_4^+ complex ion.

The effect of V(III) on spectra of methanesulfonate in 15 mM methanesulfonic acid, though noticeable, was much smaller than for sulfate, probably because of the very weak basicity of methanesulfonate. In this regard, essentially no pH dependence was observed in the spectral line width of solutions of methanesulfonate ion.

Acidity and Vanadium Associated with Intracellular Sulfate and Sulfonate. With a view to the foregoing, the average vanadium concentration of each blood cell sample was determined. These were as follows: S85, 0.099 M;[5] S86, 0.079 M; W87, 0.062 M (see Materials and Methods for details). These results validate the possibility that some part of the S-K XAS line width arising from the blood cell samples could be due to complexation, *e.g.*, of blood cell sulfate, by V(III). Thus, comparison of Figures 3b and 4b with Figure 2b indicates that the S85 and W87 sulfate features resemble more closely the shapes resulting from complexation of sulfate by V(III) (Figure 4b). Similarly, the broadening of the S85 sulfonate feature in Figure 2b can, more provisionally, be assigned to complexation by V(III), since although acidity to pH 0.0 had no effect (compare also the second derivative features in Figure 5a,b), methanesulfonate solution S-K X-ray spectra were clearly broadened by V(III).

In contrast to the above, the second derivative sulfate feature from the S86 sample shows no clear sign of V(III) complexation. However, this feature proved superimposable upon the second derivative spectrum of inorganic sulfate in pH 1.5 sulfuric acid alone. Thus the sulfate portion of all three sample spectra show the effect of either acidity or vanadium(III) complexation or both.

Figure 5a,b shows the sulfur K-edge XAS spectra, along with the second derivatives, of a series of inorganic model solutions containing mixtures of methanesulfonic acid and sulfate at pH 1.5, without and with stoichiometric V(III) ion, respectively. Comparative inspection reveals that the vanadium-containing solutions produce spectra (Figure 5b) in which the sulfate features are broadened, less intense, and shifted to higher energy, as described above. The effects of V(III) on the sulfonate spectra are less obvious, though in all cases the second derivative feature is more prominent.

Both sets of Figure 5 spectra reproduce the salient high-valent sulfur features of all the blood cell absorption spectra of Figures 1 and 2a, as well as the second derivative spectra of Figure 2b. The presence of both sulfate and sulfonate within *A. ceratodes* is thus strongly corroborated.[5,8] The greater similarity of the S85 and W87 sulfate features of Figure 2 to those of Figure 5b, exhibiting the effect of V(III), is again evident.

In direct comparisons with the model solution spectra, the S85 sample spectra are most closely approximated by the 1:1 sulfate:sulfonate spectra of Figure 5b. However, both the sulfonate and sulfate second derivative features in the blood cell spectra are

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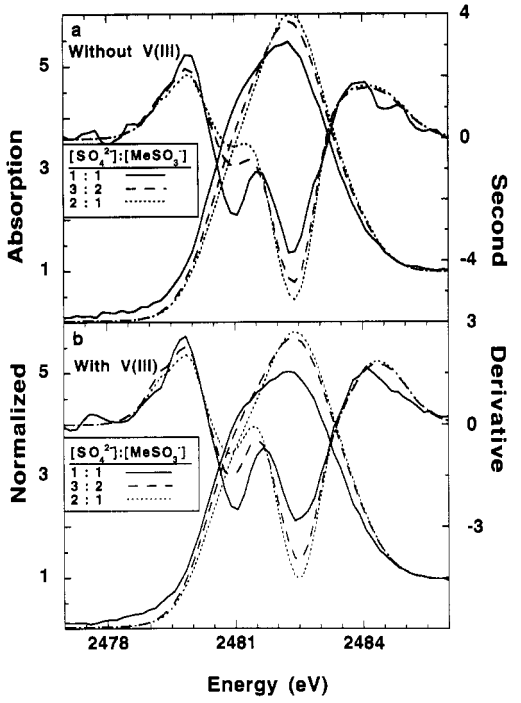

Figure 5. Sulfur K-edge XAS spectra of inorganic model solutions containing varying ratios of sulfate and methanesulfonate in 15 mM acid. The ratios are given in the box inset to each part. (a) Absorption and second derivative spectra of these ions in the absence of V(III). (b) The same as (a) except in the presence of stoichiometric V(III). The V(III)-containing solution spectra are in each case broader. Total [S] was 0.1 M. These spectra clearly reproduce the essential features of the blood cell X-ray spectra shown in Figure 2.

broader than the model solution spectra. This may imply that the complexation of sulfate by V(III) is more complete in the blood cells than in the stoichiometric model solutions. Such could arise, *e.g.*, if the blood cell concentration of vanadium(III) were more than stoichiometric with respect to sulfate. Other endocytic counterions, such as Cl^-, would then be necessary. We have detected appreciable intracellular chloride ion from Cl K-edge XAS spectra from blood cell preparations well washed with chloride-free isotonic buffer (unpublished data). Alternatively, if produced by acidity alone, the extra line width would imply an intracellular concentration of about 0.5 M sulfuric acid. This level seems biologically unlikely and is not consistent with the intracellular pH 1.8 derived from earlier vanadyl ion EPR studies.[5,6] In addition, as discussed above, the S85 sulfate line shape is broadened in a fashion more suggestive of V(III) complexation than of protonation.

In virtually complete contrast to the foregoing, the X-ray spectrum of sample S86 most closely matched the vanadium-(III)-free pH 1.5 2:1 model solution spectrum of Figure 5a. The second derivative sulfate feature in the S86 sample spectrum was in fact found to be superimposable over that of a pH 1.5 inorganic sulfate solution. The second derivative line of sulfate in pH 1.5 acid is readily discernible from that of sulfate in neutral solution (*cf.* Figure 3b). Therefore, the V(III) present in these cells must have been stored away from contact with the intracellular sulfate or sulfonate. Although acidic metal-free ascidian blood cells have been reported,[10,17,36] no judgment can be made here as to the respective locales of vanadium and sulfate or sulfonate in this blood cell sample. Given the likely difficulty of wholesale *inter*cellular transfer of quantities of materials, however, the colocation of sulfate, sulfonate, and vanadium in the same cells seems more likely. This point is discussed further below.

Finally, like comparisons for the S-K X-ray spectrum of sample W87 suggested a sulfate-to-sulfonate ratio closer to 3:2. However, whereas the sulfonate feature resembled that of the vanadium-free pH 1.5 inorganic model solution spectrum of Figure 5a, the sulfate feature was again broader than that in the spectrum of the analogous pH 1.5 model solution containing vanadium(III) (Figure 5b). The discussion presented above with regard to V(III) and sulfate in the S85 sample is pertinent here. Thus for the W87 sample, it appears that V(III) and sulfonate are sequestered apart but that V(III) and sulfate are stored together in solution.

The observed line broadenings permit the clear differentiation of three very different allocation regimes for blood cell vanadium and high-valent sulfur within the three cell samples. These are (sample (sulfonate, sulfate)) S85 (+V, +V), S86 (–V, –V), and W87 (–V, +V). These assignments match the differentiations made with regard to line width in the section on high-valent sulfur above. All the sulfate data are uniformly consistent with the intracellular presence of acid. The case for acidic blood cell sulfonate in the S86 and W87 spectra is more ambiguous, since pH 1.5 acid does not broaden the S-K edge spectrum of methanesulfonate.

Gaussian Curve-Fitting Experiments. The high-valent sulfur portions of the blood cell XAS spectra were fit with standard Gaussian curves in order to define, in an independent and more quantitative manner, the sulfonate/sulfate ratios in the three blood cell samples. To illuminate the basis for these experiments, some discussion of the nature of X-ray absorption edge features is warranted.

Following the 1s-to-valence electronic transitions, multiple Rydberg-like excitations can contribute significant XAS intensity at energies just below the ionization edge jump.[30c,37,38] The actual shape of absorption lines in a K-edge spectrum results from a convolution of the Lorentzian transition envelope,[30b] and the Gaussian imposed by the X-ray spectrometer optics.[26b,28,39] In practice the Gaussian largely dominates, though the convolved Lorentzian contributes heavily to the wings of the spectrum. Consequently, the Gaussian curves used herein to fit the X-ray spectra adequately reproduced the body, but not the tails, of the measured absorption envelope. This effect shows up particularly strongly in the second derivative spectra.

Two sets of two unique standard Gaussian curves were successfully obtained to fit the XAS features of sulfonate and sulfate: one set of two for these anions in the presence of V(III) and one set for the absence of V(III). It was therefore possible to determine through the fits whether blood cell sulfonate or sulfate was stored in the presence or absence of endogenous V(III). Rather than explicitly fit each of the above-mentioned Rydberg lines, it was found that two extra Gaussians could adequately represent the Rydberg envelope. Thus, the high-valent portion of each blood cell XAS spectrum was fit with a total of four standard Gaussian curves: one each for sulfonate and sulfate and two more to fit the Rydberg intensity. Fitting protocols and criteria, the method used to obtain the standard Gaussians, and the standard Gaussian parameters are described in the section on Materials and Methods.

In all the fits to the sulfonate/sulfate portions of the XAS spectra from the blood cell samples, a linear constant offset was included in order to account for the edge jump at 2476 eV of the low-valent sulfur. Of the three blood cell sample XAS spectra, only the spectrum of sample S85 could be well fit using the standard Gaussians. The standard sulfonate Gaussian was not able to completely reproduce the detailed shape of the inflection at 2481.5 eV in the XAS spectra of samples S86 and W87.

However, successful fits of the S86 and W87 spectra were obtained following a small adjustment of the line width or position

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Table 2. Gaussian Fit Results for *A. ceratodes* Whole Blood Cell Samples

Gaussian Fitting Results

	S85		S86		W87	
Gaussian[a]	fwhm	eV	fwhm	eV	fwhm	eV
sulfate	1.70(+)[b]	2482.4	1.58(–)	2482.2	1.70(+)	2482.4
sulfonate	1.90(+)	2481.1	1.60(–)	2480.9	1.70(–)	2480.9
I^c_{total}(emp, pred)[c]	(0.42,0.40)		(0.30,0.42)		(0.33,0.30)	

Blood Cell Sulfur Ratios

	S85	S86	W87
sulfate:sulfonate	1.0:0.9[d]	1.0:0.5[e]	1.0:0.44
(high:low) valent sulfur	5.2:1.0	1.0:1.0	6.1:1.0
av blood cell [V]	0.099 M[f]	0.079 M[g]	0.062 M[g]

[a] Full width at half-maximum (±0.01) and position (±0.01) in eV. Compare these values to the standard values in Table 1. [b] Interaction with V(III), (+); absence of V(III) interaction, (–), as indicated by the Gaussian fwhm (see Table 1). [c] The empirical (emp) and predicted (pred) total high-valent sulfur edge-jump intensities of each blood cell S-K XAS spectrum (see text). [d] Previously estimated to be 1:0.92 using a difference method.[8] [e] This ratio is less reliable since the predicted I_{total} is incorrect (see text). [f] See Reference 5. [g] This work.

of the standard sulfonate Gaussian (see below). The standard sulfate Gaussians were successfully used in all the fits. The line widths and positions of all the Gaussians used to fit the blood cell spectra are given in Table 2, and the results are discussed below.

Since the standard sulfonate Gaussian was modified in order to fit the S86 and W87 blood cell sample XAS spectra, it was deemed necessary that an independent way of judging the adequacy of these fits be devised. Thus it was decided to compare the calculated high-valent blood cell sulfur XAS edge-jump intensity, as predicted by each of the fits, with the corresponding empirical high-valent sulfur edge-jump intensity, as measured from each respective blood cell XAS spectrum. A close correspondence between the predicted and the measured edge jumps would indicate a well-behaved fit, independent of the other criteria described in the Materials and Methods section above.

The total high-valent sulfur edge-jump intensity, I^c_{total}, for each blood cell XAS spectrum is just the sum of the individual edge-jumps of blood cell sulfate and sulfonate; that is, $I^c_{total} = I^c_{sulfonate} + I^c_{sulfate}$. For each of the two sets of standard Gaussians used in the fits, it was determined that the ratio (I/A) between the area (A) of a standard Gaussian in any given fit and the edge-jump intensity (I) due to the component represented by that Gaussian is a constant within the limits of error for the fits (Table 1). Thus, the predicted blood cell XAS spectrum edge-jump intensity, for, *e.g.*, blood cell sulfate, $I^c_{sulfate}$ could be obtained using the simple ratio

$$I^c_{sulfate} = A^c_{sulfate}\left(\frac{I^s_{sulfate}}{A^s_{sulfate}}\right) \qquad (2)$$

where $A^c_{sulfate}$ is just the area of the standard Gaussian used to fit the sulfate feature of each given blood cell XAS spectrum. The value of $(I^s_{sulfate}/A^s_{sulfate})$ is the ratio of edge jump intensity to the sulfate standard Gaussian area, as given in Table 1. The same calculation can be made for blood cell sulfonate. The total high-valent sulfur edge-jump intensity for each blood cell XAS spectrum can thus be predicted from the fit to that spectrum.

The empirical high-valent sulfur edge-jump intensities can be measured directly from each blood cell XAS spectrum as the difference between the low-valent sulfur edge intensity at 2476 eV and the total edge-jump at 2488 eV. If a given fit adequately represents the high-valent portion of a blood cell spectrum, the predicted intensity, I^c_{total}, should equal the empirical intensity, within the error limits of the fit (±10%).

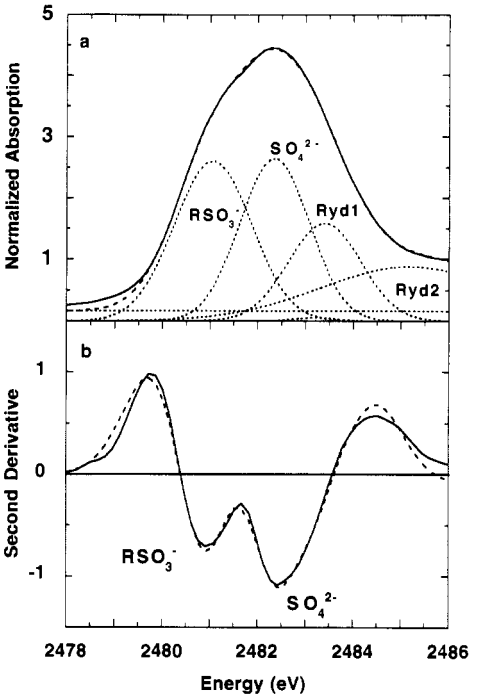

Figure 6. Gaussian fit to the S85 blood cell sulfur K-edge XAS spectrum. (a) The X-ray absorption spectrum (—) and the fit to the spectrum (- - -) are shown along with all the Gaussian components (···). Ryd1 and Ryd2 include the so-called Rydberg features which are transitions to weakly bound states (see text). In order to fit the second derivative asymmetry at 2483.2 eV, a Gaussian of fwhm = 1 eV was added, equivalent to about 1% of the total area of the sulfate Gaussian. (b) The second derivative of the X-ray spectrum (—) and of the fit to the spectrum (- - -). The fits to blood cell samples S86 and W87 were of comparable quality.

The results of this analysis are given in Table 2. The predicted edge-jump intensities for S85 and W87 reproduce the empirical intensities within the error limits of the fit. That for the S86 sample deviates markedly however, implying a less reliable fit.

In Figure 6a is shown the fit to the S85 blood cell spectrum. The fit to the second derivative is shown in Figure 6b. The Gaussian components of the fit are also shown and labeled. The features marked "Ryd1" and "Ryd2" incorporate the intensity due to the Rydberg-like transitions discussed above. The fits to the blood cell XAS spectra of samples S86 and W87 were comparable in quality.

The relative proportions of all the sulfur types as deduced from the fits (qualified as noted above) are gathered in Table 2 for each blood cell sample. These reveal that each of the three blood cell samples is unique with respect to the other two. The average blood cell vanadium concentrations are also given. Both the sulfate:sulfonate ratios and the assessment of an interaction of these anions with V(III) closely match the results, discussed in the previous section above, following direct comparisons with model solution mixtures. The variation in the ratios of blood cell sulfur types from sample-to-sample, and thus from time-to-time, is striking.

Low-Valent Sulfur. In Figure 7 the low-valent sulfur K-edge spectrum of the S86 whole blood cell pack is compared with the spectra of a variety of biologically-relevant sulfur-containing compounds. The analogous spectra of the S85 and W87 collections are comparable. Cysteine appeared to match the maximum, but not the low-energy shoulder, of the blood cell XAS spectrum.

The second derivatives of the low-valent portions of the X-ray spectra of all the blood cell samples are shown in Figure 8. Structure not resolved in the absorption spectra is evident. All the ascidian spectra display multiple minima not matched by cysteine, which produces only a single minimum at 2473.5 eV (not shown). A variety of model compound second derivative

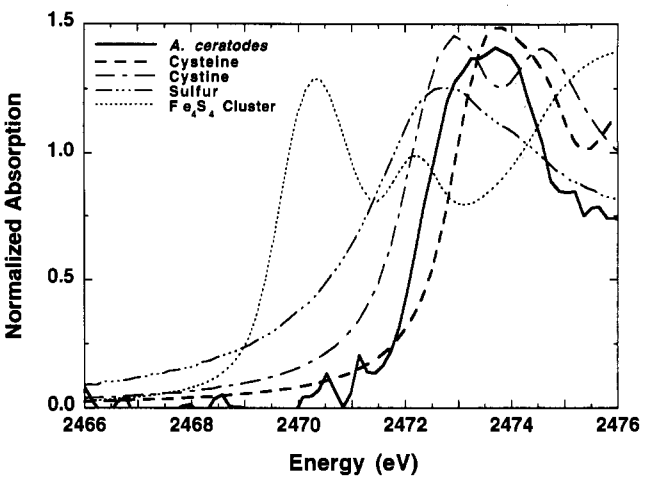

Figure 7. Sulfur K-edge XAS spectra of the low-valent sulfur within sample S86 and of several model compounds. All samples were prepared as finely ground solids, except $(Et_4N)_2[Fe_4S_4(SC_6H_5)_4]$, which was in DMF solution. None of the inorganic model XAS spectra match that of the blood cell sample in shape or edge position. Numerical mixtures of cysteine and cystine spectra did not reproduce the blood cell spectrum. There was no sign of iron–sulfur cluster sulfide in any of the blood cell sample spectra.

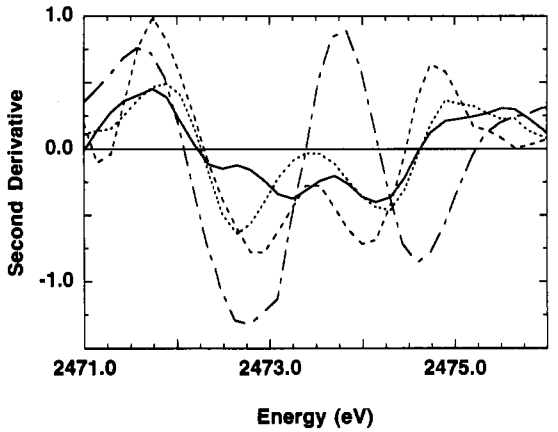

Figure 8. Second derivative K-edge XAS spectra of the low-valent sulfur within each of the *A. ceratodes* blood cell preparations and that of cystine: S85 (—); S86 (- - -); W87 (···); cystine (– · –) as a finely ground solid. The S86 and W87 spectra are ×1.5 for clarity. All three blood cell spectra have the multiple features characteristic of disulfides but are not reproduced by cystine. No two spectra are identical, indicating either a different disulfide, a mix of disulfides, or a different chemical environment for the disulfide, in each blood cell preparation. The S85 spectrum contains three minima rather than two. The source of these latter features is unknown, but the spectrum is not reproduced by that of, *e.g.*, diphenyl trisulfide.

spectra were examined for similarity, including cysteine, cystine, diphenyl disulfide, and thiourea. Somewhat more biologically exotic compounds, such as elemental sulfur, dibenzyl trisulfide, sodium hydrogen sulfide, 2-thiohydantoin, and 3,3′-diethylthiacyanine bromide among others, were also investigated. Of these only the disulfides showed the doublet minima evident in the tunicate blood cell spectra (Figure 8); however, none precisely matched the splittings. In addition the second derivatives of numerical mixes of cysteine and cystine spectra did not match the triplet shape of the S85 spectrum.

Disulfides yield two absorption peaks possibly due to orbital mixing.[40,41] If so, the magnitude of the splitting should be influenced by the dihedral angle of the relevant orbitals. Cyclic disulfides might then yield different doublet widths than linear disulfides in a second derivative sulfur K-edge XAS spectrum. Experiments are planned to test this idea.

As a tentative conclusion, it thus appears that blood cells from *A. ceratodes* are rich in a disulfide of presently unknown structure. It is not a typical linear disulfide, such as cystine, dibenzyl disulfide, or diphenyl disulfide; nor is it similar to elemental sulfur. Second derivative spectra also appear to exclude large amounts of cysteine from among those low-valent sulfur materials present. Finally, the relative amounts and types of low-valent sulfur species vary in a time-dependent manner (Table 2, Figures 1 and 8). Particularly noteworthy in this regard is the cyclic-like variation of the ratio of low-valent sulfur to total sulfur in the three samples.

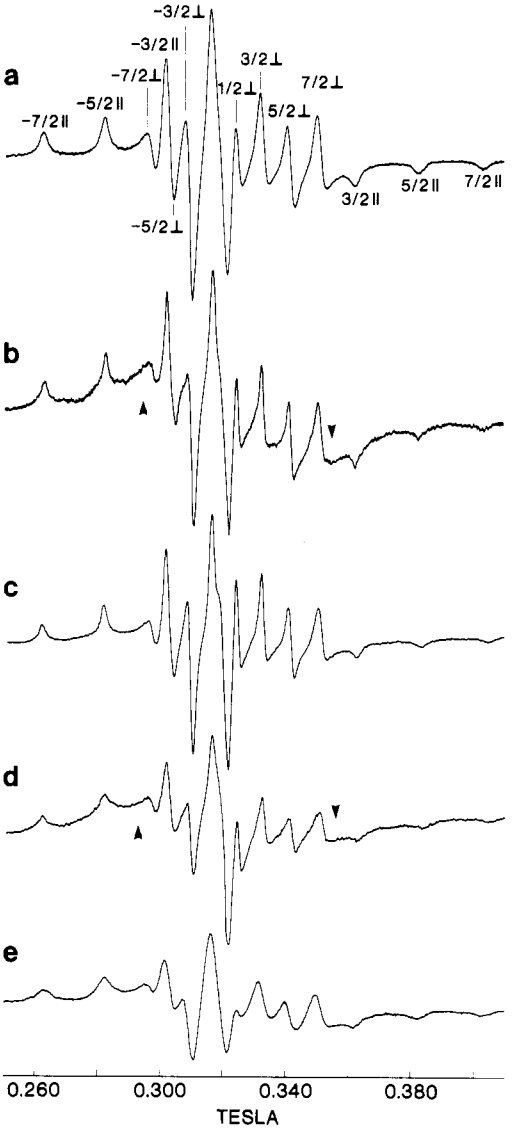

Figure 9. Vanadyl ion frozen-solution EPR spectra at liquid-nitrogen temperatures. Key: (a) 1 mM $VOSO_4$ in 6 mM sulfuric acid (pH 1.9), with line assignments taken from ref 43. (b) 1 mM $VOSO_4$ in 1 mM sulfuric acid with 50 mM sodium sulfate (pH 3.0), showing the broad baseline feature due[5] to excess sulfate (arrows). Note the generally poorer resolution and greater noise level of the spectrum relative to (a), despite the same vanadyl ion concentration. In the absence of excess sulfate, the individual vanadyl lines would be completely unresolved.[5] (c) Well-resolved whole blood spectrum from a single W87 specimen, $A_o = 0.01090$ cm^{-1}, $g_o = 1.973$. (d) Whole blood spectrum from a second single W87 specimen, $A_o = 0.01098$ cm^{-1}, $g_o = 1.972$, broadened in a manner similar to (b) (arrows), implying interactions between endogenous vanadyl ion and sulfate. The magnetism derived from the blood cell spectra are consistent with pentaaquovanadyl ion.[5,6,43] (e) 1 mM $VOSO_4$ dissolved in 3 mM sulfuric acid (pH 2.3). Line broadening due to vanadyl ion hydrolytic dimerization characterizes this spectrum; an effect clearly different from that of sulfate (compare (b)).

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EPR Experiments. In Figure 9 are vanadyl ion EPR spectra comparing inorganic solutions with two whole blood cell samples taken from individual animals of the W87 collection.

Comparing Figure 9a with Figure 9b reveals the broad feature (arrows) reported previously[5] to underlie the relatively sharp hyperfine lines of aquovanadyl ion in moderately acidic solutions containing excess sulfate. At pH 3.0 (*cf.* legend) in the absence of added sulfate, none of the vanadyl ion hyperfine lines appearing in Figure 9b would be well resolved.[5,42,43] Thus the effects of two processes, as described before,[5] are here illustrated: sharp hyperfine lines due to suppression by acid or excess sulfate of vanadyl ion hydrolytic dimers and the contrary appearance of sulfate-induced broadening.

In Figure 9c,d are shown the vanadyl ion EPR spectra of whole blood cell samples taken from two separate individual animals. The weak-field magnetism in each spectrum is consistent with endogenous aquovanadyl ion (*cf.* Figure 9 legend). The Figure 9c spectrum is quite similar to that in Figure 9a, and indeed the width at half maximum of the $-^7/_2 \parallel$ line (28 G) implies an intracellular pH of 1.8.[5] The spectrum in Figure 9d, however, shows evidence of sulfate broadening (arrows), similar to, but somewhat less pronounced than, that of Figure 9b. Of the vanadyl ion EPR spectra obtained from six single animal whole blood preparations, in our hands two have thus far shown sulfate broadening. Similar sulfate broadening is also evident in the reported EPR spectra of both whole blood and signet-ring preparations from *Ascidia gemmata*.[10]

In Figure 9e is shown the EPR spectrum of vanadyl sulfate in pH 2.3 (3 mM) sulfuric acid. Comparison with the Figure 9a spectrum reveals the dramatic influence of acidity on line width in this pH range.[5,10] That is, the features in the Figure 9e spectrum are homogeneously broad and blunt exemplifying the appearance of hydrolytic dimerization equilibria.[42,43] This effect is readily discernible from the sharp hyperfine lines over a broad underlying resonance characteristic of excess sulfate.

Since both hydrolytic and sulfate-induced broadening are suppressed at low pH,[5] any intracellular interaction between sulfate and vanadyl ions will be visible only in cells storing these ions in a relatively higher pH milieu in the presence of excess sulfate. For the cells producing the spectrum of Figure 9d this pH is about 2.1, as estimated from the 22-G low field half-width at half-maximum of the $-^7/_2 \parallel$ line. This vanadyl ion spectrum evidences the presence of an intracellular pool of sulfate.

More to the point, however, both intracellular vanadyl ion and V(III) are apparently able to each interact directly in solution with excess sulfate ion. Therefore the biological colocation of these two vanadium oxidation states in solution with sulfate can be inferred. Since vanadyl ion is the intermediate oxidation state between assimilated vanadate and V(III), this is a reasonable assumption. However, the X-ray absorption edge and EPR data reported here represent the first experimental support for this idea and alike support a common conclusion as regards the coexistent presence of endogenous acid, vanadium, and sulfate in blood cells from *A. ceratodes*. With this, the intracellular pH of 1.8, as determined by EPR[5,6] for the vanadyl ion locale within *A. ceratodes* blood cells, is more readily applied to the region wherein resides dissolved V(III).

Summary and Conclusions

In summary, sulfur K-edge X-ray absorption spectra reflect the interaction between sulfate ion and protons or complexing metals (noting in passing that this method makes a synchrotron X-ray facility by far the world's most expensive pH meter). Application to whole blood cell packs from *Ascidia ceratodes* has revealed an interaction between endogenous sulfate and most likely vanadium(III) in two of three independent collections. All three whole blood cell collections yielded sulfur K-edge XAS spectra consistent with acid sulfate. A direct endocytic interaction between dissolved V(III) and sulfate is also commensurate with the results of prior proton NMR,[6,9] S-K XAS,[7,8] and EXAFS[4] experiments on whole blood cell packs.

EPR experiments on whole blood taken from single animals of the W87 collection further indicated an interaction between endogenous vanadyl ion and sulfate. Similar spectra have been reported for blood cells from *A. gemmata*.[10] It therefore seems reasonable that dissolved V(III) and VO^{2+} in *A. ceratodes* are cellularly colocated with a single pool of sulfate.[5,9,16,17,20] The intracellular pH of 1.8 derived from whole blood vanadyl ion EPR experiments reported here and earlier[5,6] can thus be associated with the environment which includes dissolved V(III).

A similar intracellular pH is derivable from the work of Anderson and Swinehart,[23] who investigated whole blood cell preparations from *A. ceratodes* collected from Bodega Bay, CA. Using visible spectroscopy, they found that 15% of the intracellular vanadium was represented by the $[V(OH)_2V]^{4+}$ aquo dimer. Using both eq 4 and the data in Table VI in the work of Newton and Baker,[44] and assuming similar chemical environments, this result allows a calculated estimate of about pH 1.5 for the V(III) milieu in these blood cells. These data further support the notion that dissolved V(III), in the blood cells of *A. ceratodes*, exists in acidic solution primarily as a mixture of the $[VSO_4(H_2O)_{5-6}]^+$ complex and $V(III)_{aq}$, rather than within a chelated matrix.

Given this, the question arises as to why sulfate is the counterion of choice within the cell, rather than the more readily available chloride. One possibility can be advanced, based upon the data of Kimura *et al.*,[34] who investigated the equilibrium complexation reaction between V(III) and sulfate in concentrated acidic solution. Using their $K_{assoc} = 29\ M^{-1}$ and a solution concentration of, *e.g.*, 0.5 M $V_2(SO_4)_3$, the concentration of $[(SO_4)^{2-}]_{free} = 0.58$ M, $[V(III)]_{free} = 0.083$ M, and $[(VSO_4)^+] = 0.92$ M.

The ionic strength produced by these species is 2.0 M, compared with 7.5 M if the $V_2(SO_4)_3$ salt were completely dissociated or 6.0 M for 1.0 M VCl_3. Therefore, the choice of sulfate as the endogenous counterion has the effect of dramatically lowering the ionic strength at high intracellular [V(III)], thereby reducing the osmotic gradient against the cytosol or surrounding plasma.

No evidence of any interaction between sulfate and V(III) was detected within the S86 whole blood sample. However the available data are consistent with sulfate within a vanadium-free pH 1.5 milieu. This blood cell sample was bracketed in time by those which did show a V(III)–sulfate interaction, one of which followed the S86 sample by only 6 months. Since all three blood cell samples contained appreciable average vanadium concentrations, the mobilization of V(III) between sulfate-containing solution and some other storage regime is indicated.

This regime could be the vanadium-containing granules observed in other ascidian species by Rowley,[20] by De Vincentiis *et al.*,[21,22] and by Bell and co-workers,[16,17] using electron microprobe X-ray analysis. Alternative modes of vanadium storage are thus apparently possible, even within a single species such as *A. ceratodes*. The variation in results and conclusions contained in the literature regarding the cellular disposition of vanadium[5,9-23,45] would then follow.

Of three whole blood cell preparations from *Ascidia ceratodes* specimens collected over a period of 18 months, no two were completely alike. The variations included the presence or absence of a vanadium(III)–sulfate interaction, the ratio of sulfate-to-sulfonate, and the relative amounts and types of low-valent sulfur species. These findings taken together are suggestive of a rich, surprisingly rapid, and relatively[19-22] unanticipated sulfur metabolism.

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As a final comment, we would like to emphasize the significance of these very marked and surprising variations in blood sulfur chemistry. All the *A. ceratodes* collections described here were gathered from Monterey Bay, CA, and the two *least* similar groups were obtained almost exactly 1 year apart. The shifts in cellular chemistry and contents are significant, and do not seem to reflect any trends phased to annual seasonal cycles.[46] Whether the observed shifts are coupled to other rhythms or to environmental cues or stresses remains unknown. However it is clear that complicated and profound changes in blood chemistry and/or blood cell population can readily occur through time within this, and by analogy other, species.

A particularly pertinent example of this is represented by the reported blood chemistry of *A. ceratodes* from Bodega Bay, CA, an estuarine environment about 250 km north of Monterey. An average blood cell [V] of both 0.1 M [12] and 0.2 M [23] has been found for these animals, which are comparable to the ~0.1 M reported for those from Monterey Bay. As described above, the acidities of the respective intracellular V(III) environments are also apparently similar. However, the [V] in cell-free plasma was reported to be 10–60 μM in *A. ceratodes* from Bodega Bay,[23] relative to 140 μM in those from Monterey.[5] The facile suggestion[23] that inadvertent cell lysis represents this difference is not supported by the reported[5] plasma:cellular iron or zinc ratios. Therefore the difference in plasma [V] between *A. ceratodes* specimens collected from the two regions is most likely real and certainly not inconsistent with the other time-related intraspecific variations in blood chemistry discussed herein. The reported differences in blood colors[5,6,23] should be similarly viewed.

Rather than due to variations in sulfur chemistry within single cell types, the variations described in this work may reflect changes in blood cell populations. It is known that significant variation in blood cell distribution, especially between morula and signet-ring cell counts, can occur over the course of 1 year in *A. ceratodes*.[46] Further study, combining specific cell counts within a blood cell sample with sulfur K-edge X-ray absorption spectra, would be necessary to resolve these questions.

These considerations are in addition to the variations in blood chemistry in and between species pointed out before.[6] Therefore it seems wise that possible variations in blood chemistry due to collection time, locale, and most especially species should be kept in mind when interpreting the results from any single set of experiments. Indeed, for lasting insights, investigations should sample each species over an extended time period.

Acknowledgment. We thank Dr. Trevor A. Tyson for his helpful discussions and interest in this work. In addition, we thank Prof. James E. Penner-Hahn, Department of Chemistry, University of Michigan, for providing the curve-fitting program Fitcur and Mr. David Eliezer and Dr. Soichi Wakatsuki for improving this program. This work was supported by grants NSF CHE 91-21576 and NIH RR-01209 (to K.O.H.). SSRL is supported by the Department of Energy, Office of Basic Energy Sciences, Divisions of Chemical and Materials Sciences. Further support is provided by the National Institutes of Health, National Center for Research Resources, Biomedical Research Technology Program, and by the Department of Energy, Office of Health and Environmental Research.

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